ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 25
July 7, 2010
World J Gastroenterol
2010 July 7; 16(25): 3091-3218

Online Submissions
www.wjgnet.com/1007-9327office
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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2010-2013

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 25 July 7, 2010

EDITORIAL

3091 Impairment of pre-mRNA splicing in liver disease: Mechanisms and consequences

Berasain C, Goñi S, Castillo J, Latasa MU, Prieto J, Ávila MA

3103 Malignant colorectal polyps

Bujanda L, Cosme A, Gil I, Arenas-Mirave JI

TOPIC HIGHLIGHT

3112 Heme oxygenase-1 as a therapeutic target in inflammatory disorders of the gastrointestinal tract

Vijayan V, Mueller S, Baumgart-Vogt E, Immenschuh S

ORIGINAL ARTICLE

3120 Comprehensive and innovative techniques for liver transplantation in rats: A surgical guide

Hori T, Nguyen JH, Zhao X, Ogura Y, Hata T, Yagi S, Chen F, Baine AMT, Ohashi N, Eckman CB, Herdt AR, Egawa H, Takada Y, Oike F, Sakamoto S, Kasahara M, Ogawa K, Hata K, Iida T, Yonekawa Y, Sibulesky L, Kuribayashi K, Kato T, Saito K, Wang L, Torii M, Sahara N, Kamo N, Sahara T, Yasutomi M, Uemoto S

3133 Cetuximab plus FOLFOX6 or FOLFIRI in metastatic colorectal cancer: CECOG trial

Ocvirk J, Brodowicz T, Wrba F, Ciuleanu TE, Kurteva G, Beslija S, Koza I, Pápai Z, Messinger D, Yilmaz U, Faluhelyi Z, Yalcin S, Papamichael D, Wenczl M, Mrsic-Krmpotic Z, Shacham-Shmueli E, Vrbanec D, Esser R, Scheithauer W, Zielinski CC

3144 Celiac disease serology in patients with different pretest probabilities: Is biopsy avoidable?

Sugai E, Moreno ML, Hwang HJ, Cabanne A, Crivelli A, Nachman F, Vázquez H, Niveloni S, Argonz J, Mazure R, La Motta G, Caniggia ME, Smecuol E, Chopita N, Gómez JC, Mauriño E, Bai JC

3153 Promoter hypermethylation and loss of *CD133* gene expression in colorectal cancers

Jeon YK, Kim SH, Choi SH, Kim KH, Yoo BC, Ku JL, Park JG

Contents

World Journal of Gastroenterology
Volume 16 Number 25 July 7, 2010

BRIEF ARTICLE

3161 Diffusion-weighted magnetic resonance imaging to predict response of hepatocellular carcinoma to chemoembolization
Chung JC, Naik NK, Lewandowski RJ, Deng J, Mulcahy MF, Kulik LM, Sato KT, Ryu RK, Salem R, Larson AC, Omary RA

3168 Impact of pain on health-related quality of life in patients with inflammatory bowel disease
Schirbel A, Reichert A, Roll S, Baumgart DC, Büning C, Wittig B, Wiedenmann B, Dignass A, Sturm A

3178 Use of isolated Roux loop for pancreaticojejunostomy reconstruction after pancreaticoduodenectomy
Ballas K, Symeonidis N, Rafailidis S, Pavlidis T, Marakis G, Mavroudis N, Sakantamis A

3183 Technical problems produced by the Bravo pH test in nonerosive reflux disease patients
de Hoyos A, Esparza EA

3187 Thiopurine S-methyltransferase polymorphisms and thiopurine toxicity in treatment of inflammatory bowel disease
Dong XW, Zheng Q, Zhu MM, Tong JL, Ran ZH

3196 Role of diffusion-weighted magnetic resonance imaging in the diagnosis of extrahepatic cholangiocarcinoma
Cui XY, Chen HW

3202 Perfusion CT findings in liver of patients with tumor during chemotherapy
Zhang Q, Yuan ZG, Wang DQ, Yan ZH, Tang J, Liu ZQ

CASE REPORT

3206 Annular pancreas associated with duodenal carcinoma
Brönnimann E, Potthast S, Vlajnic T, Oertli D, Heizmann O

3211 Surgical treatment of hepatocellular carcinoma with severe intratumoral arterioportal shunt
Ishii H, Sonoyama T, Nakashima S, Nagata H, Shiozaki A, Kuriu Y, Ikoma H, Nakanishi M, Ichikawa D, Fujiwara H, Okamoto K, Ochiai T, Kokuba Y, Sakakura C, Otsuji E

3215 Hepatocellular carcinoma occurring in a Crohn's disease patient
Ishida M, Naka S, Shiomi H, Tsujikawa T, Andoh A, Nakahara T, Saito Y, Kurumi Y, Takikita-Suzuki M, Kojima F, Hotta M, Tani T, Fujiyama Y, Okabe H

World Journal of Gastroenterology
Volume 16 Number 25 July 7, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
July 7, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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http://www.wjgnet.com

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3091

World J Gastroenterol 2010 July 7; 16(25): 3091-3102
ISSN 1007-9327 (print)

EDITORIAL

Impairment of pre-mRNA splicing in liver disease: Mechanisms and consequences

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Author contributions: All authors contributed equally to this paper; Berasain C and Ávila MA wrote the paper.
Supported by The Agreement between FIMA and the "UTE project CIMA"; Red Temática de Investigación Cooperativa en Cáncer RD06 00200061 (to Berasain C and Ávila MA), and Ciberehd (to Prieto J) from Instituto de Salud Carlos III; Grants FIS PI070392 and PI070402 from Ministerio de Sanidad y Consumo; Goñi S was supported by a fellowship from Ministerio de Sanidad y Consumo; Latasa MU and Castillo J were supported by a Ramón y Cajal contract and a Torres Quevedo contract from Ministerio de Educación y Ciencia, respectively
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Received: February 22, 2010 Revised: April 12, 2010
Accepted: April 19, 2010
Published online: July 7, 2010

Abstract

Pre-mRNA splicing is an essential step in the process of gene expression in eukaryotes and consists of the removal of introns and the linking of exons to generate mature mRNAs. This is a highly regulated mechanism that allows the alternative usage of exons, the retention of intronic sequences and the generation of exonic sequences of variable length. Most human genes undergo splicing events, and disruptions of this process have been associated with a variety of diseases, including cancer. Hepatocellular carcinoma (HCC) is a molecularly heterogeneous type of tumor that usually develops in a cirrhotic liver. Alterations in pre-mRNA splicing of some genes have been observed in liver cancer, and although still scarce, the available data suggest that splicing defects may have a role in hepatocarcinogenesis. Here we briefly review the general mechanisms that regulate pre-mRNA splicing, and discuss some examples that illustrate how this process is impaired in liver tumorigenesis, and may contribute to HCC development. We believe that a more thorough examination of pre-mRNA splicing is still needed to accurately draw the molecular portrait of liver cancer. This will surely contribute to a better understanding of the disease and to the development of new effective therapies.

Key words: Cell signalling; Hepatocarcinogenesis; Pre-mRNA splicing; Splicing factors; Targeted anticancer therapy

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Berasain C, Goñi S, Castillo J, Latasa MU, Prieto J, Ávila MA. Impairment of pre-mRNA splicing in liver disease: Mechanisms and consequences. *World J Gastroenterol* 2010; 16(25): 3091-3102 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3091.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3091

INTRODUCTION

Hepatocellular carcinoma (HCC) is a deadly disease, and is currently ranked as the fifth most common cancer worldwide[1]. Most cases of HCC emerge on a background of chronic liver injury and inflammation induced by viral infection (hepatitis B and C viruses), chronic alcohol abuse, exposure to hepatotoxins (such as aflatoxin B), and genetic or metabolic conditions (such as haemochromatosis, 1-antitrypsin deficiency, obesity or diabetes)[2]. The end-stage of chronic liver disease is liver cirrhosis, characterised at the histological level by the massive deposition of extracellular matrix in fibrous septa that surround regenerative nodules. In these regenerative

nodules, partially dedifferentiated hepatocytes are driven by a pro-inflammatory milieu to divide in an attempt to restore the lost functional mass, frequently giving rise to foci of dysplastic cells[3,4]. Accumulating evidence indicates that this microenvironment favours the development of mutations and genetic alterations that are at the origin of the malignant transformation of the liver[5-7]. In view of this, chronic hepatitis and cirrhosis are considered as pre-neoplastic conditions. Nevertheless, a small but sizeable proportion of HCCs develop in normal liver tissues, in the absence of any known risk factor for liver cancer. The mechanisms leading to cancer in this small but sizeable minority of HCC cases are not known[1].

HCCs are complex tumors from the molecular point of view[8,9]. Over the past decade many efforts have been made to identify the molecular alterations that take place during the development of HCC. High throughput array-based techniques evaluating gene expression profiles have provided valuable clues for the identification of key molecular pathways driving the neoplastic conversion of the liver[10-12]. Such studies have significantly contributed to the definition of accurate prognostic genetic signatures, and to the identification of relevant therapeutic targets, some of which are currently being validated in the clinical setting[13-15]. However, conventional microarray approaches are not robust enough to detect the subtle differences in the transcriptome that arise through alternative splicing mechanisms, and therefore potentially relevant alterations involved in the carcinogenic process can be missed[16,17]. Indeed, a large proportion of the diversity within the transcriptome is generated by alternative splicing, a mechanism through which multiple mRNAs and structurally different proteins can be produced from a single gene and that may affect more than 70% of human genes[18,19]. The protein products generated by alternative splicing can have different or even antagonistic biological roles, and therefore their relative levels may impact significantly on cell function. Alterations in mRNA splicing are important cause of disease, as illustrated by the fact that single-point mutations affecting splicing represent at least 15% of all disease-causing point mutations[20]. The relationship between alternative splicing and cancer has also been clearly established, cancer-specific splice variants and cancer-associated changes in the relative levels of spliced isoforms of genes with an established role in carcinogenesis have been observed[21-25]. Moreover, in some cases the pro-tumorigenic effects of these changes have been directly demonstrated, and the mechanisms leading to the appearance of these tumor-associated alterations in normal mRNA splicing are now being elucidated[24,25]. Together these findings attest to the importance of dysregulated pre-mRNA splicing in cancer. However, compared to other types of tumors, less information is available on the disruption of normal splicing in liver cancer and its biological significance. Here we discuss representative cases that illustrate the importance that perturbations in this process may have from the early stages of hepatocarcinogenesis.

OVERVIEW OF THE BASIC MECHANISMS OF PRE-MRNA SPLICING AND ITS REGULATION

Most eukaryotic pre-mRNAs contain noncoding sequences (introns) that need to be removed to generate the correct concatenation of exonic sequences[18,19]. Introns represent more than 90% of the length of pre-mRNAs, and the spliceosome, a complex nuclear machine, needs to accurately identify specific nucleotide sequences at intron-exon boundaries to carry out intron excision and exon joining. The spliceosome is composed of five types of small nuclear ribonucleoproteins (snRNPs), plus a large number of ancillary proteins. This complex is able to recognize a 5' donor splice site beginning with a GU dinucleotide, and a 3' acceptor site ending with an AG dinucleotide at the boundaries of introns, as well as the so-called branching sequence that precedes the 3' acceptor site. The 5' splice site is recognized by the U1 snRNP, while the branching sequence and the 3' acceptor site are recognized and bound by the U2 snRNP and the auxiliary factor U2AF, respectively (Figure 1A)[21]. Subsequent interaction with the U4, U6 and U5 snRNPs leads to the formation of the catalytically active complex that carries out the trans-esterification reactions, resulting in the cleavage and religation of the mRNA chain.

The accurate excision of introns requires the recognition of the above-mentioned consensus sequences that identify the splice sites[26]. Deviations from these consensus sequences generate weak sites that have less affinity for their respective snRNPs, resulting in less efficient exon recognition and therefore allowing the alternative selection of exons[21,24]. In these cases an additional class of sequence elements present both in introns and exons may come into play and modulate the selection of splice sites by the spliceosome machinery. These sequences, known as splicing enhancers and silencers, are short conserved elements of about 10 nucleotides that can enhance or repress exon recognition. The specific binding of a wide range of splicing regulatory proteins, such as SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNP), to these elements in pre-mRNAs influences (positively or negatively) the placement of the spliceosome on the appropriate splice sites (Figure 1B). This mechanism allows the facultative use of weak splice sites and mediates the generation of alternatively spliced mRNAs[19,24]. Nevertheless, many if not all exons contain exonic splicing enhancers that tend to be clustered around splice sites, and these elements are of particular high density within constitutive exons. As discussed by Jensen *et al*[26], this suggests that enhancers are important for the control of constitutive exon splicing, even in the presence of consensus splice sites. Exonic splicing silencers also help to define exon boundaries, and strongly affect splicing when there are cryptic or multiple splice sites of similar strength. These elements are thought to play a more prominent role in the control of alternative splicing[26,27]. Figure 2 summarizes

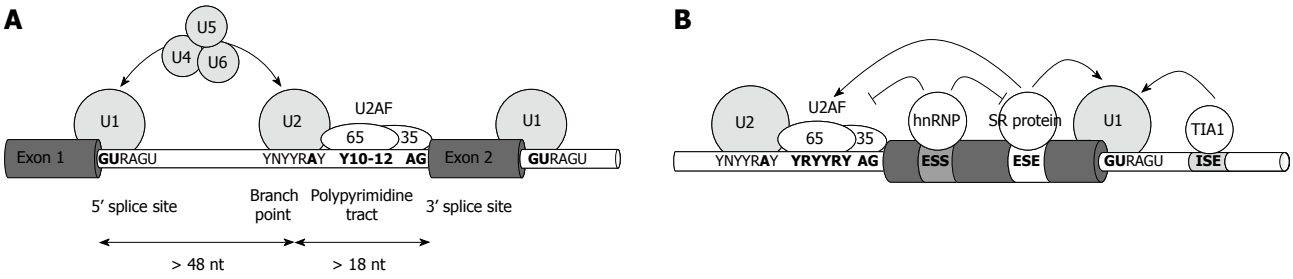

Figure 1 Splicing consensus sequences and interactions with small nuclear ribonucleoproteins (snRNPs), SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). A: Arrangement of donor and acceptor sites in a eukaryotic gene and interaction with snRNPs during splicing. Core elements necessary for pre-mRNA splicing include the 5' and 3' splice sites and a branch point sequence and a polypyrimidine-rich tract located upstream of the 3' splice site. The splicing factor U2AF (U2 auxiliary factor) consists of two subunits which bind to the 3' splice site and the polypyrimidine tract. U2AF promotes the binding of U2 snRNA in the U2 snRNP complex to the branch site. The U1 snRNP particle binds to the upstream and downstream 5' splice sites through base pairing of the U1 snRNA. The additional assembly of the snRNPs U4, U5 and U6 is required for the constitution of the spliceosome and the removal of introns; B: Interaction of splicing regulatory proteins with exonic and intronic target sequences. SR (Ser-Arg) proteins bind to exonic splicing enhancers (ESEs) to stimulate the binding of U2AF to a weak 3' splice site, which here is interrupted by purines (R). They also stimulate the binding of the U1 snRNP to the downstream 5' splice site. SR proteins antagonise the negative effect on splicing of hnRNPs bound to exonic splicing silencers (ESSs). In many cases, U-rich sequences situated immediately downstream of 5' splice sites known as intronic splicing enhancers (ISEs) are bound by factors such as T cell-restricted intracellular antigen 1 (TIA1) to facilitate U1 binding.

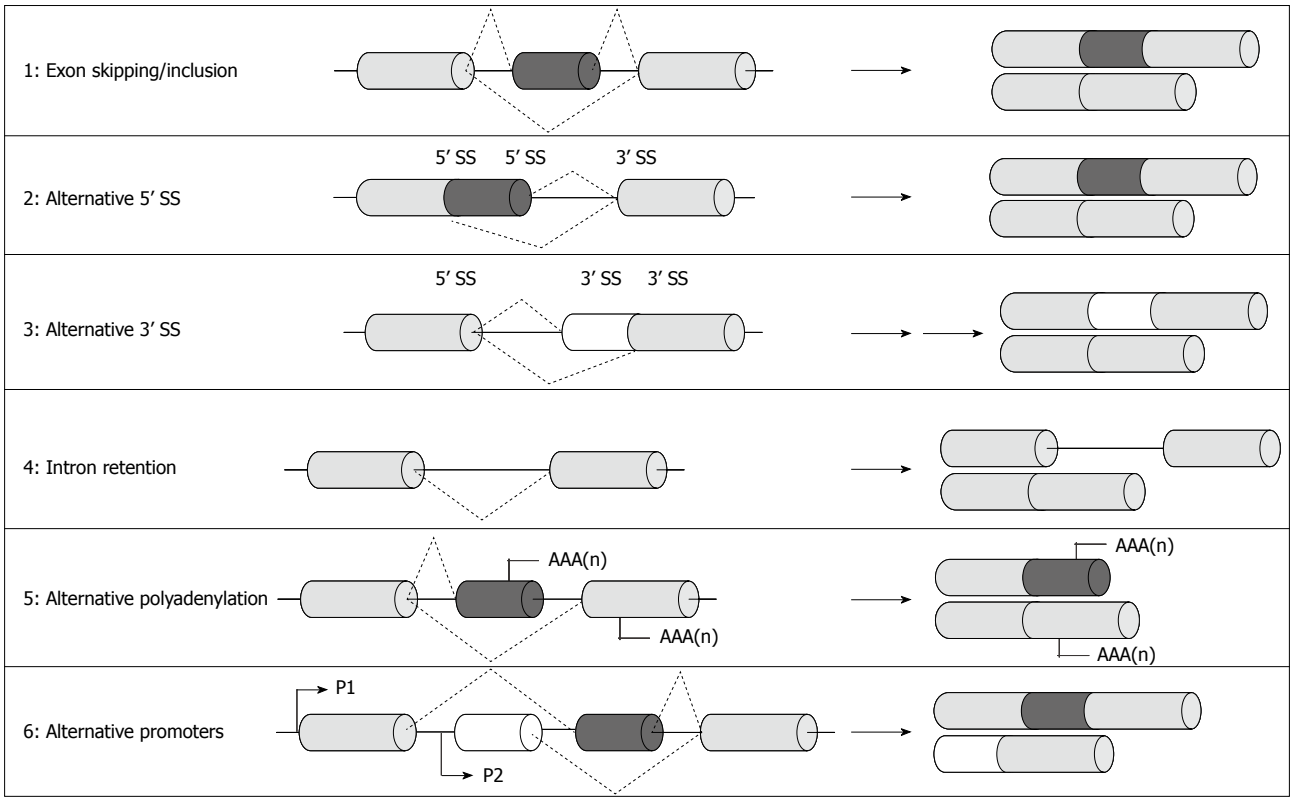

Figure 2 Different modes of alternative splicing. After pre-mRNA processing a single gene can encode multiple mRNA isoforms that possess distinct coding and regulatory sequences. Alternative splicing isoforms can result from: 1: The skipping or inclusion of alternative exons; 2: The selection of alternative 5'; or 3: 3' splice sites (SS); 4: The inclusion of introns; 5: The selection of different polyadenylation sites [AAA(n)]; and 6: The transcriptional initiation at an alternative promoter associated with the inclusion of specific exons.

the different alternative splicing events observed, these include: (1) exon skipping (cassette exons), in which the exon can be spliced from the transcript together with its flanking introns; (2) alternative 5' and (3) 3' splice site selection, where two or more splice sites can be recognised in an exon; (4) intron retention, in which an intron can remain in the mature mRNA; (5) alternative polyadenylation signals, which allows the generation of isoforms with 3' untranslated regions (UTRs) of different length; and (6) the alternative usage of promoters, with the generation of isoforms with promoter associated exons[18,19].

SR proteins are highly conserved RNA-binding proteins that facilitate splice site recognition and promote the inclusion of exons in the mature mRNA. The SR protein family members mostly interact with exonic splicing enhancers (ESE), stabilising the interaction of snRNPs and other factors at splice sites. Among other factors this family includes the ASF/SF2, 9G8, SC35, SRp30c, SRp20,

SRp40 and SRp46 proteins[27]. In contrast to the SR proteins the hnRNPs, such as hnRNPA1 and hnRNP I, have been mainly implicated in exon skipping. hnRNPA1 binds to exonic splicing silencers (ESS) and is able to prevent the binding of SR proteins to their specific sites in the exonic region, while hnRNPI competes with the U2AF auxiliary factor for its binding sequence close to the 3' splice site[24,28]. Variations in the expression levels of these splicing factors are known to affect the selection of splice sites, and therefore are thought to significantly influence the relative levels of splice variants among different tissues and also between normal and transformed cells[23,24].

Interestingly, accumulating evidence points also to the influence of extracellular stimuli on the regulation of alternative splicing[28,29]. In this respect, the activity of splicing factors is known to be affected by their phosphorylation status[30]. For instance SR proteins can be extensively phosphorylated on Ser residues, and their phosphorylation status affects protein-protein and protein-RNA interactions[28,30]. Moreover, extracellular signals can also impinge on the subcellular localisation of splicing factors, and consequently modulate their ability to interact with pre-mRNAs. This has been shown for different members of the SR protein family[29], and also for the splicing factor Slu7[31]. Several kinases and phosphatases responsible for the post-translational modification of splicing factors have been identified, and these include for instance the SR protein kinases (SRPKs), Clk/Sty, CDC-2 related protein kinase-7, and more recently, Akt and Fas-activated Ser/Thr kinase (FASTK)[28,29,32,33]. Among the phosphatases able to dephosphorylate splicing factors of the SR family we can cite PP1 and PP2A[29]. Less is known regarding the extracellular signalling pathways that control the activity of splicing factors, however, recent experimental efforts are beginning to pay off and relevant pathways are being delineated[29]. For example, insulin treatment has been shown to increase SRp40 phosphorylation and to stimulate protein kinase CβII alternative splicing *via* Akt2 activation[34]. Similarly, growth factors like the epidermal growth factor (EGF) have been demonstrated to modulate the splicing of the surface antigen CD44 in HeLa cells through the activation of Ras/mitogen-activated protein kinase (MAPK) cascade[35]. More recently, the activation of the EGF receptor (EGFR) was also shown to regulate the alternative splicing of the tumor suppressor genes *Krüppel-like zinc finger transcription factor 6* (*KLF6*) and *p73* in HCC cells. Although this will be discussed later in more detail, alternative splicing of *KLF6* pre-mRNA triggered by EGFR stimulation involved the Ras/phosphatidylinositol 3-kinase (PI3K)/Akt cascade, while that of *p73* specifically depended on c-Jun N-terminal kinase (JNK) activation[36,37]. Complex interactions between intracellular signalling pathways in the control of alternative splicing are also emerging. Such as the case of the antagonistic effect of JNK signalling on PI3K-mediated splicing regulation of the fibronectin gene in response to extracellular stimuli[38]. Taken together, these observations underscore the dynamic nature of alternative splicing, and also the high sensitivity to environmental signals displayed by this fundamental mechanism of gene expression regulation.

MECHANISMS AND SIGNIFICANCE OF ALTERATIONS IN PRE-MRNA SPLICING IN HEPATOCARCINOGENESIS

As previously mentioned, alterations in pre-mRNA splicing patterns, including changes in the normal tissular patterns of alternative splicing and the appearance of tumor-specific aberrantly spliced mRNAs, are increasingly being reported and implicated in cancer[39,40]. The mechanisms responsible for these alterations observed in cancer cells are still not well known. Nevertheless, some of these mechanisms are currently being elucidated and include: (1) mutations that create or disrupt splice sites or splicing enhancers or silencers[21]; (2) the abnormal expression of splicing factors[22,23,41,42]; and (3) the activation of cell signalling pathways that affect the activity of the splicing machinery[21,29]. In spite of recent advancements a major challenge in the field is still to discriminate whether these changes in alternative splicing can be the cause or are the consequence of human diseases. This is especially difficult in neoplastic diseases like HCC, if we take into account the multiple and heterogeneous genetic alterations that occur along the multi-step process of hepatocarcinogenesis, and the profound effects that the tumor microenvironment may have on transformed cells. Nevertheless, in some cases direct evidence has been provided on the oncogenic potential of liver cancer-associated splice variants and on the mechanisms involved in their generation. Interestingly, some cancer-associated isoforms have already been detected at pre-neoplastic stages, suggesting their potential early contribution to liver malignisation. Below we discuss some examples that are summarised in Table 1.

One interesting case of dysregulated alternative splicing occurring early during human hepatocarcinogenesis was reported by Saito *et al*[43]. These authors described the overexpression of a splice variant of DNA methyltransferase 3b (*DNMT3b*), namely *DNMT3b4*, in liver tissues showing chronic hepatitis and cirrhosis, as well as in HCC tissue samples. In contrast to *DNMT3b3*, the major variant in normal liver tissues, the *DNMT3b4* splice variant lacks the conserved methyltransferase motifs IX and X in exon 21, and probably also lacks this enzymatic activity. An elevation of the ratio of *DNMT3b4* to *DNMT3b3* mRNA was significantly correlated with the degree of DNA hypomethylation on pericentromeric satellite regions in precancerous conditions and HCC[43]. Hypomethylation of pericentromeric satellite regions may induce chromosomal instability, and is considered an early event during hepatocarcinogenesis. The authors hypothesised that the protein product of *DNMT3b4* transcript might compete with the active variant DNMT3b3, behaving as a dominant negative isoform. They confirmed this possibility by transfecting HEK293 epithelial cells, which express the DNMT3b3 active variant, with a DNMT3b4 expres-

Table 1 Examples of genes with altered splicing in liver carcinogenesis

Gene	Function	Selected ref.
DNMT3b4	DNA methyltransferase	[43,44]
MDM2	E3 ubiquitin ligase	[47]
Aurora kinase B Sv2	Serine/Threonine kinase	[45,46]
Hugl-1	Tumor suppressor	[49]
Tensin 2var3	Focal adhesion	[48]
Cadherin 17	Cell surface adhesion	[55]
MAD1 (β)	Spindle-assembly checkpoint	[50]
KLF6	Tumor suppressor	[36]
SVH	Unknown	[52]
p73	Tumor suppressor	[37,72]

sion vector and observed satellite DNA demethylation[43]. Furthermore, subsequent observations demonstrated that DNMT3b4 transfection resulted in enhanced growth rate and increased expression of transformation-related genes, even before chromosomal alterations appeared, underscoring the profound biological consequences of this altered splicing event[44].

Other examples of aberrantly-spliced genes detected in the preneoplastic cirrhotic liver include the serine/threonine kinase aurora kinase B (*AURKB*), and the E3 ubiquitin ligase and p53-antagonistic protein *MDM2*. AURKB, which is expressed in normal liver tissue, had been shown to be overexpressed in HCC correlating with tumor recurrence and a poor prognosis[45]. It was subsequently observed that over 60% of metastatic liver cancer tissues expressed the *AURKB* splice variant 2 (*AURKB Sv2*), which was also present in about 16% of the cirrhotic tissues tested regardless of their aetiology, but was absent in the normal liver parenchyma[46]. Interestingly, *AURKB Sv2* variant-positive HCC samples were mostly obtained from younger patients, and correlated with a poor outcome and short disease-free period[46]. From a mechanistic point of view the contribution of AURKB Sv2 to hepatocarcinogenesis is not known. This splice variant lacks parts of the kinase domain, and it could compete with the full length AURKB in a dominant negative manner, but experimental data on this aspect are not available yet. Regarding *MDM2*, two splice variants with tumorigenic potential, which are absent in the normal liver tissue, were found in alcoholic and autoimmune liver cirrhosis. However, for still unknown reasons these variants were not detected in hepatitis C virus (HCV)-infected cirrhotic samples[47].

As previously mentioned, the experimental demonstration of the pro-oncogenic effects of the splice variants found in transformed tissues is important to establish a cause and effect relationship. This is commonly done by expressing the splice variant under evaluation in cultured cell lines, as presented above for DNMT3b4, and more recently also by the generation of transgenic animals with targeted expression of the spliceoform in question. Although these approaches can be limited by the generally unphysiological expression levels obtained, relevant mechanistic information may be drawn from these studies. For instance, Yam *et al*[48] found that the Tensin 2 splice variant 3 (*TENSIN 2sv3*) was overexpressed in 46% of HCC tissues and most HCC cell lines examined, and that the expression of this variant correlated with a more aggressive tumor phenotype and other pathological features of poor prognosis. Tensins constitute a new family of focal adhesion proteins that link the extracellular matrix to the actin cytoskeleton and to intracellular signalling pathways. To directly assess the consequences of *TENSIN 2sv3* overexpression, the authors generated stable clones in a HCC cell line with low *TENSIN 2sv3* expression. These clones displayed increased growth, invasive properties, and tumorigenicity in an orthotopic model of HCC[48]. Together these findings identified a novel determinant in the metastatic behaviour of HCC which is generated by alternative splicing. Another cytoskeletal protein recently shown to be aberrantly spliced in HCC is the product of the *Hugl-1* gene, a tumor suppressor mainly expressed in the cytoplasm and involved in the regulation of cell polarity. Lu *et al*[49] found that 32.5% of HCCs displayed aberrant spliced variants of this gene that lacked a conserved repeat motif involved in protein-protein interactions. The presence of these Hugl-1 variants correlated with poor differentiation and large tumor size, and their overexpression in HCC cells resulted in enhanced invasion and tumorigenicity when injected into nude mice. Similar findings were recently reported for the *mitotic arrest deficient 1* (*MAD1*) gene. Sze *et al*[50] identified a novel *MAD1* splice variant in human HCC samples which they named *MAD1β*, while the original wild-type isoform was renamed *MAD1α*. MAD1α is a key protein in the mitotic checkpoint complex that monitors the status of kinetochore-microtubule attachment and the formation of the connections to the mitotic spindle. Defective mitotic checkpoint leads to DNA aneuploidy and chromosomal instability, which are features of HCCs[51]. MAD1β was overexpressed in 24% of the HCC tissue samples compared to the surrounding parenchyma, and interestingly more than 50% of the cases expressed MAD1β both in the tumoral and nontumoral tissue. MAD1β lacks the exon 4 of MAD1α, and is localised to the cytoplasm instead of the cell nucleus. MAD1β overexpression also affected the subcellular location and protein levels of other key components of the mitotic checkpoint such as MAD2. Functional studies overexpressing MAD1β in HCC cell lines demonstrated that the presence of this splice variant resulted in severe chromosome aberrations, thus demonstrating that MAD1β induces mitotic checkpoint incompetence[50]. Finally, there are also examples in which the specific targeting and downregulation of an aberrant splice variant found in HCC cells and tissues results in reduced tumorigenesis. This is the case of the "specific Splicing Variant involved in Hepatocarcinogenesis" (*SVH*) gene, an armadillo repeat domain containing gene of still unknown biological function that can produce four splice variants (*SVH-A, -B, -C* and *-D*). Only the splice variant *SVH-B* was upregulated in HCC tissues and hepatoma cell lines, and only the overexpression of this variant in non-transformed liver cell lines resulted in accelerated cell growth and tumorigenicity in nude mice[52]. Interestingly, the specific inhibition of *SVH-B* with antisense oligode-

oxynucleotides reduced HCC cell growth and survival[52]. This observation also suggests the possibility of specifically targeting aberrantly spliced mRNAs to quell liver cancer, as will be discussed later.

The observations described above illustrate the oncogenic potential of aberrantly spliced isoforms in HCC tissues. However, for all these cases no information is so far available regarding the molecular events involved in their generation[43,48-50,52]. In fact, the mechanisms that lead to the appearance of aberrantly-spliced genes in hepatocarcinogenesis have been so far elucidated for a limited number of genes. Among them we find *Cadherin 17* (*CDH17*), also known as liver-intestine cadherin (LI-Cadherin), a non-classic member of the cadherin family of cell-cell adhesion proteins overexpressed in about 90% of HCCs[53,54]. CDH17 expression was shown to confer tumorigenic potential to premalignant liver progenitor cells[53]. Interestingly, it was later found that 50% of HCC samples, and 30% of peritumoral tissues, also expressed a CDH17 splice variant lacking exon 7[55]. The expression of this splice variant was strongly associated with decreased overall survival of the patients[55]. While the reason why overexpression of this splice variant may have a pathogenic role remains speculative, the mechanisms behind its generation have been characterised. Wang *et al*[55] detected a base change at position 651 in exon 6, and another single nucleotide polymorphism (SNP) in a putative branch point at intron 6 position 35 (IVS6+35) was also found. The mutation at position 651 in exon 6 could affect exon 7 inclusion by generating an ESS, or by disrupting an ESE located in exon 6[21]. GG and AG polymorphisms were identified in 73% of the patients at IVS6+35, and in the same patients TT and CT polymorphisms were also observed at 651 (exon 6), in the control group normal livers showed the wild-type phenotype 651 CC, IVS6+35 AA. The functional role of these two SNPs was confirmed in a minigene assay, in which the presence of 651 T and IVS6+35 G SNPs resulted in exon 7 skipping[55]. It was later observed that the 651 T IVS+35 G haplotype, specially 651 TT and IVS6+35 GG homozygotes, seemed to be strongly associated with HCC, and therefore could be considered as a genetic susceptibility factor for HCC development in a Chinese population[56].

While point mutations in splice or regulatory sites are perhaps the most common alterations leading to aberrant splicing in cancer, the role of signalling pathways is gaining recognition. This is very well illustrated by the study of fibronectin alternative splicing regulation. Fibronectin is a glycoprotein found in blood, body fluids and tissues that plays a key role in cell adhesion and migration. Fibronectin has a domain structure consisting of three internally homologous repeats, termed type Ⅰ, Ⅱ and Ⅲ domains. There are two type Ⅲ domains that can be alternatively spliced, and incorporated or not in the mature transcript, the extra domains A and B, denominated EDA and EDB[57]. In addition, the so-called type Ⅲ connecting segment (ⅢCS) can also be alternatively spliced, generating in total up to 20 fibronectin isoforms in humans[57]. Fibronectin is biologically classified in two forms, namely plasma and cellular fibronectin, and only cellular fibronectin contains the EDA and EDB sequences. Plasma fibronectin is secreted by the hepatocytes, but these cells, as other normal adult cells, express limited amounts of EDA-containing fibronectin, which is expressed by foetal hepatocytes[57]. Several functions have been described for the EDA domain, including cell adhesion, wound healing, matrix assembly, matrix metalloproteinase expression, cell differentiation and tissue injury and inflammation[57]. Alternative splicing of the EDA exon is controlled by a bipartite element comprising an ESE and an ESS[29,57]. Srebrow and co-workers[38,58,59] have characterised the effect of extracellular matrix components and growth factors like EGF, hepatocyte growth factor and fibroblast growth factors, on fibronectin splicing. These authors observed that extracellular matrix components such as laminin and collagen Ⅳ downregulated EDA inclusion, while growth factors promoted the inclusion of EDA in fibronectin mRNA[38,59]. The signalling pathways connecting the cell surface with the nuclear splicing machinery controlling fibronectin splicing were also investigated. Exposure to a laminin-rich basement membrane resulted in JNK activation and sustained extracellular signal-regulated kinase (ERK) dephosphorylation, and these events were mechanistically linked to the described downregulation of EDA inclusion[38]. On the other hand, growth factor-activated Ras-PI3K-mediated signalling was shown to alter the phosphorylation levels of the SR proteins SF2/ASF and 9G8, and direct evidence was also provided on the importance of these events in mitogen-stimulated EDA inclusion[32,38].

All the mechanistic observations on the modulation of fibronectin splicing described above were carried out in mammary epithelial cells, however, they can be highly relevant to the alterations of fibronectin splicing found in liver disease. Indeed, EDA and EDB containing variants have been detected in the rodent liver during acute and chronic injury, and during liver regeneration after partial hepatectomy[60-63]. In models of liver injury, EDA-containing fibronectin was mainly produced by sinusoidal endothelial cells, while extracellular matrix-producing cells expressed EDB fibronectin[60,63]. In contrast, in human chronic hepatitis and in liver fibrosis EDA-containing fibronectin was detected not only in non-parenchymal cells, but also in hepatocytes[63,64]. Moreover, both EDA- and EDB-containing fibronectin variants were detected in human HCC tissues, and were localised to hepatoma cells[62,65]. The molecular mechanisms involved in fibronectin alternative splicing in liver injury and transformation are currently unknown. However, the pro-inflammatory milieu characteristic of chronic liver injury and HCC activates the same intracellular pathways that promote fibronectin splicing in mammary epithelial cells. Therefore, it is likely that cytokines and growth factors upregulated during hepatocarcinogenesis promote the alternative splicing of fibronectin in the liver. In support of this is the positive effect of transforming growth factor-β (TGFβ) on EDA inclusion observed in isolated mouse liver endothelial cells, or that of TGF and EGF found in human HCC cells in culture[58,59,62]. The expression of EDA-containing fibronectin during liver injury has been shown to contribute to the activation of ECM-

producing cells[60]. This effect, together with its ability to stimulate cell cycle progression and cellular migration observed in other cell types[57], suggests its likely involvement in the neoplastic conversion of the liver.

Together with fibronectin, other relevant examples of genes for which extracellular signals are known to affect their normal alternative splicing are the tumor suppressor genes *KLF6* and *p73*. As previously mentioned KLF6 is a ubiquitously expressed Krüppel-like zinc finger transcription factor, and tumor suppressor gene that is functionally inactivated in a number of cancers including HCC[66]. The *KLF6* pre-mRNA can give rise to three alternative splice variants, and at least one of them acts as a dominant-negative protein that antagonises the full length KLF6. This variant, named KLF6 SV1, lacks the three zinc finger DNA binding domains but retains the activation domain that mediates protein-protein interactions essential for the biological activity of this family of transcription factors. KLF6 SV1 is overexpressed in several human cancers, correlating with poorer outcome and reduced survival[67]. Increased *KLF6* alternative splicing, expressed as the ratio of *KLF6 SV1* to full length *KLF6*, was observed in 76% of the HCC samples examined[36]. While full length KLF6 overexpression in HCC cells inhibited growth and promoted cell differentiation[68], the overexpression of KLF6 SV1 partially restored the decreased cell proliferation induced by inhibition of the Ras pathway[36]. Mechanistically, the first cause that was identified for the generation of KLF6 SV1 in cancer cells was the presence of a SNP in intron 1 of the *KLF6* gene. This SNP abrogates a binding site for the splicing factor ASF/SF2 and generates a binding site for the SR protein SRp40, provoking the use of two cryptic splice sites in exon 2[66]. The presence of this SNP in association with HCC development has not been tested so far. However, it was known that *KLF6* could still be alternatively spliced into *KLF6 SV1* in normal and cancerous tissues without this SNP[66]. One relevant mechanism for the generation of KLF6 SV1 in HCC cells was recently outlined by Yea *et al*[36]. It involved the oncogenic activation of the Ras/PI3K/Akt pathway and the splice regulatory protein ASF/SF2. Interestingly, upstream of Ras the authors identified the EGFR tyrosine kinase activity as a signal that could trigger KLF6 SV1 generation. These findings may be of special relevance for the biology of HCC, they link a signalling pathway hyperactive in liver cancer[69] to the functional inactivation of a tumor suppressor gene through dysregulated alternative splicing.

p73 is another tumor suppressor gene which is frequently altered in different cancers, including HCC[70]. This gene is structurally related to *p53*, sharing common transcriptional targets that promote cell cycle arrest, apoptosis and limit anchorage-independent cell growth[70]. However, at variance with *p53*, no inactivating mutations have been described for *p73* in cancer cells. Inhibition of p73 tumor suppressive activity derives from the coexpression of N-terminally truncated isoforms. Two of these isoforms are known as ΔEx2p73 and ΔEx2/3p73, and are splice variants of the full length *p73* pre-mRNA that lack the transactivation domain, but retain the DNA binding domain and a C-terminal oligomerisation domain. ΔEx2p73 and ΔEx2/3p73 behave as dominant negative inhibitors of both p53 and p73, and are not expressed in normal tissues, but are frequently up-regulated in HCC[37,71-73]. Forced expression of these variants induces malignant transformation in fibroblasts and chemotherapy resistance in cancer cells[74,75]. Moreover, transgenic mice overexpressing human ΔEx2/3p73 in hepatocytes spontaneously developed HCC, attesting to the *in vivo* oncogenicity of aberrantly spliced p73 variants for liver cells[76]. The mechanisms regulating the generation of protumorigenic p73 variants are not known, however, a recent study by Castillo *et al*[37] identified a signalling pathway that promoted the generation of ΔEx2p73 in liver cells. It was observed that the stimulation of the EGFR by its ligand amphiregulin (AR) in an autocrine manner triggered the splicing of p73 into its ΔEx2p73 variant[37]. AR expression is undetectable in the healthy liver, but it is upregulated during injury and inflammation, contributing to tissue repair but also to the progression of liver disease[77-79]. Overexpression of AR is detected in liver tumors, and significantly participates in the maintenance of the neoplastic phenotype of HCC cells[79]. Interestingly, the expression of AR significantly correlated with the presence of ΔEx2p73 transcripts not only in tumor tissues, but also in cirrhotic livers. Furthermore, this correlation was also observed in the non-neoplastic normal parenchymal tissue of livers that developed HCC in the absence of any risk factor for this neoplasia[37]. These findings suggest that AR-mediated dysregulation of *p73* pre-mRNA splicing can be an early event during hepatocarcinogenesis, including those cases in which cancer develops in a tissue not chronically injured. Mechanistically it was demonstrated that downstream of the EGFR the activation of JNK1 resulted in the downregulation of the splicing factor Slu7 in HCC cells[37]. Slu7 is involved in the correct selection of the 3' splice site during the second step of splicing, and it has been demonstrated that Slu7 knockdown results in exon skipping of endogenous genes[80]. Inhibition of Slu7 expression by AR/EGFR/JNK1 signalling involved the activation of the transcription factor Elk-1, a previously recognised repressor of *Slu7* transcription[80]. Of importance, the expression of Slu7 was also found to be significantly decreased in chronic liver disease and HCC. Together, these observations identify a cancer-related extracellularly triggered signalling pathway that culminates in the transcriptional knockdown of a splicing factor, and the consequent generation of an oncogenic splice variant.

As mentioned before, abnormally expressed splicing factors can induce the production of mRNA isoforms nonexistent or present in low amounts in normal cells. Increasing evidence points to the significant contribution of this phenomenon to malignant progression, as recently demonstrated by the oncogenic consequences of SF2/ASF overexpression[41,81], or the contribution to the cancerous phenotype of hnRNP proteins upregulation[42]. However, a decrease in the expression of splice factors is less frequently observed in cancer cells, although some cases have been reported[23]. For instance reduced expression of

U2AF was found in pancreatic cancer[82], and siRNA-mediated inhibition of its expression in HeLa cells increased the level of the oncogenic CDC25B phosphatase[83]. In view of this, the downregulation of Slu7 gene expression in the cirrhotic liver may have mechanistic implications for the progression towards HCC.

POTENTIAL THERAPEUTIC INTERVENTIONS TARGETING SPLICING DEFECTS

The appreciation that dysregulated splicing may influence the development of human disease makes this process a potential therapeutic target. Different strategies are currently being explored to correct or inhibit pathological splicing events. They may be grouped in two major categories: molecules that can change alternative splicing and antisense strategies[39,84-86].

High throughput screens and individual studies on specific molecules have identified a number of compounds that can change splice site selection. For instance two different natural products with broad anticancer activity, pladienolide and spliceostatin A, have been recently reported to bind and interfere with the essential splicing protein SF3b, a core component of the U2 snRNP, inhibiting the splicing of a number of transcripts resulting in growth inhibition[87,88].

Targeting the expression and the post-translational modifications of splice modulators have also been attempted with promising results. As mentioned before, there are several kinases that phosphorylate the SR proteins in their arginine/serine domains (RS), affecting their intracellular localisation and their interaction with other splice factors and the pre-mRNAs. These kinases include topoisomerase I (Topo I) and members of the Clk/Sty and SRPK families[84,85]. Several antineoplastic compounds that target Topo I, such as diospyrin and indole derivatives like NB-506, can modify splice site selection. NB-506 inhibited the phosphorylation of SF2/ASF and altered the splicing pattern of several target genes including the apoptosis regulator Bcl-X in tumor cells[89]. The Clk1 kinase inhibitor TG003 also interferes with SF2/ASF-dependent splicing and can suppress alternative splicing in reporter genes[90]. The specific targeting of SRPK1 may be of special relevance, given its prominent role in cancer cells. Downregulation of SRPK1 expression by siRNA was reported to inhibit cancer cell proliferation and increase the sensitivity to chemotherapeutics[91], therefore the inhibition of this kinase may be an effective pharmacological strategy. Another approach to modify the activity of splice modulators is to target protein phosphatases (PPs). PP1 can bind directly to a conserved motif in the RNA-recognition region of at least nine splicing-regulatory proteins[84]. Modulation of PP1 activity shows a strong influence on alternative splicing, as illustrated by the lipid ceramide which activates PP1 and promotes the alternative splicing of the apoptosis regulator Bcl-X into its pro-apoptotic variant Bcl-Xs[92]. Activation of PP1 expression and upregulation of Bcl-Xs levels have also been demonstrated to occur in response to S-adenosylmethionine (AdoMet) and 5'-methylthioadenosine (MTA) treatment in HCC cells[93]. Interestingly, these two naturally occurring compounds show antitumoral effects in HCC and can induce growth inhibition and apoptosis in liver cancer cells, while normal hepatocytes are spared from this effect[94,95]. The reasons why AdoMet and MTA promote PP1 expression and Bcl-Xs upregulation only in HCC cells are still unknown, but this fact represents an advantage over other experimental approaches in which splicing can be altered both in normal and cancerous cells.

The selective modulation of splicing reactions is a big challenge, and the specific target of one splicing event is difficult to achieve with the above-mentioned compounds. This can be addressed with antisense oligonucleotides that can complementarily bind to a target site in pre-mRNAs and regulate the splicing process. Antisense oligonucleotides can block cryptic splicing sites created by a mutation, and redirect splicing back to the correct splice site. They can be used to induce exon skipping, and can also be targeted to regulatory sequence elements within exons and introns, like ESEs and ESSs[86]. These antisense oligonucleotides must be stable in biological fluids, and also need to be efficiently delivered inside target cells. To achieve the desired stability, chemical modifications are introduced that prevent degradation. To increase cellular delivery, formulations coupling oligonucleotides to arginine-rich cell penetrating peptides have been devised[39]. Moreover, intracellular delivery of antisense oligonucleotides to correct splicing defects has also been attained using adenoviral vectors[96]. The new generation antisense oligonucleotides include: phosphoroamidate morpholino oligomers, peptide nucleic acids (PNA), and locked nucleic acids (LNA)[86]. Morpholino oligonucleotides have been used to inhibit splicing silencers and to activate otherwise repressed exons. This has been shown for instance for the α exon of fibroblast growth factor receptor 1 (FGFR1), which is excluded in glioblastoma cells[97]. PNAs are oligonucleotide analogs in which the nucleobases are bound to a polyamide backbone that is highly stable *in vivo*[98]. They have been tested in cultured cells and *in vivo* with promising results, inducing the splicing of Bcl-X and promoting apoptosis in cancer cells[99]. LNA antisense oligonucleotides contain a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon. This modification increases the serum stability of oligonucleotides, prevents their degradation by RnaseH, and imparts high specificity toward their target sequences[100]. LNAs have been successfully used to modulate the splicing of tumor necrosis factor receptor 2 (TNFR2) in the liver, switching the endogenous expression of the membrane bound functional form of TNFR2 to a soluble secreted form lacking exon 7 (Δ7TNFR2). The secreted form acted as a decoy receptor for TNF-α, and mice were protected from liver damage in a model of acute inflammation and injury[101]. LNAs have also been used to target and promote the degradation of specific oncogenic splice forms. This has been shown recently by Emmrich and co-workers[102], who designed LNA antisense oligo-

nucleotides directed against the *ΔEx2p73* and *ΔEx2/3p73* oncogenic isoforms of the tumor suppressor p73, which as previously described are upregulated in HCC. Specificity was obtained by targeting the splice junction of each exon deletion variant. This strategy reduced tumorigenic p73 transcripts, without affecting wild-type p73, and inhibited malignant melanoma growth. Additional examples illustrating the potential of antisense oligonucleotide-based strategies to correct splicing defects have been recently reviewed in detail by Khoo and Krainer[103]. Among other cases, these authors describe how the splicing of liver apolipoprotein B 100 (APOB100), a key player in the development of atherosclerosis, can be engineered without affecting the expression of the intestinal isoform APOB48, involved in fat absorption from the gut. Specific targeting of *APOB100* pre-mRNA with antisense oligonucleotides induced the skipping of exon 27, leading to the generation of a shorter variant that has been associated with reduced cholesterol and LDL levels[103].

Also within these targeted strategies, a recent report by Alló and co-workers demonstrated that small interfering RNAs (siRNAs) targeting gene sequences surrounding an alternative exon (the flanking introns) can modulate its alternative splicing, resulting in exon inclusion[104]. It was found that upon siRNA transfection there was an increase in the levels of facultative heterochromatin in the vicinity of the siRNA target sites. The authors proposed that the condensed chromatin structure slows down Pol II elongation and facilitates the inclusion process. Regardless of the mechanistic details underlying this phenomenon, these findings open the door to the application of siRNA technology to correct splicing defects.

Although further studies are needed, together these observations demonstrate that targeting the splicing machinery is feasible and can also be achieved *in vivo*. Moreover, the application of antisense-based therapeutic strategies may be especially effective to treat liver diseases, as suggested by the preferential accumulation of antisense oligonucleotides in this organ when administered to mice[105].

HCC is a molecularly complex type of tumor, however, the identification of key splicing defects that may be corrected or interfered with targeted molecules as outlined above could help to develop new therapeutic strategies. These strategies may be used in combination with other targeted therapies, or with conventional chemotherapeutic approaches.

CONCLUSION

The detection of splicing aberrations from early stages of hepatocarcinogenesis together with the validation of their functional significance, suggests the likely implication of splicing defects in the oncogenic transformation of the liver. The realisation of this is just a start point for future research aimed at the development of effective therapeutic interventions in a deadly disease like HCC. There are different fronts in which action is needed. For instance, a thorough knowledge of the splicing alterations in pre-neoplastic and transformed liver tissue is still lacking. The use of improved splice-sensitive microarray platforms, and the implementation of new technologies like deep sequencing of transcriptome (RNA-seq), that allows both known transcript quantification and novel transcript discovery, are likely to yield valuable information on all splicing events[106,107]. These approaches will allow the identification of new splicing defects with potential pathological significance, and the generation of splicing signatures that may have prognostic value. The characterisation of functional splice regulatory elements in the genome that can be targeted by RNA binding proteins is also a challenge. New methods combining immunoprecipitation of RNA-protein complexes with high-throughput sequencing of reverse transcribed tags are currently being devised[19,106]. From the therapeutic perspective, it is possible that alterations in pre-mRNA splicing resulting from hyperactive intracellular signalling pathways could be reversed in part by the emerging targeted therapies that block these pathways[6,15,36,37]. However, when aberrant splicing is the consequence of genetic mutations that affect splicing reactions, and are not due only to the dysregulation of signalling pathways that impinge on pre-mRNA splicing, different approaches must be used. In these cases that involve structural changes due to mutations, the inhibition of hyperactive signalling pathways may not be effective, and the antisense oligonucleotides described above can be applied. Nevertheless, some aspects such as their potential off-target effects and their efficient delivery to the target tissues still need to be further elaborated[86]. In summary, the integration of pre-mRNA processing alterations in the molecular portrait of liver cancer will surely contribute to the understanding of the disease and the development of new effective therapies.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3103

World J Gastroenterol 2010 July 7; 16(25): 3103-3111
ISSN 1007-9327 (print)

EDITORIAL

Malignant colorectal polyps

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Supported by Instituto Salud Carlos III
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Received: February 5, 2010 Revised: April 7, 2010
Accepted: April 14, 2010
Published online: July 7, 2010

Abstract

Nowadays, the number of cases in which malignant colorectal polyps are removed is increasing due to colorectal cancer screening programmes. Cancerous polyps are classified into non-invasive high grade neoplasia (NHGN), when the cancer has not reached the muscularis mucosa, and malignant polyps, classed as T1, when they have invaded the submucosa. NHGN is considered cured with polypectomy, while the prognosis for malignant polyps depends on various morphological and histological factors. The prognostic factors include, sessile or pedunculated morphology of the polyp, whether partial or *en bloc* resection is carried out, the degree of differentiation of the carcinoma, vascular or lymphatic involvement, and whether the polypectomy resection margin is tumor free. A malignant polyp at T1 is considered cured with polypectomy if it is a pedunculated polyp (Ip of the Paris classification), it has been completely resected, it is not poorly differentiated, the resection edge is not affected by the tumor and there is no vascular or lymphatic involvement. The sessile malignant polyp (Is of the Paris classification) at T1 is considered not cured with polypectomy. Only in some cases (e.g. older people with high surgical risk) local excision (polypectomy or endoscopic submucosal dissection or conventional endoscopic mucosal resection) is considered the definitive treatment.

Key words: Favourable histology; Follow-up; Malignant polyps; Non-invasive high grade neoplasia; Treatment

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Bujanda L, Cosme A, Gil I, Arenas-Mirave JI. Malignant colorectal polyps. *World J Gastroenterol* 2010; 16(25): 3103-3111 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3103.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3103

INTRODUCTION

Adenomatous polyps are tumors of benign neoplastic epithelium with variable potential for malignancy. The adenoma-carcinoma sequence is well known and it is accepted that more than 95% of colorectal cancers arise from adenomas. The World Health Organisation (WHO) classifies adenomas into tubular (less than 20% villous architecture), tubulovillous and villous[1], with approximately 87% of adenomas being tubular, 8% tubulovillous and 5% villous. Only 5% of adenomas are in danger of becoming malignant. The probability of high grade dysplasia and of carcinomatous transformation increases with polyp size, especially when they are larger than 1 cm, they have a villous component, there are many polyps or the age at diagnosis is more than 60 years. The neoplasia is considered to be advanced when polyps are 1 cm or more in diameter, there is a villous component or a high degree of dysplasia. More than 25% of advanced polyps[2] and colon cancers are located in the area

proximal to the splenic flexure[3]. Mixed polyps also have the ability to become malignant, as does hyperplastic polyposis syndrome.

EPIDEMIOLOGY

The prevalence of cancerous polyps in series of endoscopically removed polyps is between 0.2% and 11%[4-7]. Nowadays, the number of cases in which malignant polyps are removed is increasing due to screening programmes. In an asymptomatic population of people over 50 years old who underwent direct colonoscopy, there was a 0.8% prevalence of adenocarcinoma of which 50% were carcinoma "*in situ*" or in stage I [8,9]. During screening programmes, adenocarcinomas have been detected in between 3% and 4.6% of those who undergo colonoscopy following a positive immunological faecal occult blood test result[1,10]. In 2006, Rubio *et al*[11] reported 10 patients with hyperplastic polyposis coli syndrome and a review of the literature showed that 50% (74/147) of patients with hyperplastic polyposis coli syndrome developed colorectal cancer (CRC).

HISTOLOGY

Carcinoma "*in situ*", intramucosal carcinoma, high-grade dysplasia or intraepithelial neoplasia is the stage at which there is no involvement of *the muscularis mucosa*. In general, this tumor stage does not cause metastasis. It is classified as pTis or Stage 0 in the TNM staging system. These terms are defined as non-invasive high grade neoplasia (NHGN) in the Vienna classification[12]. Carcinoma *in situ* or high-grade dysplasia or intraepithelial neoplasia corresponds to a carcinoma that is restricted to the epithelial layer without invasion into the lamina propria. Intramucosal carcinoma is a carcinoma characterised by invasion into the lamina propria.

When the carcinoma spreads to the submucosa, the polyp is considered to have become malignant, being able to spread to lymph nodes or distant sites. The tumors that affect the submucosa are classified as T1 and correspond to Stage I of the TNM staging system. This term is defined as submucosal carcinoma in the classification of Vienna[12] or malignant polyp.

The term pseudoinvasion refers to the presence of glandular epithelium of the mucosa beneath the muscularis mucosa in colonic polyps. These lesions have no malignant potential and should be treated in a similar way to adenomas[13]. However, this phenomenon can be mistaken for invasive carcinoma by an inexperienced pathologist. Pseudoinvasion usually occurs in large polyps (> 1 cm), especially those with long stalks, and is most commonly found in polyps of the sigmoid colon. Islands of adenomatous epithelium are displaced through the muscularis mucosa and are found within the submucosa of the stalk. The displaced glandular tissue usually has rounded, not infiltrative, contours, carries with it a small amount of lamina propria, and is cytologically identical to the overlying adenomatous component. Haemorrhage and haemosiderin deposition are commonly seen and are a clue to diagnosis. In addition, inflammation and granulation tissue can be found. Cystic dilatation of the displaced glands with mucin distention is also not uncommon in pseudoinvasion because mucin produced by the entrapped glands has no means of reaching the lumen. Occasionally, rupture of dilated glands occurs with acellular mucin extravasation and there is a subsequent inflammatory response. Distinction from mucinous (colloid) carcinoma is important and can be difficult. Specifically, in mucinous carcinoma, the mucin pools contain malignant cells, a feature lacking in pseudoinvasion. For these reasons it is highly recommended that level sections and second opinions are obtained in cases of polyps with potential pseudoinvasion[14].

All adenomas have some degree of dysplasia, be it high or low. However, low and high grade dysplasias are artificial subdivisions of a spectrum; there is no definition of "high-grade". Indeed, the WHO book on tumors of the digestive system, does not contain a list of criteria for high-grade dysplasia in adenomas[15,16]. However, in general, high-grade dysplasia entails more substantial changes and includes carcinoma "*in situ*". Among these changes we consider architectural alteration, often resembling the glandular arrangement of adenomas and cytologic abnormalities, principally cellular and nuclear pleomorphism, nuclear hyperchromatism, loss of nuclear polarity, and marked stratification of nuclei. Other authors have considered as features of high grade dysplasia: loss of normal glandular architecture, hyperchromatic cells with multilayered irregular nuclei and loss of mucin, high nuclear/cytoplasmic ratio, marked nuclear atypia with prominent nuclei and focal cribriform patterns. Not all these features are necessarily present to the same degree in all dysplastic epithelia, while low-grade dysplasia manifests these same changes but to a lesser degree[15,16].

PROGNOSTIC FACTORS

Many factors have been associated with a higher probability of residual disease or recurrent carcinoma.

Morphology

Morphology is described as polypoid (pedunculated or sessile) and nonpolypoid (flat or ulcerated) subtypes according to the Paris classification[17]. The endoscopist should be alert to some features that are suggestive of possible malignancy. These features include the size, the presence of depressed ulceration, irregular contours, deformity, a short and immobile stalk and the inability to elevate a sessile polyp when a submucosal bleb is formed. Nonpolypoid colorectal neoplasms have a greater association with carcinoma (NHGN or submucosal invasive carcinomas) compared with polypoid neoplasms, irrespective of size[7]. Attempts at diagnosis in such suspicious lesions, as well as in flat or depressed lesions, can be carried out using chromoendoscopy and magnification techniques that can highlight abnormalities of glandular cytoarchitecture, while also revealing information concerning the extent of submucosal invasion[18,19]. Kudo *et al*[20] developed

Table 1 Pit pattern classification

Type of lesion	Description
Type 1	Round pits
Type 2	Stellar or papillary pits
Type 3 L	Large tubular or roundish pits
Type 3 S	Small tubular or roundish pits
Type 4	Branch-like or gyrus-like pits
Type 5	Non-structural pits

Evaluation of the fine surface structure (pit pattern) of the mucosa. Lesions with type 1 and 2 pit patterns are nontumorous epithelial tissue, that is normal, inflammatory, or hyperplastic tissue. Tumorous lesions have type 3 S, 3 L, 4 and/or 5 pit patterns. Lesion 3 L and 4 are typical of the protruded type of tumor, whereas type 3 S and 5 are typical of the depressed type of tumor.

the pit pattern classification for colon polyps with six classes of surface pattern depicted by magnifying endoscopy after indigo-carmine dye (Table 1). Class 5 of this pit pattern classification or an unstructured surface has been shown to correlate well with a diagnosis of malignancy, and can provide important additional information prior to endoscopic treatment. However, endoscopic ultrasound using high frequency transendoscopic miniprobes currently appears to be the most accurate method for defining submucosal or further bowel wall invasion, enabling direct referral for surgical intervention in those cases with deeper infiltration who are at the greatest risk of lymphatic spread[21].

Type of resection

When *en-bloc* removal of a polyp is performed, it is possible to assess the depth of infiltration of the tumor cells and whether the margin is affected. Pedunculated malignant polyps are easily removed using a loop snare. However, this technique frequently results in piecemeal removal when applied to sessile and flat malignant polyps. Nevertheless, around one-third of malignant polyps are removed in this way[22]. *En-bloc* removal is advantageous because it allows full histological evaluation of the complete resection and is associated with lower recurrence rates than piecemeal removal[23]. Endoscopic submucosal dissection (ESD) has been found to be particularly useful for the removal of sessile or flat adenomatous lesions. It has an advantage over other endoscopic techniques in that it allows *en-bloc* removal of large (> 2 cm) colonic lesions. In ESD an electrosurgical cutting device is used to carefully dissect the deeper layers of the submucosa to remove neoplastic lesions in the mucosa. In a meta-analysis it was found that ESD *en-bloc* resection is achieved in 84.9% of lesions, and clear vertical and lateral margins are achieved in 75.3% of cases[24].

Polypectomy resection margin

It is essential that the pathologist identifies the stalk or the depth of the diathermy burn. The risk of relapse ranges from 0% to 2% in malignant polyps with a margin of resection greater than 1 mm. When the resection margin is also involved, or is less than 1 mm, the percentage of relapse ranges between 21% and 33%[25]. Most authors believe that a resection margin of $\geqslant$ 2 mm is safe and that in such cases the probability of residual disease or recurrent carcinoma is low[4,5,25,26]. However, whether the requisite distance from cancer to margin of resection should be > 1, > 2, or > 3 mm or only a clear margin of excision is still under debate.

Stage of differentiation

Four grades were considered. Grade 1 corresponded to a well-differentiated intestinal-type adenocarcinoma and is composed of well-formed glands with open lumina or with more than 95% glandular differentiation. Grade 2 was moderately differentiated intestinal-type adenocarcinoma containing solid nests showing only focal glands or with 50%-95% glandular differentiation. In the case of Grade 3, the carcinoma is poorly differentiated intestinal-type, signet ring cell or mucinous adenocarcinoma, composed of hyperchromatic cells arranged into solid sheets and forming absorptive glands. These tumors have between 5% and 50% glandular differentiation. Undifferentiated tumors which have less than 5% glandular differentiation correspond to Grade 4. Medullary carcinomas with high microsatellite instability are classified as undifferentiated carcinomas. The prognosis correlates with the histological grade[27]. Grade 3 of differentiation is seen in 5.7% to 9.2% of patients with polyps and the risk of residual lesions or relapse in these cases is of the order of 36%-38%[25]. In most cases, grade 3 differentiation is associated with invasive adenocarcinoma cells $\leqslant$ 1 mm from a clearly visualized margin.

Level of invasion of adenocarcinoma into the polyp

Haggitt *et al*[28] assigned anatomic regions (levels) of invasion to each malignant polyp. In this study, level 1 described invasive adenocarcinoma limited to the polyp head; level 2 included neck involvement; level 3 corresponded to adenocarcinoma cells in the stalk; and level 4 to invasion, adenocarcinoma cells infiltrating the submucosa at the level of the adjacent bowel wall (Figure 1). In this system, invasive adenocarcinoma in a sessile polyp by definition had level 4 invasion. However, precise histological evaluation of Haggitt's level may be difficult, especially the differentiation between Haggitt's level 1 *vs* 2, and 2 *vs* 3. Properly marked and orientated specimens are essential. Some authors conclude that only patients with level 4 invasion require resection[29]. More recently, some authors have proposed an additional histological classification system based on the grade of cell differentiation at the lesion margins and on the size and depth of invasion of the submucosa. Accordingly, the degree of submucosal invasion has been classified into three types based on the depth of invasion. When less than one-third of the submucosa is invaded the stage is sm1, and if more than two-thirds is invaded the stage is sm3, while stage sm2 is intermediate with invasion of cancer into the middle third. Sm1 is when the depth of invasion is less $\leqslant$ 1 mm or 1000 μm from the muscularis mucosae[17]. It has been shown that penetration of cancerous cells into the lower third of the

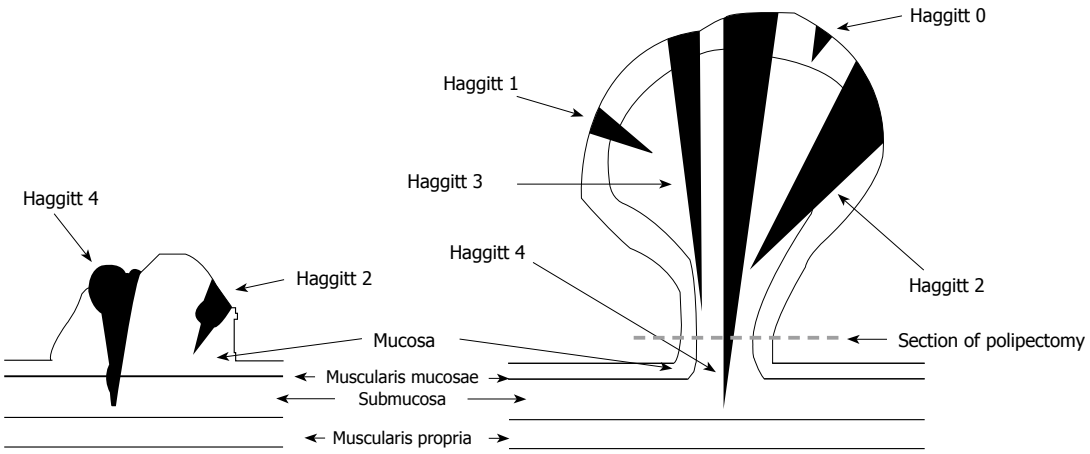

Figure 1 The Haggitt classification. Haggitt 0: Limited to the mucosa without invasion of submucosa; Haggitt 1: Invasion of submucosa but limited to the head of the polyp; Haggitt 2: Invasion extending into the neck of the polyp; Haggitt 3: Invasion into any part of the stalk; Haggitt 4: Invasion beyond the stalk but above the muscularis propria.

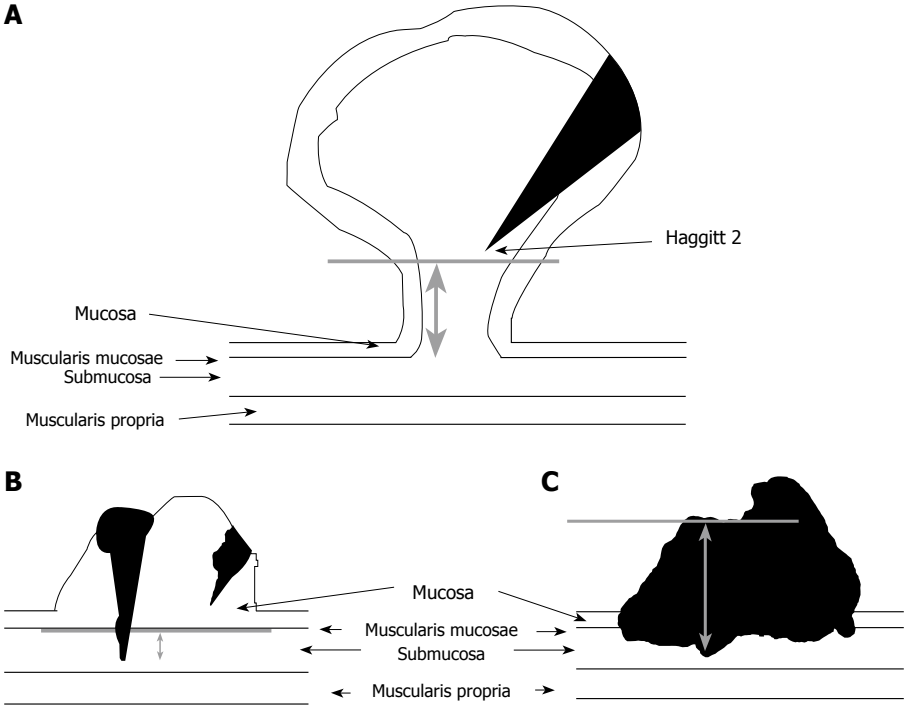

Figure 2 Kitajima's classification. The method used for measurement of submucosal invasion (SM) depth. A: For pedunculated submucosal invasive colorectal carcinoma, level 2 according to Haggitt's classification was used as the baseline (grey line), and SM depth was measured as the vertical distance from this line to the deepest portion of invasion; B: For nonpedunculated, when the muscularis mucosae could be identified in HE stained specimens, the muscularis mucosae was used as baseline (grey line) and the vertical distance from this line to the deepest portion of invasion represented with bidirectional arrow grey; C: For nonpedunculated, when the muscularis mucosae could not be identified due to carcinomatous invasion, the superficial aspect of the invasive carcinoma was used as baseline (grey line), and the vertical distance from this line to the deepest portion of invasion was determined (bidirectional arrow grey).

submucosa (sm3) of sessile lesions is associated with a greater risk of lymphatic spread than only mild penetration[27-32]. Research based on large patient series has shown a 1%-3% risk for lymph node metastases in sm1 cancers, 8% in sm2 cancers and 23% in sm3 cancers[31].

However, two problems exist in measuring submucosal invasion (SM) depth; how to measure SM depth in a lesion whose muscularis mucosae could not be identified, and how to determine SM depth in tumors demonstrating morphological differences. To solve the first problem, when the muscularis mucosae could not be identified due to carcinomatous invasion, Kitajima *et al*[33] (Figure 2) in a Japanese collaborative study defined the superficial aspect of submucosal invasive colorectal carcinoma (pedunculated, nonpedunculated with muscularis mucosae identified and nonpedunculated without muscularis mucosae identified) and measured SM depth from this baseline to the deepest portion of invasion. For pedunculated, submucosal invasive colorectal carcinoma, the level 2 proposed by Haggitt was used as baseline (neck of the adenoma or junction between adenoma and stalk). In these cases the rate of lymph node metastasis was 0% when stalk invasion was < 3 mm or 3000 μm. For nonpedunculated, the muscularis mucosae was used as baseline and the vertical distance from this line to the deepest portion of invasion represented SM depth. In nonpedunculated polyps without muscularis mucosae identified, the superficial aspect was used as baseline and the vertical distance from this line to the deepest portion of invasion was determined. The rate of lymph node metastasis was 0% if SM depth was < 1 mm or 1000 μm for nonpedunculated polyps[33].

Lymphatic invasion

The presence or absence of lymphatic invasion by cancer is defined as tumor cells within a true endothelial-lined channel in the absence of red blood cells[34]. The risk of lymphatic spread from a malignant polyp has been estimated by histological study of resected specimens. Since lymphatics do not penetrate much beyond the muscularis mucosae, focal cancer that has not invaded through this layer appears to present little or no risk of lymph node spread[35]. A plexus of lymphatic channels is normally found in the superficial submucosa and within the muscularis mucosa, with rare extensions into the lamina propria (mucosa) limited to the region at the base of the crypts. The near absence of lymphatics within the mucosa has been proposed as the reason for the observed lack of malignant potential (lymph node metastasis) observed in polyps showing only intramucosal carcinoma. However, this theory has been challenged by studies using more sensitive techniques to detect lymphatic vessels. Studies using the relatively new antibody D2-40 (Dako, Carpenteria, CA, USA), which stains lymphatic but not blood vessel endothelium, have shown that lymphatics undergo proliferation and are present in the stalk and mucosa of adenomas and early invasive cancers. In malignant polyps, lymphatic channels are often present near nests of infiltrating tumors[36,37]. From a practical point of view, detecting lymphatic invasion by expert pathologists using light microscopy is difficult. There are no recognised guidelines for establishing the presence of lymphatic invasion (for example, the number of sections or immunostains needed to identify lymphatic vessels). For example, in a study in which five pathologists assessed the lymphatic invasion of 140 malignant polyps, they agreed (4 out of 5 observers) on only 17 cases. The intra- and inter-observer variability in the interpretation of samples received among even the most expert histopathologists can be high and often leads to diagnostic uncertainties which inevitably results in a more cautious therapeutic approach being taken[38]. True lymphatic invasion is rare, although retraction of tissue creating an artificial space around tumor cells is common in paraffin sections. The use of immunohistochemistry for D2-40 may help identify lymphatic channels. However, its use is not yet routine, and technical issues such as loss of a suspicious focus in level sections limits the usefulness of special stains in this setting. The presence of lymphatic invasion in a malignant polyp has been proposed by some researchers as an indication for colectomy. However, few malignant polyps with lymphatic invasion have been reported, and most of them have had positive margins, Grade 3 invasive adenocarcinoma (as defined above), or both[5]. Approximately 12% to 16% of all polyps have lymphatic invasion, and in these cases, the risk of relapse or residual lesions ranges between 17% and 39%.

Vascular invasion

The presence or absence of venous invasion is defined as cancer in an endothelial-lined channel surrounded by a smooth muscle wall[36]. However, it is difficult to recognise venous invasion. Vascular markers, such as CD31, CD34 and factor Ⅷ may help in assessing vascular invasion. These markers strongly stain blood vessel endothelium, and, to a lesser extent, lymphatic endothelium[14]. The prevalence of venous invasion in malignant polyps varies greatly from one study to another, ranging from 3.5% to 39%[38]. Often venous invasion is associated with lymphatic invasion and/or tumors which have a resection margin of less than 2 mm and/or are poorly differentiated. In contrast to the majority of studies, Talbot *et al*[39] observed that venous invasion was not associated with poorer prognosis.

Favourable and unfavourable histology and the risk of residual disease or recurrent carcinoma

Favourable histology is defined as Grade 1 or 2 differentiated adenocarcinoma in which carcinoma cells are at least 2 mm from a clearly visualised margin, resection is carried out *en bloc* and there is an absence of vascular or lymphatic invasion.

The definition of unfavourable histology is when the distance between the invasive tumor and the cauterized biopsy margin < 2 mm or 2000 μm, there is piecemeal removal or tumor within the cauterized region constitutes a positive margin or a poorly differentiated tumor (Grade 3) or there is lymphatic or vascular invasion. In these cases, surgical resection is indicated because of the increased risk of lymph node metastasis or residual disease[14]. On the other hand, in the absence of unfavourable features, polypectomy is considered curative. Specimens that do not lend themselves to proper analysis for any reason (piecemeal removal or poor orientation) sometimes result in a default decision to resect.

In 1995, Volk *et al*[5] reviewed 20 studies in which 858 malignant polyps were analysed. They observed that in 89 patients (10%), there was residual disease or recurrent carcinoma. However, there were relapses or tumors in the area of the resection in only 8 (1%) patients with favourable histological criteria. Subsequent studies have also reported an incidence of less than 1%[38,40]. Only one study described an incidence higher than 5% in malignant polyps with favourable histology[41] and the study itself has been widely criticised and excluded from subsequent reviews[5]. By contrast, in malignant polyps with unfavourable histology, the risk of relapse or residual lesions ranges between 10% and 39%[5,14,31,40].

TREATMENT

Prior to removal of the polyp, it is difficult to know whether the polyp is malignant or not. Some features, as we have mentioned earlier, can give some indication of the degree of malignancy (Figure 3). In these cases, it is advisable to perform tattooing in order to mark the base of the resected polyp. Tattooing helps the surgeon or endoscopist to locate the base of the polyp. Regardless of the morphological characteristics, a polyp is normally removed when detected. Polypectomy should be per-

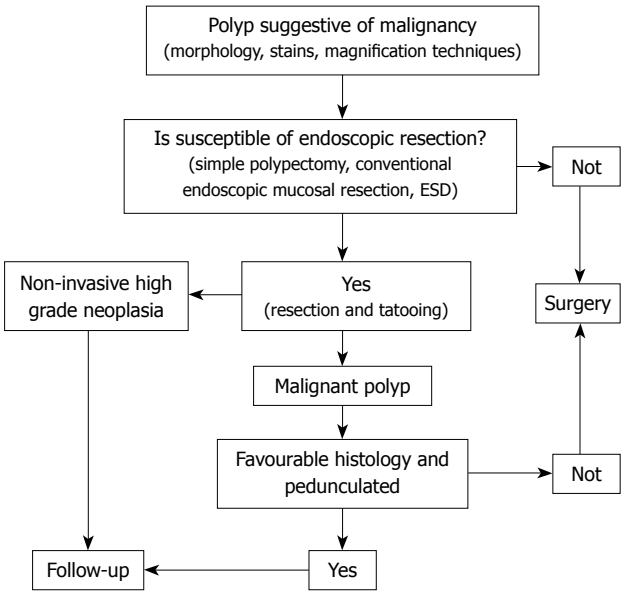

Figure 3 Diagnostic and therapeutic algorithm of malignant polyps.

formed *en bloc*, since this is essential to establish and define favourable or unfavourable histological criteria. In just a few cases, only polyp biopsies are performed. This may be due to a lack of coagulation data, the polyp being difficult to remove at that point in time, or the patient being on antiplatelet drugs or anticoagulants.

NHGN regardless of their morphology, are considered to be cured with polypectomy[42].

The indication for a malignant polyp with sessile morphology, regardless of favourable histological criteria, is surgery[1], especially in patients younger than 50 years old, who tend to present fewer surgical complications[39]. ESD has emerged as a possible technique to successfully resect malignant colonic polyps *en bloc*[24,43]. This approach is indicated for polyps larger than 2-3 cm, involving more than one-third of the colon circumference or two haustral folds, or with a flat/depressed morphology. The technique makes it possible to treat large (> 2 cm) sessile and flat polyps enabling pathological evaluation and cure in most patients. ESD is better than conventional endoscopic mucosal resection because it has higher *en bloc* resection and curative rates despite the longer procedure time and higher perforation associated with ESD[44]. Also, ESD can be an alternative to surgery for older patients and for those suffering from associated conditions that contraindicate surgery. In addition, this type of resection should be considered for malignant polyps with sessile morphology, and hence, with surgical indication, regardless of histological criteria.

Surgical treatment is also recommended for malignant polyps with pedunculated morphology which have unfavourable histological criteria (partial polyp resection, poorly differentiated carcinoma, vascular or lymphatic invasion, margin of resection < 2 mm or depth of submucosal invasion is ⩾ 3 mm from muscularis mucosae[1,33]. On the other hand, for malignant polyps with pedunculated morphology but with favourable histological criteria, polypectomy is considered to be curative.

However, until now many pathology reports did not report histological criteria. For example, at the University of Minnesota between 1987 and 2000, in 83% of patients angiolymphatic vessel invasion was not reported, in 69% the depth of invasion by cancer cells was not reported and in 22% the degree of tumor differentiation was not stated[45]. Besides the agreement among experienced pathologists was poor with respect to histological grade of differentiated carcinoma and angiolymphatic vessel invasion[45].

In recent years, various serum markers have been identified in an effort to establish which patients could benefit from surgical treatment and from a stricter follow up. These markers include metalloproteinase 7[46], vascular adhesion proteins[47], vascular endothelial growth factors[48] and cytokeratins[49]. The majority of markers have been studied in patients operated on for colon cancer with infiltration of the muscularis propria (equivalent to or higher than T2), so these results cannot readily be extrapolated to malignant colorectal polyps.

An exception to these guidelines is patients with malignant polyps, with sessile or flat morphology, that are located in the rectum. The occurence of distant metastases is correlated to T-stage and, after radical resection of T1 tumors, the 5-year rate of metastases is about 10%[50], similar to malignant polyps in other locations. An adequate preoperative evaluation of the patient's general health is essential before deciding the modality of treatment for the individual T1 rectum cancer patient. About 50% of local recurrences following local resection are curable if the patients are included in an intensive follow-up programme. T1 rectal cancer should be classified into at least two subgroups (low and high risk). The most widely accepted criteria for classifying low-risk T1 cancer are tumor diameter < 3 cm or tumor infiltration no deeper than the middle layer of the submucosa. Some authors differ as to whether T1 sm2 cancer should be classified as low-risk or high-risk cancer. Between the low risk criteria are tumor diameter < 3 cm, high or intermediate differentiation, infiltration no deeper than the middle layer of the submucosa (sm1 or sm2) and no sign of lymphovascular infiltration[51]. Low risk T1 cancers should be treated with local resection (ESD or conventional endoscopic submucosal resection or transanal endoscopic microsurgery). Also, local resection of high-risk T1 cancers may offer the best treatment for older patients (> 80 years) and for patients with comorbidity[51].

Transanal endoscopic microsurgery (TEM) is a minimally invasive surgical technique that allows the surgeon to operate on problems in the mid and upper rectum without having to make an incision through the abdomen. TEM is a safe technique with few complications. The local recurrence rate varies widely, less than 10% in patients with low-risk. The ideal patient for local excision with TEM therefore has a small T1 cancer with low-risk, located between 5 and 10 cm from the anal verge (5-15 cm up on the posterior rectal wall)[51].

FOLLOW-UP

In cases of NHGN and malignant polyp with pedunculated morphology and favourable histological criteria, it is recommended that a colonoscopy be carried out three months after taking the biopsy[1,52]. If this is normal, a further check up is advised after one year, three years and five years[48]. Some authors suggest that if the results within three months are negative, subsequent monitoring should be the same as that offered to patients with non-malignant adenomas, since there is no evidence that such patients are at a higher risk of metachronous polyps or cancers than those patients with benign adenomas[42,53]. However, recent studies estimate that 11.8% of patients who have undergone polypectomy will develop a metachronic advanced adenoma and 0.6% an invasive carcinoma. Associated risk factors include age, number of polyps (5 or more), size (greater than 1 cm), villous architecture, proximal location, and being male. The odds ratio increases progressively according to the number of adenomas, it being 1.39 for those who have had 2 adenomas and 3.87 for those who have had 5 or more. Smoking, body mass index, family history of CRC, and degree of dysplasia were not found to be associated with higher risks of advanced adenoma or cancer[54].

There have been reports of cases of malignant pedunculated polyps with unfavourable histological criteria which, despite no findings of residual carcinoma in the intestine wall or lymph node involvement, are found on follow up to have distant metastasis, even five years after surgery[4,5]. These data force us to consider the monitoring of such patients using serum levels of carcinoembryonic antigen and imaging techniques such as computerised tomography which would enable early detection of recurrence.

Published series of malignant colorectal polyps usually include fewer than 100 cases, and most of these are retrospective studies. Given this, it would be interesting for new prospective studies to be carried out to evaluate the progress of such patients and to establish the most suitable treatment and follow up regimens for them.

CONCLUSION

The prevalence of malignant polyps in series of endoscopically removed polyps is between 0.2% and 11%. Currently the number of cases in which malignant polyps are removed is increasing due to screening programmes. Carcinoma "*in situ*", intramucosal carcinoma, high-grade dysplasia or intraepithelial carcinoma is the stage at which there is no involvement of the muscularis mucosa. These terms are defined as non-invasive high grade neoplasia in the Vienna classification. When the carcinoma spreads to the submucosa, the polyp is considered to have become malignant, being able to spread to lymph nodes or distant sites. This term is defined as submucosal carcinoma in the classification of Vienna. The definition of unfavourable histology is when the distance between the invasive tumor and the cauterized biopsy margin < 2 mm, there is piecemeal removal or tumor within the cauterized region constitutes a positive margin or a poorly differentiated tumor (grade 3) or there is lymphatic or vascular invasion. In these cases, surgical resection is indicated because of the increased risk of lymph node metastasis or residual disease. Also, the indication for a malignant polyp with sessile morphology, regardless of favourable histological criteria, is surgery. In cases of non-invasive high grade neoplasia and submucosal carcinoma with pedunculated morphology and favourable histological criteria, it is recommended that a colonoscopy be carried out three months after taking the biopsy and if this is normal, a further check up is advised after one year, three years and five years.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3112

World J Gastroenterol 2010 July 7; 16(25): 3112-3119
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Kostas Pantopoulos, Associate Professor, *Series Editor*

Heme oxygenase-1 as a therapeutic target in inflammatory disorders of the gastrointestinal tract

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Received: February 22, 2010 Revised: April 1, 2010
Accepted: April 8, 2010
Published online: July 7, 2010

Abstract

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation. HO-1 not only protects against oxidative stress and apoptosis, but has received a great deal of attention in recent years because of its potent anti-inflammatory functions. Studies with HO-1 knockout animal models have led to major advances in the understanding of how HO-1 might regulate inflammatory immune responses, although little is known on the underlying mechanisms. Due to its beneficial effects the targeted induction of this enzyme is considered to have major therapeutic potential for the treatment of inflammatory disorders. This review discusses current knowledge on the mechanisms that mediate anti-inflammatory protection by HO-1. More specifically, the article deals with the role of HO-1 in the pathophysiology of viral hepatitis, inflammatory bowel disease, and pancreatitis. The effects of specific HO-1 modulation as a potential therapeutic strategy in experimental cell culture and animal models of these gastrointestinal disorders are summarized. In conclusion, targeted regulation of HO-1 holds major promise for future clinical interventions in inflammatory diseases of the gastrointestinal tract.

Key words: Antioxidant; Heme oxygenase; Hepatitis; Immunity; Inflammation; Inflammatory bowel disease; Oxidative stress; Pancreatitis

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Vijayan V, Mueller S, Baumgart-Vogt E, Immenschuh S. Heme oxygenase-1 as a therapeutic target in inflammatory disorders of the gastrointestinal tract. *World J Gastroenterol* 2010; 16(25): 3112-3119 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3112.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3112

INTRODUCTION

Heme oxygenase (HO), which was initially described more than 40 years ago, enzymatically degrades heme and produces equimolar amounts of carbon monoxide (CO), biliverdin, and iron[1] (Figure 1). In a coupled reaction, biliverdin is converted into bilirubin (BR) *via* biliverdin reductase[2]. Two distinct isozymes of HO, HO-1 and HO-2, have been identified and represent the products of two different genes with distinct tissue- and cell-specific expression patterns[3-5]. The constitutive isoform HO-2 is preferentially expressed in brain and testis[6], and is essentially not regulated by metabolic or receptor-mediated

stimuli[4,7]. In contrast, the inducible HO isozyme, HO-1, which exhibits low basal expression levels in most cells and tissues, is markedly upregulated not only by its substrate heme, but also by other oxidative stress stimuli, such as UV-light and lipopolysaccharide (LPS) or directly by reactive oxygen species, such as hydrogen peroxide. In addition, heavy metals, sulfhydryl-reactive reagents, and hypoxia are potent inducers of HO-1[8-10]. Thus, although HO-1 does not directly catalyze an antioxidant reaction, its induction is generally considered an adaptive cytoprotective response against the toxicity of oxidative stress[11-13]. In the current review, we focus our attention on recent findings that show the emerging anti-inflammatory and immunomodulatory role of HO-1 and its products. In particular, we highlight recent advances in the understanding how HO-1 might modulate the inflammatory immune response and the potential role of HO-1 for therapeutic applications in inflammatory conditions of various organs in the gastrointestinal tract.

HO-1 GENE DEFICIENCY CAUSES PROINFLAMMATORY PHENOTYPICAL ALTERATIONS

A potential link between HO-1 and inflammatory disease has been shown by Willis *et al*[14] in an animal model of carragenin-induced pleurisy, in which specific upregulation of HO enzyme activity attenuated a complement-dependent inflammation. Mice that are deficient for HO-1 not only develop chronic inflammation, but are also highly vulnerable to experimental sepsis induced by the classical proinflammatory mediator endotoxin[15]. In addition, innate and adaptive immune reactions are severely affected in these knockout mice[16-18]. In contrast, HO-2 knockout mice appear to have an intact immune regulation, but exhibit defects in their central and autonomous nervous system[19]. The phenotype of the only known human case of genetic HO-1 deficiency is very similar to that observed in HO-1 knockout mice. This HO-1 deficient patient, a Japanese boy who died at the age of six years, was initially diagnosed with anemia and a chronic inflammatory disorder[20,21].

ANTI-INFLAMMATORY EFFECTS OF HO-1 IN MONONUCLEAR PHAGOCYTES AND ENDOTHELIAL CELLS

Although HO-1 is expressed in all tissues and cells, the immunomodulatory functions of HO-1 appear to be primarily dependent on HO-1 functions in mononuclear phagocytes and endothelial cells. In the following, we describe pertinent findings that illustrate the anti-inflammatory role of HO-1 in these two cell types.

Mononuclear phagocytes

Mononuclear phagocytes, such as monocytes and macrophages, are cells with a common bone marrow lineage and have versatile functions in the innate and adaptive immune system[22,23]. As an example, macrophages ingest and kill invading microorganisms as a first line of defence and are activated by various immunological stimuli, such as microbial products or cytokines. In response to these stimuli, macrophages are able to initiate and enhance the inflammatory immune response[23]. It has been known for many years that tissue macrophages, such as Kupffer cells and spleen macrophages, are cell types in which HO-1 is highly expressed under normal conditions[24,25]. Several studies have been performed with cultured mononuclear cells from rodents. Here, HO-1 is upregulated in response to LPS[25-27], which attenuates the expression of various proinflammatory genes, such as cyclooxygenase-2, tumor necrosis factor-α, interleukin (IL)-1, and IL-6[18,28-31]. The cell-specific antiinflammatory function of HO-1 in mononuclear phagocytes was recently confirmed in a conditional HO-1 knockout mouse model. The cell-specific lack of HO-1 in mononuclear cells caused a major defect of interferon-β expression. In addition, a pathological immune response in an experimental infection with Sendai virus and in autoimmune encephalomyelitis in these animals was observed[32]. Interestingly, others have shown that HO-1 is also important for the appropriate function of human and mouse dendritic cells. It has been shown that pharmacological induction of HO-1 attenuates maturation and cell-specific functions of dendritic cells[33,34]. Moreover, HO-1 expression in mononuclear cells has been demonstrated to be essential for the functionality of regulatory T cells[17].

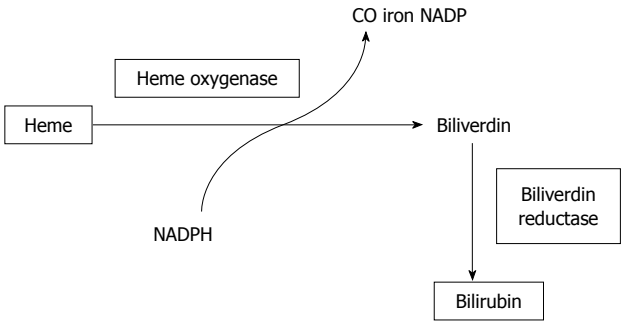

Figure 1 The heme oxygenase enzyme reaction. Heme is enzymatically degraded to yield carbon monoxide (CO), iron, and biliverdin, which is converted into bilirubin in a coupled reaction.

Endothelial cells

The endothelium plays a central role in the regulation of inflammatory reactions, because it serves as a barrier between the peripheral blood stream and inflamed tissues. More specifically, endothelial monolayers regulate the recruitment and transmigration of immunologically active blood cells, such as polymorphonuclear leukocytes (neutrophils) or T lymphocytes, to the site of an inflammation[28,35,36]. Hayashi and colleagues[37] have reported that HO-1 regulates cell-cell interactions between polymorphonuclear leukocytes and endothelial cells. These authors showed in an *in vivo* rat model that the increased

enzyme activity of HO in the endothelium of microvessels downregulated the adhesion of leukocytes during experimental oxidative stress[37]. Accordingly, others have demonstrated that the activity of endothelial HO-1 specifically modulates leukocyte recruitment into organs with an experimental inflammation[38]. Independently, in a streptozocin-induced rat model of experimental diabetes, overexpression of HO-1 has been shown to attenuate oxidative stress-dependent endothelial cell damage[39]. Similar findings in the endothelium have recently been reported for knockout mice, in which genetic deficiency of HO-1 caused major pathological alterations of the endothelial monolayer[40]. Specifically, endothelial cells from HO-1 deficient mice were shown to be more susceptible to apoptosis and denudation from the extracellular matrix. Moreover, independent groups have shown that antiinflammatory endothelial protection of HO-1 might be mediated *via* its ability to downregulate the tumor necrosis factor-α-induced expression of various adhesion molecules[41-43]. It is also remarkable that histopathological studies in the autopsy of a human patient with genetic HO-1 deficiency revealed major endothelial cell damage[21].

HOW DOES HO-1 MEDIATE ITS ANTIINFLAMMATORY FUNCTIONS

The mechanisms of how HO-1 may counteract inflammatory reactions are not understood in detail. An accumulating body of evidence, however, indicates that the HO substrate heme is a compound with major proinflammatory properties and that the HO products BR and CO have potent antiinflammatory functions.

Heme as a proinflammatory compound

The tetrapyrrole heme has contradictory biological properties. On the one hand, heme is important for oxygen and mitochondrial electron transport as the prosthetic group of various hemoproteins such as hemoglobin, myoglobin, and cytochromes[44,45]. On the other hand, heme can be toxic as it can cause oxidative stress in its "free" non-protein bound form. The prooxidant properties of heme have been shown in various animal and cell culture models[46,47]. Due to the critical role of intracellular heme levels, enzymatic synthesis and degradation of this compound is tightly controlled[48-50]. More recently, heme has also been recognized to exhibit potent proinflammatory properties[18,51]. As an example, Jeney *et al*[52] have demonstrated that heme-dependent oxidation of low-density lipoproteins is involved in inflammation-mediated tissue damage. In this report, the proinflammatory effects of heme have also been shown to be correlated with specific clinical conditions, such as atherosclerosis and hemolytic anemia[52]. Independently, others have demonstrated that intravenous administration of heme caused experimental inflammation *in vivo* with a major influx of leukocytes[38]. Heme-dependent toxicity has also been associated with its proinflammatory effects in an animal model of experimental cerebral malaria, in which heme-dependent detrimental effects were markedly more pronounced in HO-1 knockout mice[53]. Recent findings might shed light on the mechanisms that could be involved in the proinflammatory effects of heme. Figueiredo and colleagues have demonstrated that initiation of heme-dependent inflammation was mediated *via* specific interaction of heme with the central pattern recognition receptor toll-like receptor-4, in a cell culture model of mononuclear cells and in mice[54]. The role of HO-1 in maintaining intracellular heme homeostasis has been reviewed elsewhere[18,47,48,51].

Bilirubin

The role of bilirubin (BR) as a beneficial compound with potent antioxidant and antiinflammatory effects has only been appreciated in recent years[55,56]. Protection against an experimental inflammation has been shown for HO-derived BR in an animal model[37]. Moreover, it has been observed in a murine asthma model that BR specifically reduces leukocyte transmigration to the site of an experimental inflammation *via* interaction with the adhesion molecule vascular cell adhesion molecule-1[57]. More recently, it has been reported in a mouse model of sepsis that a single bolus of BR markedly blocked the toxicity of endotoxin[58]. Epidemiological studies have indicated that moderately increased concentrations of serum BR (e.g. in Gilbert's disease) are associated with a lower risk of developing cardiovascular disease[59,60] and colorectal carcinoma[61]. In conclusion, the generation of BR by HO-1 might, in part, explain the antiinflammatory effects of this enzyme.

CO

Although CO is generally considered a toxic gas, various physiological functions of CO as a major signaling molecule have been recognized in recent years[3,62,63]. In particular, HO-1-derived CO has been shown to be involved in the regulation of apoptosis, neurotransmission, coagulation, vasodilation, and inflammation. In a pioneering report on the potential protective effects of this gas, administration of exogenous CO has been shown to block the LPS-induced production of proinflammatory cytokines *via* modulation of p38 MAP kinase[29]. Similar to the signaling gas NO, CO upregulates the production of cGMP *via* activation of soluble guanyl cyclase. This mechanism has also been implicated in other functions of CO, such as vasodilation and blockage of smooth muscle cell proliferation. A remarkable development with major potential for future therapeutic applications has been the introduction of CO-releasing molecules (CORMs). CORMs are compounds that can be administered intravenously and are intended to deliver CO to its target site without the toxicity of gaseous CO[64]. Further details on CO and CORMs are given in specific reviews[63,65,66].

HO-derived iron

Iron, as the third product of HO, is an essential com-

Table 1 HO-1 and inflammatory disorders of the gastrointestinal tract

Disorder	Mechanism of protection by HO-1	Experimental model	Ref.
Viral hepatitis (HBV, HCV)	HBV: repression of HBV replication	*In vivo*: transgenic mice; *in vitro*: human HepG2 hepatoma cells	[74]
	HCV: repression of HCV replication	*In vitro*: human Huh-7 hepatoma cells	[78-80]
		In vitro: human Huh-5-15 hepatoma cells	[81]
Inflammatory bowel disease	HO-1-derived biliverdin inhibits inflammatory activity	*In vivo*: colitis in dextran sodium sulfate-treated mice	[89]
	Inhibition of IRF8 activation	*In vivo*: colitis in IL-10-/- mice	[90]
	HO-1 mediates protection of 5-aminosalicylic acid	*In vivo*: colitis in trinitrobenzene sulfonic acid-treated rats	[91]
	Inhibition of NF-κB activation	*In vivo*: colitis in trinitrobenzene sulfonic acid-treated mice	[92]
	Inhibition of interleukin-17	*In vivo*: colitis in dextran sodium sulfate-treated mice	[93]
Pancreatitis	Homing of hemin-treated macrophages in pancreas before onset of inflammation	*In vivo*: acute pancreatitis in choline-deficient diet-fed mice	[101]
	Decreased expression of proinflammatory cytokines	*In vivo*: acute pancreatitis after allograft transplantation in rat	[102]
	Inhibition of cell proliferation *via* repression of ERK activity	*In vitro*: platelet-derived growth-factor-treated rat pancreatic stellate cells	[105]

HO: Heme oxygenase; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

pound for redox-dependent enzyme reactions and bioenergetics. Iron, however, might cause the formation of toxic reactive oxygen species, if not appropriately contained by specific intracellular protective mechanisms. A key role in the protection against the toxicity of HO-1-derived iron is played by the intracellular iron storage protein ferritin[67,68]. This protection has been demonstrated in various cell culture models, in which the synthesis of HO-1 and ferritin was coordinately upregulated and prevented iron-mediated cell toxicity[12,47,69]. Remarkably, genetic deficiency of HO-1 in mice[70] and humans[20,21] is associated with a major pathological iron overload in the liver and kidney.

In summary, the HO products BR and CO have anti-inflammatory potential, which could be relevant for therapeutic interventions.

TARGETING HO-1 IN INFLAMMATORY DISORDERS OF THE GASTROINTESTINAL TRACT

HO-1 and chronic viral hepatitis

Chronic viral hepatitis mainly consists of viral hepatitis B and C, and is a major cause of liver cirrhosis and end-stage liver disease worldwide. These disorders are characterized by chronic self-perpetuating inflammation of the liver for at least six months. For example, chronic hepatitis C virus (HCV) infection affects 4 million US citizens, a third of whom will progress to liver cirrhosis and primary hepatocellular carcinoma if left untreated[71-73]. Treatment with interferon and ribavirin is effective in only 50% of patients, because many patients either have contraindications to these therapies or have failed to respond to this treatment, indicating a need for alternative or supplementary therapeutic strategies. In the following, we will focus on links between HO-1 and chronic viral hepatitis and their potential therapeutic use. Research on hepatitis B virus (HBV) and HCV infections has been prevented by a lack of appropriate animal and cell culture models to study these infections[73].

As regards HO-1 and HBV infection, Protzer and colleagues have reported that HO-1 has antiviral activity in a transgenic mouse model of chronic HBV infection. In this animal model, specific induction of HO-1 by cobalt-protoporphyrin-IX significantly reduced the levels of the viral HBV core protein. Moreover, these authors have shown that the antiviral effects of HO-1 are mediated *via* reduction of HBV core protein stability in cell cultures of stably transfected hepatoma cells[74] (Table 1).

Several reports suggest that HO-1 might serve as a specific therapeutic target for the treatment of chronic HCV infection, although the results are somewhat contradictory. On the one hand, Schmidt and colleagues have presented evidence that HCV specifically downregulates gene expression of HO-1, but not that of other antioxidant genes, such as manganese superoxide dismutase and catalase[75]. In cell cultures of hepatoma cells, inhibition of HO-1 by HCV appeared to be specifically regulated *via* the HCV-core protein[75,76]. On the other hand, it has been demonstrated that HCV leads to an increased expression of HO-1 in hepatoma cell lines, possibly *via* interactions with the HO-1 repressor, Bach1[77]. Clearly, further studies are necessary to reconcile these conflicting results.

In contrast, findings from independent reports have indicated that targeted overexpression of HO-1 had antiviral effects on HCV replication (Table 1). The group of Bonkovsky has demonstrated that upregulation of HO-1 either by a pharmacological approach or, more recently, by a microRNA-based strategy repressed HCV replication in human Huh-7 hepatoma cells[78,79]. Accordingly, Zhu *et al*[80] have shown that targeted overexpression of HO-1 led to a significant inhibition of HCV replication without affecting other parameters of cell viability in human hepatoma cells, which stably replicate subgenomic selectable HCV RNAs[80]. These studies have recently been extended by others, who have shown that the HO-1 product biliverdin interfered with HCV replication *via* direct modulation of the antiviral interferon-α response in two human hepatoma replicon cell lines[81] (Table 1).

Due to the limited success rate of current therapies for chronic viral hepatitis, it is conceivable that targeted modulation of HO-1 might be an innovative comple-

mentary strategy for the treatment of these infectious diseases.

HO-1 and inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders, which is primarily represented by ulcerative colitis and Crohn's disease. The etiology of IBD is not well understood and a complex interplay of genetic, immunological and environmental factors appears to play a role in the initiation and perpetuation of IBD[82]. The onset of IBD is characterized by an autoimmune inflammatory reaction that causes excessive production of pro-inflammatory cytokines and reactive oxygen species, which in turn damage the intestinal mucosa[83]. A number of experimental animal models of IBD have been established, in which IBD develops either spontaneously, such as in various knockout mouse models, or after treatment with specific chemical compounds, such as trinitrobenzene sulfonic acid[84]. These models are widely used to investigate potential therapeutic strategies for the treatment of IBD. HO-1 regulation has been studied in a variety of these animal models and in biopsies from human IBD patients.

In a trinitrobenzene sulfonic acid-induced model of experimental colitis, it has been demonstrated that HO-1 is prominently upregulated in various cell types of the inflamed colon[85]. These findings correspond with observations from an independent study, in which increased HO-1 expression has been observed in mucosa biopsies of a murine colitis model. Importantly, in this study, HO-1 expression has also been shown to be increased in inflamed mucosa of human IBD patients[86]. Similar findings have been reported by Takagi *et al*[87] for a Japanese population. Indirect evidence that HO-1 might play a role in the pathogenesis of IBD has been demonstrated in knockout mice for the Nrf2 gene, which is the major transcriptional regulator of HO-1. Nrf2-deficient mice were more susceptible to develop an experimental colitis in response to dextran sodium sulphate when compared with their wild-type counterparts[88].

As regards the potential therapeutic role of HO-1 in IBD, important insights have been presented in independent reports on HO products (Table 1). In a dextran sodium sulphate-induced colitis model, HO-1-dependent protection against inflammation has been attributed to the beneficial effects of biliverdin[89]. By contrast, others have indicated that CO might provide anti-inflammatory protection in mice with genetic IL-10 deficiency, which develop a chronic colitis-like disease. In these mice it is also shown that the immunosuppressive effects of CO were recapitulated by pharmacological induction of HO-1[90]. Interestingly, upregulation of HO-1 might also play a role in the antiinflammatory protective effects of some established current standard therapies of IBD. As an example, Horváth *et al*[91] have shown that 5-amino salicylic acid (5-ASA), which is one of the pharmacological standard therapies of IBD, might, at least in part, mediate its antiinflammatory effects *via* upregulation of HO-1 in a trinitrobenzene sulfonic acid-induced rat colitis model. In addition, others have demonstrated that the fungal metabolite gliotoxin and the HO-1 substrate heme mediate antiinflammatory effects in independent experimental colitis models *via* specific induction of HO-1, respectively[92,93] (Table 1).

Finally, it is important to point out that targeted HO-1 induction does not provide anti-inflammatory protection in an experimental animal model of dextran sulphate sodium-induced colitis, when HO-1 was induced after the onset of IBD[86]. Therefore, it seems questionable whether HO-1 induction is useful for treatment of established IBD, but rather might be useful as a preventive measure.

HO-1 and pancreatitis

Pancreatitis is an inflammatory disorder that is clinically categorized into acute and chronic pancreatitis[94]. The clinical stages of pancreatitis range from a transient self-limiting inflammatory reaction to a fulminant disease with necrotic lesions. Severe acute pancreatitis, which might have multiple local and systemic complications, is associated with high mortality[87]. In general, acute pancreatitis is caused by alcohol abuse and gallstones. More recently, however, genetics and obesity have been identified as independent risk factors. The pathogenesis of pancreatitis is not well understood, but it is known that proinflammatory pancreas-independent factors, such as exposure to endotoxin, can trigger the onset of the disease. Oxidative stress has repeatedly been implicated in the pathogenesis of pancreatitis[95,96]. Although the exact role of oxidative stress during the course of the disease is not well understood, antioxidant enzymes and vitamins have been shown to improve the clinical consequences of pancreatitis[97]. Other therapeutic strategies for the treatment of pancreatitis include inactivation of pancreatic enzymes and blockage of platelet factor activating receptor[94,97,98].

HO-1 is upregulated in animal models of experimental pancreatitis in a cell-specific manner[99,100]. In particular, it has been demonstrated that HO-1 gene expression in the inflamed pancreas is primarily upregulated in peripheral macrophages, which migrate into areas of inflammation[101] (Table 1). In the latter report, the authors have also asked whether HO-1 induction might protect against pancreatitis. The major finding of this study, in which experimental pancreatitis was induced with a choline-deficient diet, indicated that administration of heme decreased pancreatitis-associated lethality in mice. Moreover, administration of heme increased the pancreatic tissue secretion of chemokines, which was responsible for the infiltration of HO-1 expressing peritoneal macrophages into the pancreas. It is important to note, however, that heme-dependent HO-1 induction after the onset of pancreatitis failed to reduce the severity of the disease. This finding is similar to what has been observed in IBD, as mentioned above. Thus, although HO-1 induction might not be useful for the treatment of established pancreatitis, it might help to prevent the onset of pancreatitis in a clinical setting, in which pancreatitis is likely to develop. In a model of pancreas transplantation, in which pancreatitis develops as a consequence

of ischemia-reperfusion injury, pharmacological induction of HO-1 has been shown to be protective against pancreatitis[102] (Table 1). Similar observations have also been reported in an independent study, in which pretreatment with cobalt-protoporphyrin-IX prevented the microcirculatory dysfunction after pancreas ischemia-reperfusion injury in rat[103]. More recently, the CO donor CORM-2 has been shown to protect against acute pancreatitis in rats[104].

Finally, specific induction of HO-1 might also be applicable for treatment of chronic pancreatitis. In a recent report, Schwer *et al*[105] have shown that HO-1 induction by curcumin inhibited pancreatic stellate cell proliferation, which plays a major role in the pathogenesis of pancreatic fibrosis in chronic pancreatitis. This protection was abolished by blockage of HO activity, either by an enzyme inhibitor or by knockdown of HO-1 with a specific siRNA (Table 1).

CONCLUSION

Targeted overexpression of HO-1 has major potential for the treatment of inflammatory disorders of the gastrointestinal tract. Current knowledge on the applications of HO-1 as a therapeutic target, however, still seems precocious and critical questions remain to be answered before clinical interventions might be available.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3120

World J Gastroenterol 2010 July 7; 16(25): 3120-3132
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Comprehensive and innovative techniques for liver transplantation in rats: A surgical guide

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Supported by The work at the Mayo Clinic, Florida was supported by grants to Nguyen JH from the Deason Foundation, Sandra and Eugene Davenport, Mayo Clinic CD CRT-II, and NIH R01NS051646-01A2; the work in Kyoto University Graduate School of Medicine was supported by grants of the Japan Society for the Promotion of Science, No. C20591523 and the Uehara Memorial Foundation, No. 200940051, Tokyo, 171-0033, Japan

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Received: March 6, 2010 **Revised:** April 6, 2010
Accepted: April 13, 2010
Published online: July 7, 2010

Abstract

AIM: To investigate our learning curves of orthotopic liver transplantation (OLT) in rats and the most important factor for successful surgery.

METHODS: We describe the surgical procedures for our rat OLT model, and determined the operator learning curves. The various factors that contributed to successful surgery were determined. The most important surgical factors were evaluated between successful and unsuccessful surgeries.

RESULTS: Learning curve data indicated that 50 cases were required for operator training to start a study. Operative time, blood loss, warm ischemic time, anhepatic phase, unstable systemic hemodynamic state, and body temperature after surgery significantly affected surgery success by univariate analysis, while the anhepatic phase was the most critical factor for success by multivariate analysis.

CONCLUSION: OLT in rats is the only liver transplantation model that provides clinically relevant and reliable results. Shortened anhepatic phase is key to success in this model.

Key words: Animal model; Liver transplantation; Microsurgery; Rat; Reperfusion injury; Ultra-microsurgery

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Hori T, Nguyen JH, Zhao X, Ogura Y, Hata T, Yagi S, Chen F, Baine AMT, Ohashi N, Eckman CB, Herdt AR, Egawa H, Takada Y, Oike F, Sakamoto S, Kasahara M, Ogawa K, Hata K, Iida T, Yonekawa Y, Sibulesky L, Kuribayashi K, Kato T, Saito K, Wang L, Torii M, Sahara N, Kamo N, Sahara T, Yasutomi M, Uemoto S. Comprehensive and innovative techniques for liver transplantation in rats: A surgical guide. *World J Gastroenterol* 2010; 16(25): 3120-3132 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3120.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3120

INTRODUCTION

Orthotopic liver transplantation (OLT) has become the treatment of choice for end-stage liver disease. Split OLT shows great promise for narrowing the gap between the number of patients waiting for an OLT and the number of available deceased organ donors. Living-donor OLT also addresses the severe problem of donor shortage and should ensure donor safety. However, further investigation into small-for-size grafts and small-for-size graft syndrome associated with split and living-donor OLT is needed prior to full acceptance in the clinical practice of OLT. Murine organ transplantation models, such as cardiac, lung, and kidney grafts, have been reported[1-5] and are commonly used by transplant immunity investigators. However, OLT in mice is technically very difficult, even without reconstruction of the hepatic artery (HA). Furthermore, a validated model of murine OLT is unavailable. In contrast, OLT in rats is technically accessible, producing more clinically relevant and reliable data. Hence, a comprehensive model of OLT in rats is particularly useful.

OLT in rats was first reported in 1973 using hand-suture techniques[6], while a modified model without HA reconstruction and temporal shunt of the porto-jugular veno-venous bypass was documented in 1975[7]. However, these models were not widely used due to the prolonged surgery time and technical demand. With the cuff method being introduced in 1973[8], OLT in rats without HA reconstruction became globally accepted. The pros and cons of each model were recently reported[9-23], and a combination of hand-suture and cuff methods are deemed to be key factors for successful OLT[24].

The development of clinically relevant OLT models in rats[9-23] has advanced clinical knowledge in liver transplantation[24-26]. Therefore, we will describe detailed surgical procedures of innovative OLT in rats based on two decades of experience at our centers. We will present the learning curves and important factors associated with successful OLT in rats.

MATERIALS AND METHODS

Materials

Animals: Lewis rats (RT-1[l], 8-10 wk) were used as recipients. As donors, Lewis rats were used for syngeneic grafts. As a large vessel diameter is necessary for the anastomosis, male rats of 230-250 g body weight were most suitable. Rats > 300 g were avoided because of the large amount of intra-abdominal fat, making the surgical procedures more difficult.

Adequate hydration, achieved with solution injections, is important for successful surgery, particularly prior to the anhepatic phase and after allograft recirculation[27]. We used male rats because the penile vein was easily accessible for repeated intravenous injections.

The use of animals was institutionally approved in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

General instruments: Cotton swabs with optimal stiffness (cotton tipped applicators; Hardwood Products Company, Guilford, ME, USA) and soft clay (Color Mounting Clay; Hampton Research, Aliso Viejo, CA, USA) were prepared. A cylindrical warmer (Mantello, Ambulatory Surgical Warmer, medium; Kent Scientific Co., Torrington, CT, USA) was used immediately after surgery.

General anesthesia was performed with a rodent anesthesia machine (VetEquip Inc., Pleasanton, CA, USA), including an evacuation canister and induction chamber.

Agents and solutions: Chemical agents used were: heparin (heparin lithium salt, 100 unit/mg; MP Biomedicals, Cleveland, OH, USA); Cephalexin hydrate (MP Biomedicals); buprenorphine 300 μg/mL (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA); bicarbonate, 8.4% Sodium Bicarbonate Injection USP, 1 mEq/mL, 84 mg/mL (Hospira Inc., Lake Forest, IL, USA), and microfibrillar collagen, Avitene (C. R. Bard, Inc., Murray Hill, NJ, USA). The solutions used were: Lactated Ringer's Injection USP and Ringer's Injection USP (B. Braun Medical Inc., Irvine, CA, USA); 0.9% Sodium Chloride Injection USP (Hospira Inc.).

Surgical instruments: Basic instruments required for small animal surgery were similar to those described in previous reports[27,28]. For the anastomosis of vessels with a diameter under 0.5-1.0 mm, the instruments used were as previously described[27-29].

We have preferentially used instruments from Takasago Medical Industry Co. (Tokyo, 113-0033, Japan), Kent Scientific Co., Roboz Surgical Instrument Co., Inc. (Gaithersburg, MD, USA), and Southpointe Surgical Supply Inc. (Coral Springs, FL, USA).

Sutures used were thin silk threads (Silk Suture 7-0; Braintree Scientific Inc., Braintree, MA, USA), monofilament nylon suture (10-0 Ethilon, BV130-3, 2820G; Ethicon, Inc., Somerville, NJ, USA), monofilament polypropylene sutures (7-0 Prolene, BV-1, 8304H-X, and 8-0 Prolene, BV130-5, 8732H; Ethicon, Inc.), and absorbable thread (5-0 Coated Vicryl Plus; Ethicon, Inc.). Micro-clips (Microclip size: M, ML, and L, Horizon Ligation System; Teleflex Medical, Durham, NC, USA) and applying forceps (Microclip applicators; Teleflex Medical) were prepared.

Microscopes: A surgical loupe (2.0-3.0 × magnification) or a microscope (5-6.25 × magnification) is sufficient for microsurgery. We used a surgical microscope at 5-20 × magnification (Surgical Scope M680, Type 10445496; Leica Microsystems Inc., Bannockburn, IL, USA) for hepatic artery reconstruction at high magnifications (12.5-20 × magnification).

Micro-tubes and catheters: The micro-tubes used were polyurethane micro-tubes (Polyurethane Catheters, Straight Tip, Hydrocoat, 2 French, 20 gauge; Access Technologies, Skoki, IL, USA).

The peripheral catheters used were 14 gauge (14G Cathlon i.v. catheter; Johnson & Johnson Medical, Inc., Arlington, TX 76004-3130, USA) and 24 gauge (24 G Surflo Flush; Terumo Co., Tokyo, Japan).

Preparation of the stent tube for biliary duct and cuffs for portal vein and infra-hepatic inferior vena cava

Stent tubes for biliary duct (BD) reconstruction were made using 24 gauge peripheral catheters or 2 French polyurethane micro-tubes. A total length of 7-8 mm is sufficient.

The portal vein (PV) cuff was made using a 14 gauge peripheral catheter. First, 2-3 mm of the main body and 2 mm of the extension were made (Figure 1A).

The infra-hepatic inferior vena cava (IHIVC) cuff was similarly made using a conventional sterilized tube with a minimum inner diameter of 2.0-2.5 mm and a thin wall. The total cuff length should be 5-6 mm, with 3-4 mm main body and 2 mm extension.

Anesthesia

All operative procedures were performed under general anesthesia using isoflurane accompanied by oxygen, and inhalational anesthesia was induced and maintained. Isoflurane accompanied by oxygen flow at 5 L/min was used in the introduction phase and was reduced to 0.5-2.0 L/min in the maintenance phase.

After the introduction of anesthesia, the abdominal wall was shaved using electric clippers. The feet were fixed to the surgical table. Before skin incision, 2.0-2.5 mL/rat of lactated Ringer's solution was injected intravenously (penile vein) using a 27 gauge fine needle.

Donor operation

Laparotomy: The abdominal wall was prepped with betadine. A long midline skin incision was made extending from the xiphoid process to the pubis, followed by a transverse incision. Tractions on bilateral subcostal borders and the lower abdominal walls provided maximal exposure of the abdominal cavity. Liver damage should be avoided during laparotomy. Warm saline was arbitrarily dripped onto the intraperitoneal organs to prevent drying during laparotomy.

Preparation for graft harvest: The gastrointestinal tract was moistened with warm saline and positioned to the outside of the left abdominal cavity and coated with gauze. The liver was handled delicately by blunt and soft items such as a cotton swab.

The falciform and triangular ligaments were cut, and the left inferior phrenic vein was located. This vein was skeletonized carefully and ligated with silk to prevent massive hemorrhage after liver reperfusion. The transparent membranes around the liver which fix each lobe to the surrounding organs were cut, and the PV branch communicating to the paraesophageal vessels was ligated with silk thread.

The retroperitoneum on the IHIVC was dissected, and the IHIVC was skeletonized. The right renal vein and artery were carefully isolated, ligated with silk thread, and divided close to the renal hilum. The fat tissue around the right adrenal gland was dissected. From this side, the back of the IHIVC was skeletonized from the connective tissues and the right renal artery. The IHIVC was then tunneled using blunt micro-forceps. Both lumbar and right adrenal veins were ligated. The lower lumbar vein and left adrenal vein were ligated with silk thread, and the junction of the left renal vein and the IHIVC were skeletonized.

The hepatoduodenal ligament was cut and the extrahepatic BD was mobilized completely. Rats have no gallbladder. The BD was cut at the level of the pancreas. A biliary stent tube was inserted and fixed with silk thread. Bile was usually observed coming out of the stent tube during the donor operation.

All branches of the PV trunk were ligated by silk thread. Complete isolation and skeletonization of the PV trunk is required for the cuff method. Excess attached tissues tend to cause stenosis in the cuff.

The proper hepatic artery (PHA) and the gastroduodenal artery (GDA) were dissected. The GDA was ligated with silk thread. The PHA was further dissected toward the hepatic hilum and was isolated from the PV. The common hepatic artery (CHA) was similarly dissected from

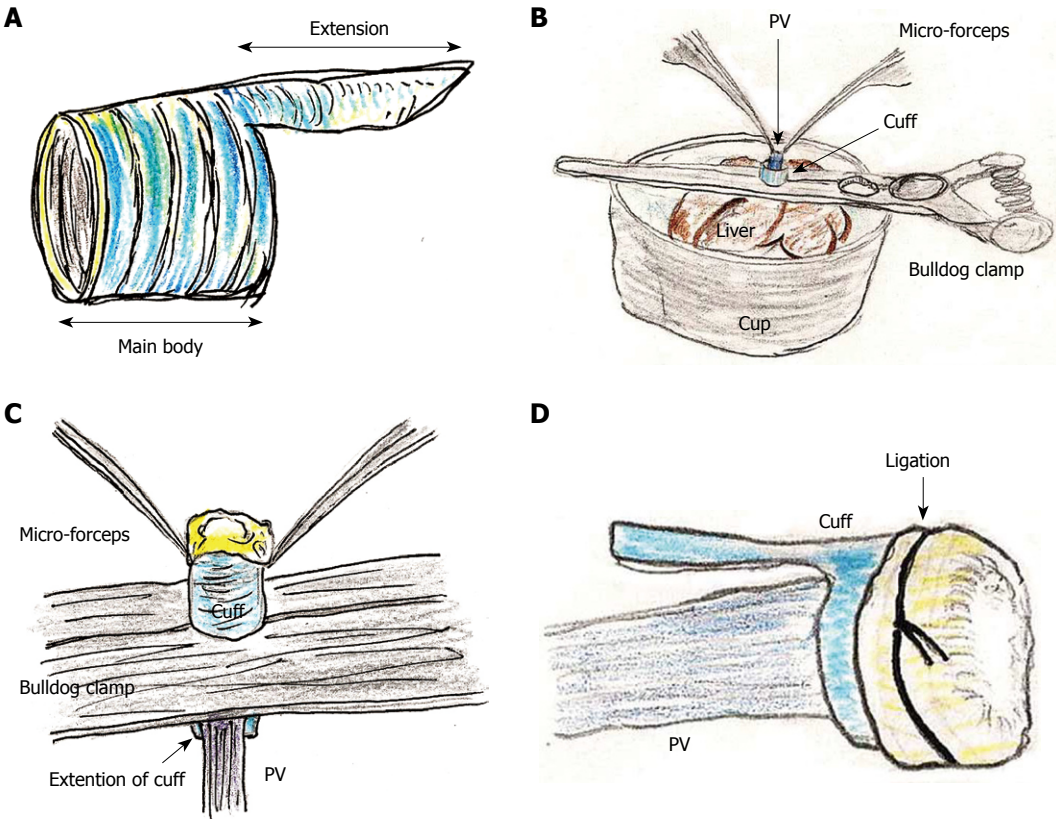

Figure 1 Portal vein (PV) cuff attachment. A: Cuff body with encircled chases and extension are made; B: PV trunk is induced through PV cuff. Cuff extension and PV trunk are grasped with a straight large-sized bulldog clamp. Cuff is set on the cup; C: Wall of PV trunk completely reversed using micro-forceps; D: Reversed PV wall is fixed to chase on cuff by ligation of silk thread.

the connective tissue around the celiac and superior mesenteric arteries.

Graft harvest: Heparinization (500 units/rat) was administered intravenously. One minute after heparin injection, the CHA was ligated at the aorta. Next, the IHIVC was clamped at an upper point of the right renal vein using a micro-clamp. The mesenteric branches of the PV trunk were clamped using micro-clamps, and one of superior mesenteric branches was opened using the cut-down method. A 24 gauge peripheral catheter was subsequently inserted into the PV trunk. After confirmation of the tip position in the PV trunk, 10 mL of cold Ringer's solution (4℃) was injected to start the hypothermic perfusion of the donor liver. A thoracotomy was performed immediately, and the thoracic supra-hepatic inferior vena cava (SHIVC) was divided. The IHIVC clamp was maintained during the cold perfusion and after the cuff attachment. The cold flush was slowly continued without high pressure, and the PV was clamped using a micro-clamp at the hepatic hilus.

After the cold wash-out, liver procurement was performed in the order of the PV trunk, diaphragm, remnant ligaments behind the SHIVC, hepatic inferior vena cava (HIVC), IHIVC, and the renal vessels. Note that the IVIHC was cut in a branch patch-fashion using the IHIVC and the left renal vein for an easy insertion of the IHIVC cuff (Figure 2A). The PV trunk was also cut in a branch patch-fashion using the portal vein trunk and the splenic vein (Figure 3A). Finally, the whole liver was harvested and immediately placed into cold Ringer's solution (4℃).

Back table benching of donor liver graft

All procedures should be performed on crushed ice. Cold Ringer's solution (4℃) was used as the preservation solution.

Plasty of the SHIVC: The anterior wall of the SHIVC was cut as near to the diaphragm as possible, leaving enough margin for the suture. The anterior diaphragm was completely trimmed. The posterior wall of the SHIVC was carefully detected, and the bilateral edge of the white tendon was removed. Stay sutures using polypropylene sutures were then made on the bilateral edges of the SHIVC. The bilateral stay sutures were held separately with curved bulldog clamps, and retention was achieved using the stay sutures. A key technique for SHIVC plasty involves leaving enough margin in the wall for retention with stay sutures (Figure 4A). Figure 4B illustrates the corresponding liver anatomy including the three lobes (right, left, and caudate lobes) arranged into seven segments including the right median, left median, left lateral, right superior, right inferior, superior caudate and inferior caudate segments[30,31].

Attachment of cuffs: Any fat tissue on the wall of the PV was completely removed, particularly in the portion

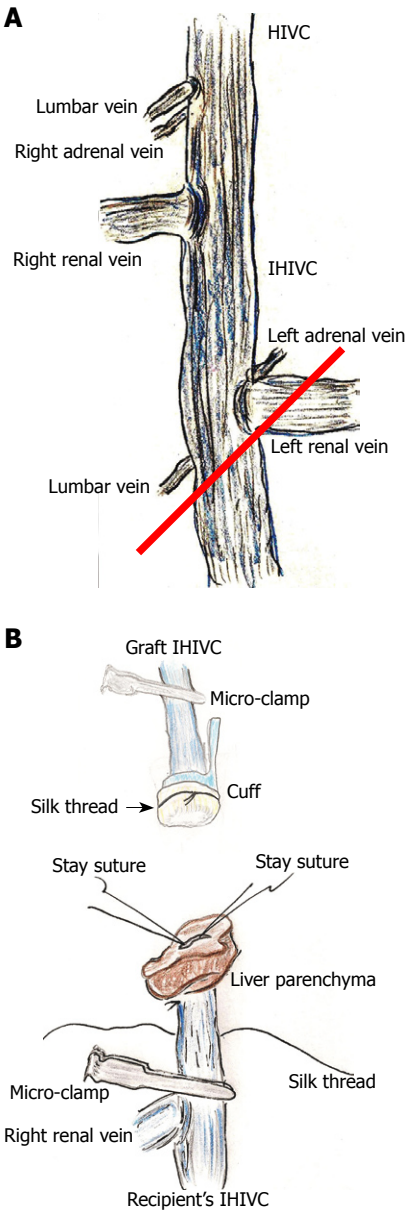

Figure 2 Infra-hepatic inferior vena cava (IHIVC) branches and reconstruction. A: Right adrenal and lumbar veins flow directly into IHIVC at point of lowest edge of right inferior segment. Left adrenal and the lower lumbar veins flow into the IHIVC at the junction of the left renal vein and the IHIVC. In donor operation for IHIVC reconstruction using the cuff method, IVIHC is cut in a branch patch-fashion (red line); B: Inner side of recipient's IHIVC is easily detected by adherent liver parenchyma. Stay sutures are made bilaterally on posterior wall, and anterior wall has some allowance for cuff insertion. Stay sutures are held by a bulldog clamp and are pulled to the cranial side. Silk thread is set behind the recipient's IHIVC beforehand. Confirmation of quality of graft IHIVC is confirmed using saline flush. Cuff is led towards the recipient's IHIVC, and cuff is inserted into IHIVC.

of the cuff, to prevent considerable cuff stenosis. The PV trunk was induced through the PV cuff. The cuff extension and the PV trunk were grasped with a large straight bulldog clamp. The cuff was set on the cup/glass using the verge of the preservation cup/glass and a long bulldog clamp (Figure 1B). The wall of the PV trunk was completely reversed using micro-forceps (Figure 1C). The reversed PV wall was fixed onto the cuff with a silk ligation (Figure 1D). A clip on the hilar PV trunk was maintained during this procedure.

The IHIVC cuff was also attached using a similar procedure to that for the PV cuff. A clip on the distal side was maintained during the procedure. After attachment of the cuffs to the PV and the IHIVC, the patency of the cuffs and the closure of branches up to the clamp points were checked using a flush of Ringer's solution through a 24 gauge catheter or dull-tip injector.

Recipient operation

Anesthesia: The induction of anesthesia and the injection of lactated Ringer's solution were the same as for the donor operation. However, shaving was omitted or performed only over as small an area as possible to maintain body temperature after surgery.

Laparotomy: The skin incision was made only by long midline incision. Too great a volume of saline on the intraperitoneal organs causes a low body temperature after recipient surgery. As such, only warm saline was used when necessary. Temporary retention of the abdominal wall by retractors was performed sparingly to prevent limitation of thoracic movements. Direct touch was possible in the recipient operation as the native liver was not used as a graft. The gastrointestinal tract was kept moistened with warm saline.

Preparation before anhepatic phase: The procedures used to mobilize the whole liver were basically the same as for the donor operation. The cut-off point of the BD was the hepatic hilus, and the BD was isolated to the upper side of the pancreas. The GDA was ligated at the point of the root. The CHA was not dissected up to the root, but the PHA was isolated sufficiently from the PV. The PHA was ligated at the hepatic hilus. Dissection of the hepatic hilus was performed more clearly than in the donor operation to provide enough length and good mobility of the BD, PHA, and PV.

The PV trunk should be isolated to provide enough length for cuff insertion. Skeletonization of the PV trunk was the same as for the donor operation, except for the splenic vein. The PV trunk was mobilized by ligating the PV branches, as for the donor operation.

For the IHIVC procedures, the portion from the HIVC to the right renal vein was completely isolated with preservation of the renal vessels. The right adrenal and lumbar veins were ligated. After cutting the dorsal membrane of the HIVC, the connective tissues at the boundary line between the SHIVC and the diaphragm were carefully dissected to provide satisfactory extensibility of the SHIVC.

Removal of native liver: A total of 2.0-2.5 mL of lactated Ringer's solution was administered intravenously. The vascular clamps at the proximal sides were applied in the order of IHIVC and PV, starting the anhepatic phase. Anesthesia was stopped. The hilar PV was ligated. SHIVC was clamped partly including the diaphragm. The SHIVC was cut at the liver parenchyma. The PV

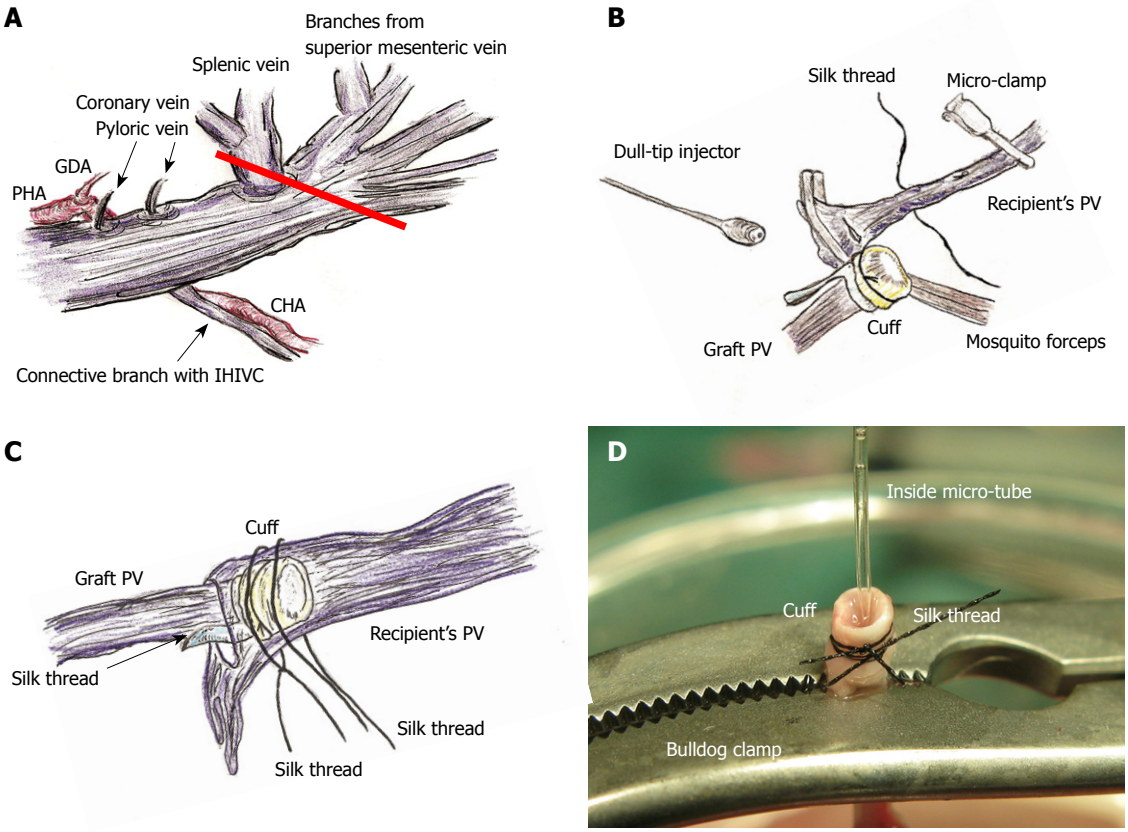

Figure 3 PV preparation and reconstruction. A: The splenic vein and coronary vein flow into PV trunk at the left or posterior sides of the PV. The pyloric vein flows into PV trunk at the left side of PV. The posterior side of PV trunk has a branch that connects with the IHIVC. The common hepatic artery (CHA) is located at the back of PV trunk, and the proper hepatic artery (PHA) and gastroduodenal artery branches are located at the left side of PV trunk. The PV trunk and these arteries are encased together in a thin sheath. The PV trunk is cut in a branch patch-fashion using PV trunk and splenic vein in the donor operation (red line); B: Retention of recipient PV is performed using mosquito forceps. Silk thread is set behind the recipient's PV trunk beforehand. The natural form of the PV is confirmed using saline flush. The cuff is led onto recipient's PV. The PV is opened using the cut-down method at the point nearest the hepatic hilus, and patency of the inner side is confirmed by saline flush; C: Cuff is inserted into recipient's PV avoiding any torsion; D: At the back table a bulldog clamp holds PV trunk, micro-tube, and cuff extension. Because of the micro-tube inside, detection of the inner side is simple. GDA: Gastroduodenal artery.

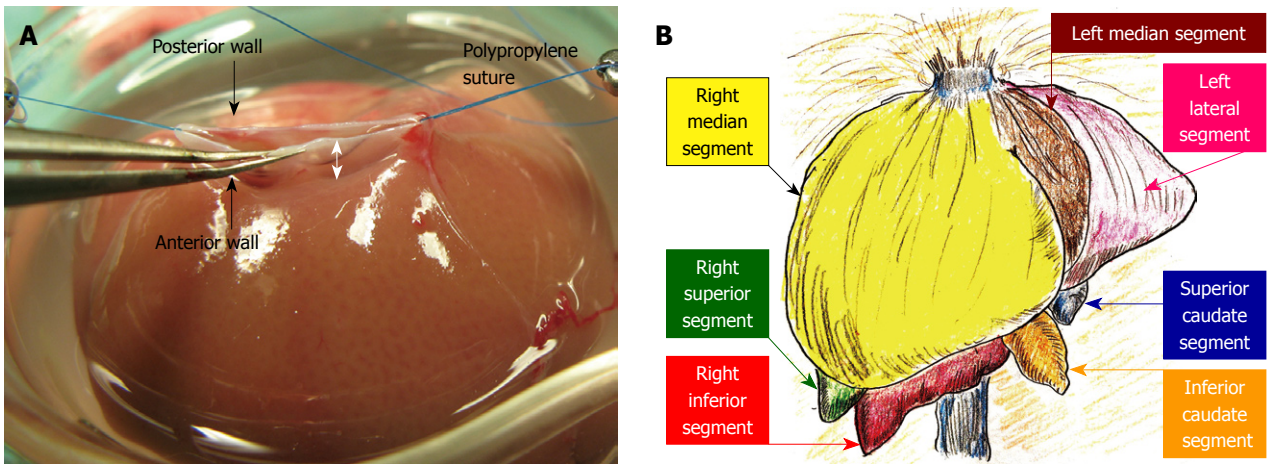

Figure 4 Hepatic segments and hepatic vein flows. A: The hepatic vein itself has no extra-hepatic margins for suture. The most important techniques for SHIVC plasty are (1) ensuring enough margin of the wall, and (2) retention using stay sutures. In particular, sufficient margin of the SHIVC wall (white arrow) is indispensable for confirmation of optimal out-flow; B: The liver comprises 3 lobes, which are subdivided into 7 segments. In basic anatomy, the left median segment is joined with the right median segment, and an incomplete lobulation is often detected in those segments.

was divided close to the hilum. The HIVC in the right inferior segment was cut at the upper point at 3-5 mm from the border line of the IHIVC and the HIVC. The native hepatectomy was completed.

Allograft implantation: The presence of bleeding points should be carefully checked using a cotton swab because secure hemostasis is very difficult after liver insertion. Placement of the graft liver is important, and should be

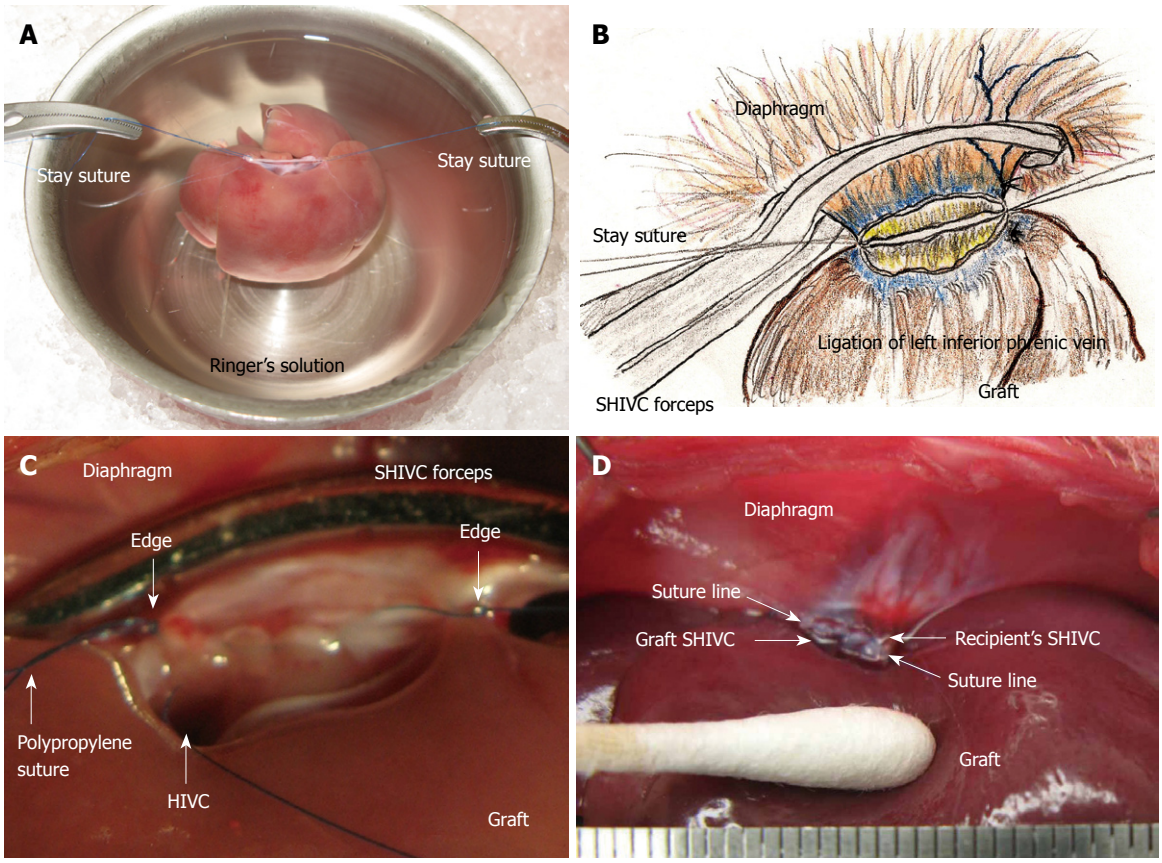

Figure 5 Supra-hepatic inferior vena cava (SHIVC) reconstruction. A: Note that an out-flow block can make the model unusable, and points of stay sutures without the axis torsion should be checked again before allograft implantation; B: The stay suture is placed bilaterally after careful consideration of the setup. The posterior wall straightens and the anterior wall is set as an arch; C: Finding after suture of the posterior wall is shown. HIVC is confirmed to enter into the right lobe. Too tight a ligation causes stenosis of the HIVC and disturbance of the flow. Ligations with stay sutures on both sides should be completed, although not too tightly; D: Completed SHIVC reconstruction is shown.

performed based on the anatomical characteristics of the graft liver. Note that an out-flow block can make the model unusable, and the points of the stay sutures without the axis torsion should be checked again before the allograft implantation (Figure 5A).

SHIVC reconstruction: A 5 or 10 mL syringe is deployed under the back of the recipient at the point of the SHIVC. In some cases, the movement of the thorax stops; however, the heart rate remains stable. A retractor is used for the retention of the costal bows if the respiratory movement is satisfactory. Forceps are used to grasp the diaphragm and expose the ventral surface, then to pull caudally. The forceps are fixed by soft clay at an adequate point for easy and stable sutures, and sutures are placed bilaterally. The posterior wall becomes straight and the anterior wall is set as an arch (Figure 5B). The left side is ligated, and the posterior wall is sutured from the left side using 5-6 stitches of continuous sutures. The last suture is ligated with a stay suture from the right side, avoiding over-tightening (Figure 5C). The anterior wall is then sutured from the right side using 15-20 stitches of continuous suture. The SHIVC cavity is filled with saline containing heparin by using an L-shaped injector before the complete closure. The anterior suture is then finished, and this thread is ligated not too tightly with the stay suture from the left side (Figure 5D). The back syringe and clay fixation are removed, and the retractors are released.

PV reconstruction: Retention of the recipient PV from the right side was achieved with mosquito forceps, and the forceps are fixed with soft clay. Note that too strong a retention makes it difficult to insert the cuff, despite increasing the PV length. The recipient PV was encircled beforehand with silk thread, and one knot is made for cuff fixation. The cuff was led onto the recipient PV. The PV was opened using the cut-down method at the nearest point of the hepatic hilus, and the inner side of the PV was confirmed with a saline flush (Figure 3B). The cuff was inserted into the recipient PV, avoiding any torsion of the PV (Figure 3C).

Allograft recirculation: The clamps were released in the order of the SHIVC, then the PV, and the allograft recirculation then starts. The anhepatic phase ceases. Cardiac and respiratory movements were allowed to recover, particularly in the case of a whole liver graft, and anesthesia is resumed.

IHIVC reconstruction: The inner side of the IHIVC was easily detected by the adherent liver parenchyma. Stay sutures were made bilaterally on the posterior wall, and the anterior wall had some allowances for the cuff inser-

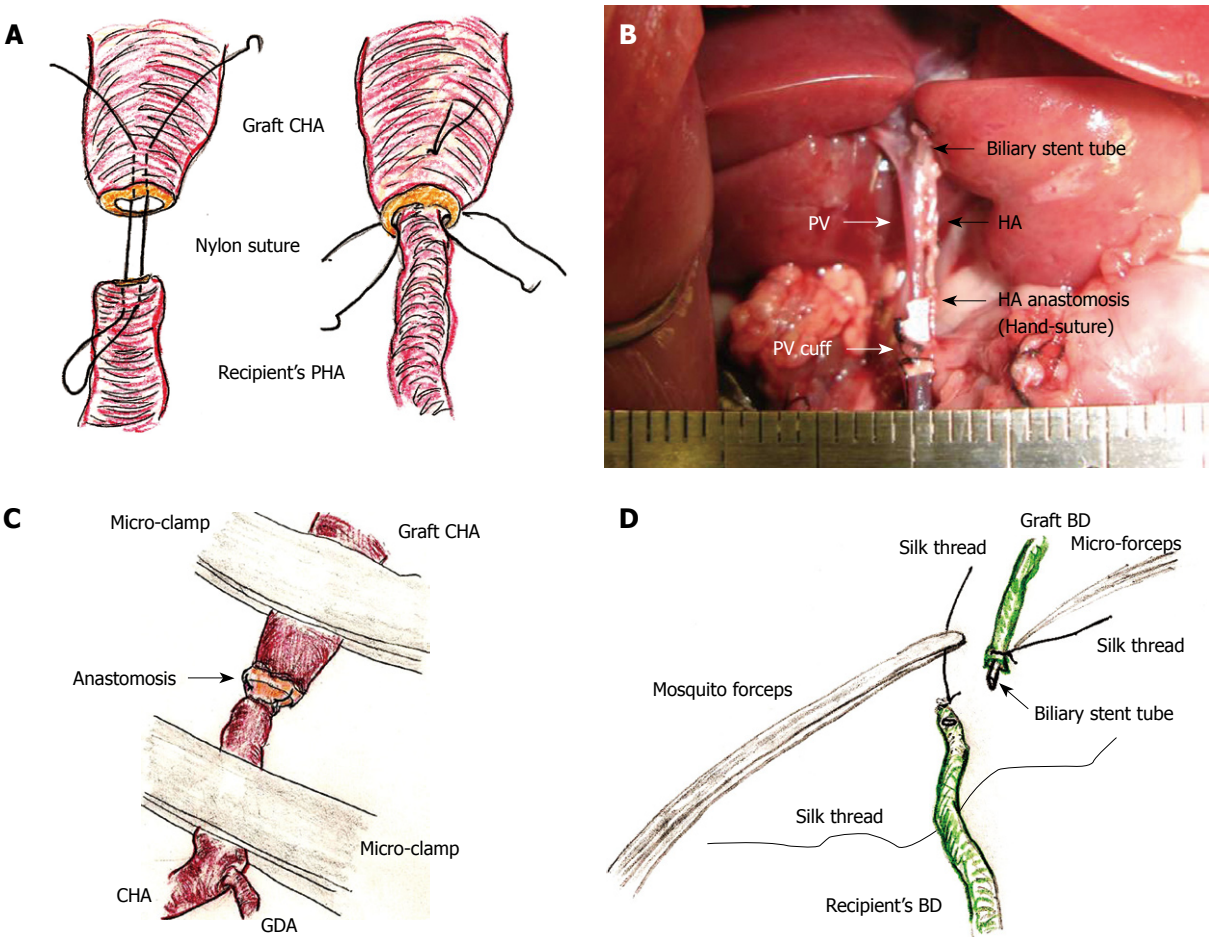

Figure 6 Hepatic artery (HA) and biliary duct (BD) reconstruction. A: An initial suture is made through the whole layer of the CHA from the outside to the inside, and a thrusting is then performed through the whole layer of the PHA from the inside to the outside. Subsequently, reverse thrusting from the PHA to the CHA is performed with the same thread. The recipient's PHA is then led into the graft CHA. One or two superficial stitches can be added if bleeding occurs; B: The diameter of the recipient's PHA is approximately 0.2 mm. Complete ultra-microsurgery allows the use of end-to-end anastomosis in HA reconstruction; C: Intermittent and alternate clamping of the HA also achieves hemostasis; D: The previously ligated silk thread of the recipient's BD is held using mosquito forceps. The recipient's BD is encircled beforehand with silk thread. The biliary stent tube is led into the recipient's BD. The recipient's BD is open using the cut-down method, and the stent tube is inserted.

tion. Stay sutures were held with a bulldog clamp and were pulled to the cranial side. The recipients' IHIVC is encircled beforehand with silk thread (Figure 2B), and one knot is made for cuff fixation. The inner side of the graft IHIVC was filled with saline. The cuff was led onto the IHIVC of the recipient liver. The cuff was inserted into the IHIVC, avoiding any IHIVC torsion. Fixation of the cuff to the IHIVC was performed. The IHIVC clamps were released in the order of the donor, then the recipient. Congestion of the right kidney and dilatation of the IHIVC were immediately resolved, and the liver color improved.

After IHIVC reflow, 2.0-2.5 mL of lactated Ringer's solution was injected *via* the penile vein. If required, a total of 0.3-1.0 mEq of bicarbonate can be injected simultaneously to offset metabolic acidosis.

HA reconstruction: An atraumatic small-sized clamp was placed on the recipient's PHA. A clamp was not needed on the graft CHA, as there was usually no backflow. The connective tissues were completely removed. A sharp cut surface is made at the ends of the recipient's PHA and graft CHA. An initial nylon suture was threaded through the whole layer of the CHA from the outside to the inside, and a thrusting was carried out through the whole layer of the PHA from the inside to the outside. A reverse thrusting from the PHA to the CHA is then carried out using the same thread. Next, due to the difference in vessel diameter, the recipient PHA was fed into the graft CHA, and the vessel clamp was released. One or two superficial stitches were added if bleeding occurred. This completed the "vest and pant" method (Figure 6A). Graft color was slightly improved after HA reconstruction.

In contrast, complete ultra-microsurgery allows the use of end-to-end anastomosis (Figure 6B). First, the sharp surfaces were joined as the vessel diameter in the graft CHA and the recipient CHA are similar. Next, three or four stitches were made through the whole layer using interrupted nylon sutures. Although additional superficial sutures are possible if bleeding occurs, the initial use of a cotton-like hemostatic agent with subtle compression is better. Intermittent and alternant clamping on the graft CHA and the recipient's CHA also achieves hemostasis (Figure 6C).

Biliary reconstruction: The previously ligated silk thread of the recipient's BD was held using mosquito forceps, and the mosquito forceps were fixed to a clay holder. The recipient's BD was encircled with silk thread and one knot was made beforehand. The biliary stent tube was led into the recipient's BD. The recipient's BD was opened using the cut-down method (Figure 6D), and the stent tube was inserted. The stent tube was then fixed with ligation of silk thread, and one edge of the ligated silk thread was also reserved for the donor operation. To prevent removal of the biliary stent tube, the preserved silk threads in the donor and the recipient were ligated together.

Abdominal closure: The intraperitoneal cavity and organs were washed with warm saline, and the point of BD anastomosis was covered with the greater omentum to prevent biliary complications. The peritoneum and fascia were closed with continuous sutures using absorbable thread, and the skin layer was closed separately using the same method.

Postoperative care and observation

The recipient was warmed on a hot pad immediately after surgery. A total of 2.0-2.5 mL of lactated Ringer's solution or maintenance solution was injected *via* the penile vein. An analgesic agent (0.1 mg/kg) was routinely given intramuscularly every 8 h for 3-5 d after surgery. Use of antibiotics was normally not required, although can be administered intravenously (30 mg/kg) if required.

Each transplanted recipient was allowed to recover in an individual cage to prevent injury by cage mates. Survival checks were made every 2 h until 48 h after surgery.

Statistical analysis

Data are presented as mean ± SD. Univariate and multivariate analyses were used for the between-group comparisons as follows: Mann-Whitney *U* test and χ^2 test for unpaired variables between two groups, Kaplan-Meier method (the log-rank) for survival rates, and logistic regression analysis for factors important for surgery success. Statistical calculations were performed using SPSS Software Version 16.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Learning curves

We examined the learning curves of some surgeons at our institutions. A success rate ⩾ 0.80 in whole liver transplantation was considered enough to learn the basic procedures for this model.

The importance of learning curves in producing reliable data using the rat OLT model has been previously reported[17]. In our experience, we observed that 30 cases are required for an initial successful OLT, and that 50 cases are required to be technically proficient for reproducible outcomes of OLT in rats. The survival curves of the first 50 cases from one surgeon can be seen in Figure 7.

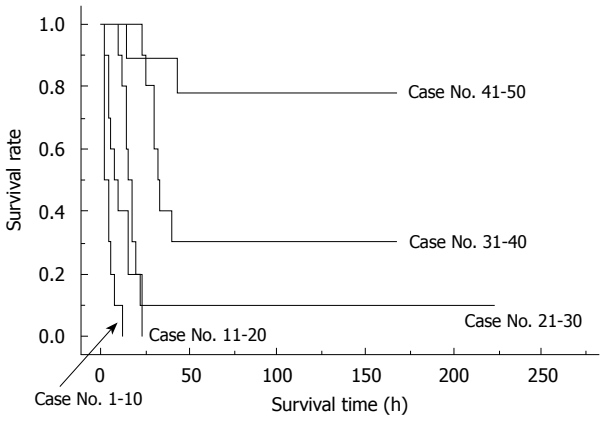

Figure 7 Learning curve in a surgeon. The initial 50 trial surgeries by one surgeon are shown. Learning curves of each surgeon showed similar patterns. Whole liver grafts were used in all cases. Preservation solution and cold ischemic time are unified as Ringer's solution for 2 h.

Critical factors for successful surgery

To determine the factors that were important for successful surgery, body weight (g), age difference between donor and recipient (d), abstinence before surgery, anesthesia method, operative time (min), blood loss (g), cold ischemic time (range: 0.5-4 h), organ preservation solution, warm ischemic time (the time from liver insertion to allograft recirculation; min), anhepatic phase (the time from portal clamp to allograft recirculation; min), unstable systemic hemodynamic state (the time from clamping of the IHIVC to IHIVC reflow), HA reconstruction, and body temperature immediately after surgery (℃) were collected in 100 OLT cases with whole liver grafts. The surgical timetable can be seen in Figure 8. The survival observation time was at least three days after surgery, and three day survivors were considered confirmation of successful surgery, as surgical and technical problems resulted in early deaths in the OLT model[17].

By univariate analyses, we found that operative time, blood loss, warm ischemic time, duration of anhepatic phase, unstable systemic hemodynamic state, and body temperature had a significant impact on the successful outcome of OLT in rats. Body weight, the age difference between donor and recipient, abstinence before surgery, anesthesia method, cold ischemic time, preservation solution, and hepatic artery reconstruction on the other hand, showed no difference (Table 1). When the univariate variables that showed a statistical significance were subjected to multivariate analysis, only the duration of anhepatic phase remained statistically significant in determining the successful outcome of an OLT in rats (Table 1). Furthermore, we compared the survival rates between the following groups: (1) anhepatic phase > 20 min; (2) anhepatic phase 15-20 min; and (3) anhepatic phase < 15 min. OLT recipients with an anhepatic phase > 20 min had a poor survival outcome, while those with an anhepatic phase of 15-20 min often died due to surgical issues. In contrast, recipients of an OLT with an anhepatic phase < 15 min all survived (Figure 9).

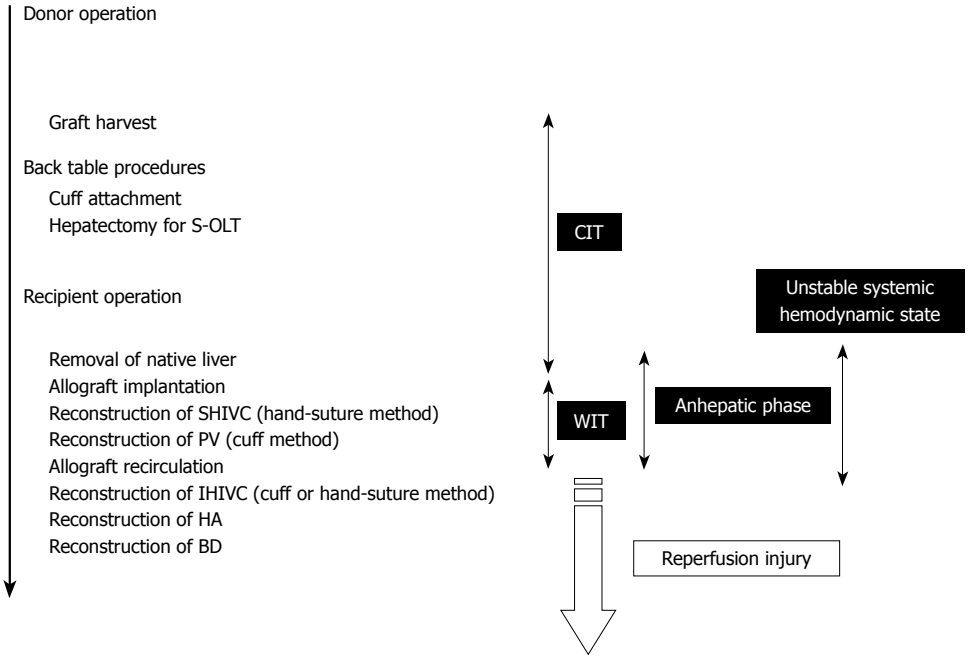

Figure 8 Time table of OLT in rat. OLT: Orthotopic liver transplantation; CIT: Cold ischemic time; WIT: Warm ischemic time.

Table 1 The important factors for successful OLT

	Metric variable (failure *vs* success)	*P* value
Univariate analysis		
Body weight (g)	231.6 ± 21.5 *vs* 224.7 ± 13.0	0.0528
The difference of age between donor and recipient (d)	2.4 ± 3.4 *vs* 1.9 ± 3.3	0.3704
Abstinence before surgery (yes *vs* no)	-	0.5678
Anesthesia (isoflurane *vs* diethylether)	-	0.2861
Operative time (min)	89.8 ± 25.7 *vs* 59.1 ± 4.7	< 0.0001[a]
Blood loss (g)	8.8 ± 2.7 *vs* 7.6 ± 3.4	0.0466[a]
CIT (min)	116.3 ± 60.8 *vs* 111.0 ± 42.9	0.6743
Organ preservation solution (Ringer's solution *vs* others)	-	0.3474
WIT (min)	30.7 ± 13.7 *vs* 12.9 ± 1.7	< 0.0001[a]
Anhepatic phase (min)	41.2 ± 14.3 *vs* 17.2 ± 2.5	< 0.0001[a]
Unstable systemic hemodynamic state (min)	54.3 ± 15.5 *vs* 25.7 ± 4.8	< 0.0001[a]
HA reconstruction (with *vs* without)	-	0.2861
Body temperature immediately after surgery (℃)	34.1 ± 1.6 *vs* 36.4 ± 0.3	< 0.0001[a]
Multivariate analysis		
Operative time (min)		0.6534
Blood loss (g)		0.9788
WIT (min)		0.1006
Anhepatic phase (min)		0.0137[a]
Unstable systemic hemodynamic state (min)		0.9185
Body temperature immediately after surgery (℃)		0.2984

[a]$P < 0.05$. OLT: Orthotopic liver transplantation; CIT: Cold ischemic time; WIT: Warm ischemic time.

DISCUSSION

The cuff method cannot be used in SHIVC reconstruction. The setting for the hepatic venous flow after allograft implantation should be carefully considered, as the initial setting impacts on all subsequent procedures. An important consideration for the SHIVC setting is that it is different from humans, as the HIVC flows into the right lobes. Bilateral retention using accurate stay sutures at both edges is indispensable from the start of the procedure. With respect to operative time, a running suture of the anterior wall is faster than an interrupted suture. The thread used in the running suture should be ligated at both edges with some allowance to prevent stenosis, although if too loose a ligation causes bleeding after allograft recirculation. The clamp for the SHIVC needs an optimal bite on the thoracic side, as a shallow bite totally disturbs the SHIVC suture and slows down the surgery, although too deep a bite results in cardiac and respiratory arrest. SHIVC reconstruction should be completed using only thin SHIVC walls, and never include the liver or diaphragm in order to prevent out-flow block and thrombosis. As for hepatic

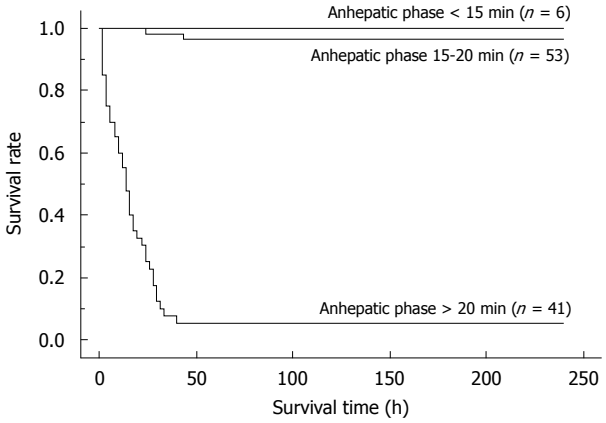

Figure 9 The differences in survival rates based on the anhepatic phase length. Recipients with a long anhepatic phase had poor survival rates, while recipients with an anhepatic phase of 15-20 min often died due to surgical issues. In contrast, recipients with an anhepatic phase < 15 min all survived. Whole liver grafts were used in all cases. All cases were accompanied by HA reconstruction. Preservation solution and cold ischemic time are unified as the Ringer's solution for 2 h. Anhepatic phase < 15 min *vs* anhepatic phase 15-20 min: $P = 0.0830$; Anhepatic phase < 15 min *vs* anhepatic phase > 20 min: $P < 0.0001$.

vein reconstruction, in contrast to humans, rats have no extra-hepatic margins in each hepatic vein. Thus, there is no choice except for anastomosis of the SHIVC to the SHIVC. A twist of the SHIVC causes out-flow block, and out-flow complications destroy the experimental setup. Even when attempting rapid surgery due to the limitation of the anhepatic phase, all IHIVC reconstruction procedures, including adequate initial setting, smooth hand-suture, and optimal ligation, should be perfectly completed.

The cuff procedures of PV are often difficult due to its thin wall and small diameter, while the IHIVC cuff procedures are comparatively easy due to its larger diameter. In some cases of small PV, we performed the following procedure: The PV trunk is clamped using the micro-clamp near the hepatic hilus to prevent air embolism, and a soft micro-tube is inserted into the portal venous trunk from the point of the wash-out injection. Next, another micro-clamp is placed on the PV trunk and the micro-tube. At the back table, a bulldog clamp holds the PV trunk, micro-tube, and cuff extension. Due to the micro-tube inside, detection of the inner side of the vessel is very easy, even in thin and small PV cases (Figure 3D). However, care must be taken to avoid air embolism and injury of the PV trunk during this procedure. The same procedure can also be applied for the attachment of the IHIVC cuff if required.

We consider that the cuffs may cause the occlusion of blood flow in long-term survivors, although based on our experience, the cuff method produces no survival problems up to 14 d recovery. Since the PV is thin and small, kinking of the trunk, torsion of the axis, and incorrect insertion can easily occur. Our studies clearly demonstrate that shortening the anhepatic phase to less than 15 min is critical during both PV reconstruction and SHIVC reconstruction. Although we and others use a hand-suture even for PV reconstruction[15], we consider the cuff method indispensable for keeping the anhepatic phase < 15 min and for obtaining reliable data. Based on the results of the present study, we perform the late phase of the recipient operation on a hot pad to prevent hypothermia. Furthermore, we currently keep transplanted rats covered with a cylindrical warmer to reduce heat loss immediately after surgery.

The importance of training for liver transplantation was previously reported[17,18]. In the first study, 65 rat OLTs were performed by a single investigator for training. The first 39 OLT were required to master the technique, and included 23 recipients that died in the first 24 h due to technical deficits and 16 OLT to learn the technique. In our experience, 20 OLTs are required to obtain an overnight survivor, while 40 OLTs are required to obtain a one week survivor. Thus, we suggest that 40-50 OLTs per surgeon are necessary for complete learning, while more OLTs are required for an amateur microsurgeon or non-surgeon. As previously reported[17,18] surgical issues can occur, even in long-term survivors, and we consider a reliable sampling rate for assays of approximately 0.6-0.9, even when experienced microsurgeons perform the surgery. Strict elimination of unsuitable rats at the sampling time point is also important for reliable data in this model. Although a large amount of time and labor is required for even a small number of reliable samples, this model produces valuable results.

A previous study investigating the regenerative capacity of individual lobes after hepatectomy in a murine hepatectomy model demonstrated that the caudate lobes work as well as the remnant liver, especially after a 75% hepatectomy[32]. Furthermore, successful survivals were reported in recipients receiving 20% grafts without HA reconstruction[33]. The 30% graft is achieved using the caudate segments, while the 10% graft is possible in this model by using the caudate lobe. However, we did not use the caudate lobe for creating 70% or 40% grafts, as the caudate lobe showed reduced regenerative capacity in the 30% and 60% hepatectomy model compared with other lobes[32]. Based on the behavior of the caudate lobe in large hepatic remnants, we performed a 60% graft without right superior, right inferior, superior caudate, and inferior caudate segments. The set-up of 20% and 30% grafts is well established in our model for studies of small-for-size grafts.

The 40% graft is clinically important in liver regeneration due to split and pediatric grafts from cadaveric donors for the donor shortage in the United States and the shift to left-lobe grafts in a living-donor for donor safety in Japan. It should be noted that when using our experimental model, there are distinct differences between basic anatomy and surgical anatomy. In basic anatomy, the left median segment is aggregated with the right median segment, and an incomplete lobulation is often detected in those segments (Figure 4B). However, dominating vessels of the left median segment make a common channel with those of the left lateral segment (Figure 4D). Thus, the left median segment should be handled together with the left lateral segment with respect to in-flow and

out-flow in surgery. No-margin hepatectomy by the clip method causes the SHIVC/HIVC twist, especially during removal of the right median segment. The hepatic margin in the clip method prevents the SHIVC/HIVC twist and common channel injury. The parenchymal margin has no hepatic circulation (these areas are insignificant as a functional volume), and we currently use right median and left median segments as a 40% graft accompanied by the margin method. Although removal of the right median segment requires several micro-clips, the left lateral segment can usually be treated with only one micro-clip. Thus, for procedure simplicity, we recommend right median and left median segments as a 40% graft using the clip method of hepatectomy accompanied by a margin, although a 40% graft of the left median and left lateral segments without margin is ideal based on surgical anatomy. This simple and useful method with margin areas prevents unintended injury of the common channel, avoids twisting of the SHIVC/HIVC, and allows easy application to incomplete lobulation. However, the percentage of the actual graft weight to the recipient's native liver cannot be calculated due to margin weight. Therefore, we estimated the percentages of each segment in 100 rats. A previous detailed report in a rat hepatectomy model used a ligation method with a hepatic margin[30], and we recommend the clip method with a hepatic margin in the rat S-OLT model.

With regard to another feature of the hepatic venous flow in rats, the hepatic veins of the right superior, right inferior, superior caudate, and inferior caudate segments flow directly into the HIVC (Figure 4D). Note that we never opened the IHIVC clamp during the wash-out procedure in the donor operation, as this can cause incomplete wash-out due to drainage from the PV to the HVC *via* these direct pathways.

Co-instantaneous reconstruction of the HA is ideal for studies focused on liver regeneration, although the omission of HA reconstruction is fine for studies focused on transplant immunity. HA reconstruction requires the most skillful ultra-microsurgery (approximately 0.1-0.3 mm vessel diameter), and extended anesthesia and operative times. For the surgeon, both finely-honed concentration and non-nervous distraction are required in microsurgery and ultra-microsurgery. If a microscope is employed, we recommend limiting its use to reconstructive procedures only to avoid fatigue. In particular, ultra-microsurgery is introduced only at limited times, while continuous microsurgery during all steps is inadvisable due to the likely loss of mental focus. If possible, preparation of the surgical equipment is better than repeating surgery in the same day to obtain enough samples[27,28].

In summary, although the rat OLT model with HA reconstruction is difficult and complicated, the model without HA reconstruction is well established[9-23]. Moreover, the model with HA reconstruction provides clinically relevant data.

COMMENTS

Background

Due to the clinical situation of liver donor shortage and donor safety, the main focus of the liver regeneration field is on patients with small-for-size liver syndrome and liver reperfusion injury. The development of clinically relevant rat orthotopic liver transplantation (OLT) models has advanced the clinical liver regeneration field. However, pseudo models of fake OLT/S-OLT, such as temporal clamp or simple hepatectomy, are still used experimentally for assessing reperfusion injury and/or small-for-size syndrome after liver transplantation due to the technical demands of the rat OLT/S-OLT model.

Research frontiers

The cold ischemic time (CIT) is a critical factor in producing reliable data using the OLT model, and also plays an important role in the mechanism of true reperfusion injury and small-for-size syndrome. Data from pseudo models that omit CIT are clinically irrelevant, and should not be translated into the actual OLT field. Clinical OLT is made possible by the phenomena of immunological tolerance even after allograft transplantation and the ability of the liver to regenerate even after initial insufficient volume. As such, these factors form the prominent focus of studies attempting to further develop the OLT field. Murine organ transplantation models, such as cardiac, lung, and kidney grafts, are well established, and are commonly used by transplant immunity investigators. New insights into the mechanisms of graft injury after OLT have also been established from experiments in small animal models. Mice are particularly suitable for laboratory assays due to the growing availability of gene-altered or knock-out animals and the development of specific agents and antibodies. However, murine OLT is the most technically difficult animal transplantation model, even when reconstruction of the hepatic artery (HA) is omitted. Furthermore, a validated model of OLT in mice is unavailable, and the authors consider that the rat OLT model produces more clinically relevant and reliable data. Hence, there is a requirement for a complete rat OLT model, including S-OLT, particularly in the field of liver regeneration.

Innovations and breakthroughs

OLT in rats is the only liver transplantation model that provides clinically relevant and reliable results. Shortened anhepatic phase is an important key to success in this model.

Applications

In summary, although the rat OLT/S-OLT with HA reconstruction model is difficult and complicated, only this model provides clinically relevant data. The authors have established this model and hope that their surgical guide will also help young researchers with an interest in the liver transplantation field.

Peer review

The article itself is well written and describes the different techniques and requirements for a successful outcome in animal liver transplantation. The illustrations are excellent and clearly portray the techniques.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3133

World J Gastroenterol 2010 July 7; 16(25): 3133-3143
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Cetuximab plus FOLFOX6 or FOLFIRI in metastatic colorectal cancer: CECOG trial

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Received: February 15, 2010 Revised: April 15, 2010
Accepted: April 22, 2010
Published online: July 7, 2010

Abstract

AIM: To investigate efficacy and safety of cetuximab combined with two chemotherapy regimens in patients with unresectable metastatic colorectal cancer (mCRC).

METHODS: Randomized patients received cetuximab with 5-fluorouracil (5-FU), folinic acid (FA) and oxaliplatin (FOLFOX) 6 (arm A, *n* = 74) or 5-FU, FA and irinotecan (FOLFIRI) (arm B, *n* = 77). *KRAS* mutation status was determined retrospectively in a subset of tumors (*n* = 117).

RESULTS: No significant difference was found between treatment arms A and B in the progression-free survival (PFS) rate at 9 mo, 45% *vs* 34%; median PFS, 8.6 mo *vs* 8.3 mo [hazard ratio (HR) = 1.06]; overall response rate (ORR) 43% *vs* 45% [odds ratio (OR) = 0.93] and median overall survival (OS), 17.4 mo *vs* 18.9 mo (HR = 0.98). Patients with *KRAS* wild-type tumors demonstrated improved PFS (HR = 0.55, *P* = 0.0051), OS, (HR = 0.62, *P* = 0.0296) and ORR (53% *vs* 36%) and in arm A, improved PFS (HR = 0.49, *P* = 0.0196), OS (HR = 0.48, *P* = 0.0201) and ORR (56%

vs 30%), compared with patients with *KRAS* mutated tumors. In arm B no significant differences were found in efficacy by *KRAS* mutation status. Treatment in arms A and B was generally well tolerated.

CONCLUSION: This study confirms that combinations of cetuximab with FOLFOX6 or FOLFIRI are effective and significantly improve clinical outcome in *KRAS* wild-type compared with *KRAS* mutated mCRC.

Key words: Cetuximab; 5-fluorouracil folinic acid and oxaliplatin; 5-fluorouracil folinic acid and irinotecan; *KRAS*; Metastatic colorectal cancer

Peer reviewers: Pascal Gervaz, PhD, Department of Surgery, University Hospital Geneva, 4, Rue Gabrielle Perret Gentile, Geneva, 1211, Switzerland; Luca Stocchi, MD, Desk A 30, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States; Dr. Abdel-Majid Khatib, PhD, INSERM, UMRS 940, Equipe Avenir, Cibles Thérapeutiques, IGM 27 rue Juliette Dodu, 75010 Paris, France

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INTRODUCTION

Colorectal cancer (CRC) accounted for 529 000 deaths worldwide in 2002[1]. Up to 25% of CRC patients present with metastatic disease (mCRC) with five-year survival rates of approximately 10% reported[2,3]. The standard treatment for unresectable mCRC has been to administer first-line 5-fluorouracil (5-FU) with folinic acid (FA)[2,4,5], with improvements in clinical outcome being demonstrated for infusional 5-FU/FA combined with oxaliplatin (FOLFOX)[6,7] or irinotecan (FOLFIRI)[8,9]. However safety profiles differ, with grade 3/4 neutropenia and neurotoxicity more common with FOLFOX, and grade 3/4 mucositis and nausea/vomiting more common with FOLFIRI.

Cetuximab [Erbitux, developed by Merck KGaA Darmstadt, Germany (under license from Imclone, NY USA)] is an immunoglobulin G1 monoclonal antibody that specifically targets the epidermal growth factor receptor (EGFR), competitively inhibiting ligand binding and ligand-dependent downstream signaling[10,11]. Cetuximab first gained approval for use in Europe and the United States in the treatment of EGFR-expressing mCRC following failure of irinotecan-containing regimens[12]. More recently, the randomized CRYSTAL study demonstrated improved progression-free survival (PFS) in EGFR-expressing mCRC patients receiving FOLFIRI plus cetuximab compared with FOLFIRI alone[13]. In addition, the phase Ⅱ OPUS trial reported a trend towards improved overall response rate (ORR) in EGFR-expressing mCRC patients receiving FOLFOX4 plus cetuximab compared with FOLFOX4 alone[14].

An accumulating body of data from studies of chemorefractory mCRC patients receiving cetuximab as monotherapy or in combination with chemotherapy suggests that clinical responses are confined to those patients whose tumors do not harbor mutations in codons 12 or 13 of the *KRAS* gene (*KRAS* wild-type)[15-19]. The *KRAS* gene encodes a GDP/GTP binding protein which, following ligand binding to receptor tyrosine kinases including EGFR, activates downstream intracellular signaling cascades promoting cellular growth and proliferation[20,21]. *KRAS* mutations (in codons 12 or 13) occur in 40%-50% of CRCs and circumvent the cellular requirement for receptor activation of the KRAS protein[20,21]. Metastatic colorectal tumors harboring *KRAS* mutations are therefore hypothesized to be refractory to EGFR-targeting monoclonal antibodies. Data from retrospective analyses of the CRYSTAL and OPUS studies confirmed that the efficacy of cetuximab in combination with FOLFIRI or FOLFOX was restricted to patients with *KRAS* wild-type tumors[13,14], indicating tumor *KRAS* mutation status to be a predictive biomarker for the efficacy of cetuximab in combination with chemotherapy.

In the current Central European Co-operative Oncology Group (CECOG)-sponsored randomized phase Ⅱ trial, the efficacy and safety of cetuximab in combination with either FOLFOX6 or FOLFIRI was investigated first-line in patients with mCRC. In addition, a retrospective subgroup analysis of clinical outcome according to tumor *KRAS* mutation status was performed.

MATERIALS AND METHODS

Main patient eligibility criteria

Patients (≥ 18 years old) with histologically confirmed adenocarcinoma of the colon or rectum, with metastatic disease unsuitable for resection with curative-intent, an Eastern Co-operative Oncology Group (ECOG) performance status < 2, and adequate organ function were eligible for inclusion.

Exclusion criteria included: previous chemotherapy for metastatic disease; prior EGFR-targeted therapy; adjuvant chemotherapy with oxaliplatin or irinotecan (5-FU-based adjuvant chemotherapy was allowed provided the chemotherapy treatment-free interval was > 6 mo). Patients with brain metastases; concurrent malignancy and those with a previous malignancy within the last 5 years (excluding non melanoma skin cancer and in situ carcinoma of cervix); coronary artery disease or a history of myocardial infarction within 12 mo of study entry; pre-existing neuropathy > grade 1; intestinal occlusion or a history of inflammatory bowel disease; a ≥ grade 3 allergic reaction to study treatment components; those

undergoing surgery (excluding biopsy) or irradiation within 4 wk of study entry were also excluded, as were pregnant or lactating patients.

The study was approved by independent ethics committees at each center and was conducted in accordance with the principles of the Declaration of Helsinki and the Note for Guidance on Good Clinical Practice. All patients provided written informed consent.

Study design

This was a two-arm randomized multicenter, open-label, parallel-group phase Ⅱ study involving 28 participating centers across 13 countries (CECOG/CORE1.2.001). Eligible patients were centrally randomized 1:1, using a minimization technique, stratifying patients according to study site, the number of organs involved and prior neo-adjuvant/adjuvant therapy. Patients received cetuximab (400 mg/m^2 initial infusion day 1, then 250 mg/m^2 weekly), then either in arm A: oxaliplatin (day 1, 100 mg/m^2) with FA [400 mg/m^2 (racemic) or 200 mg/m^2 (L-form)] plus 5-FU (400 mg/m^2 bolus plus 2400 mg/m^2 as a 46-h continuous infusion) every 2 wk (FOLFOX6), or in arm B: irinotecan (180 mg/m^2) with the 5-FU/FA regimen described (FOLFIRI). Patients received 6 mo of combination therapy, after which cetuximab was continued. Study treatment was discontinued in the case of progressive disease (PD). Patient follow-up was every 12 wk until treatment end or clinical cut-off date. The primary endpoint was PFS at 9 mo, secondary endpoints included ORR, PFS at 3, 6 and 12 mo, overall survival and safety.

Dose modifications

Dose reductions, treatment delays and the omission of a maximum of two consecutive doses of cetuximab were permitted in cases of grade 3 skin reactions. Two dose reductions for irinotecan or oxaliplatin were permitted after which the drug was discontinued (in either case cetuximab could be continued). Dose reductions were permanent.

Assessments

Computed tomography or magnetic resonance imaging of chest, abdomen and pelvis was performed at baseline and weeks 6, 12, and every 12 wk thereafter during treatment, and at the end of the study or upon PD. PFS rate was defined as the percentage of patients in each arm alive and free of tumor progression at analysis from the time of randomization, using response evaluation criteria in solid tumors (RECIST). Tumor response was evaluated according to RECIST guidelines. Survival was defined as time from randomization until death (patients lost to follow-up were censored at the time they were last determined to be alive). Adverse events (AEs) were assessed at treatment visits using National Cancer Institute Common Terminology Criteria for Adverse Events (version 3) and coded using the Medical Dictionary for Regulatory Activities (MedDRA; version 10.1).

Tumor DNA was extracted and purified from formalin-fixed paraffin-embedded tissues as previously described[14]. The presence of *KRAS* mutations in codons 12 and 13 was determined by allele-specific real time polymerase chain reaction assays using validated methodology (DxS Ltd Manchester UK)[22,23]. EGFR expression was determined using the DAKO EGFR pharmDx™ test. Tumor *KRAS* mutation status and EGFR expression were assessed centrally by one pathologist.

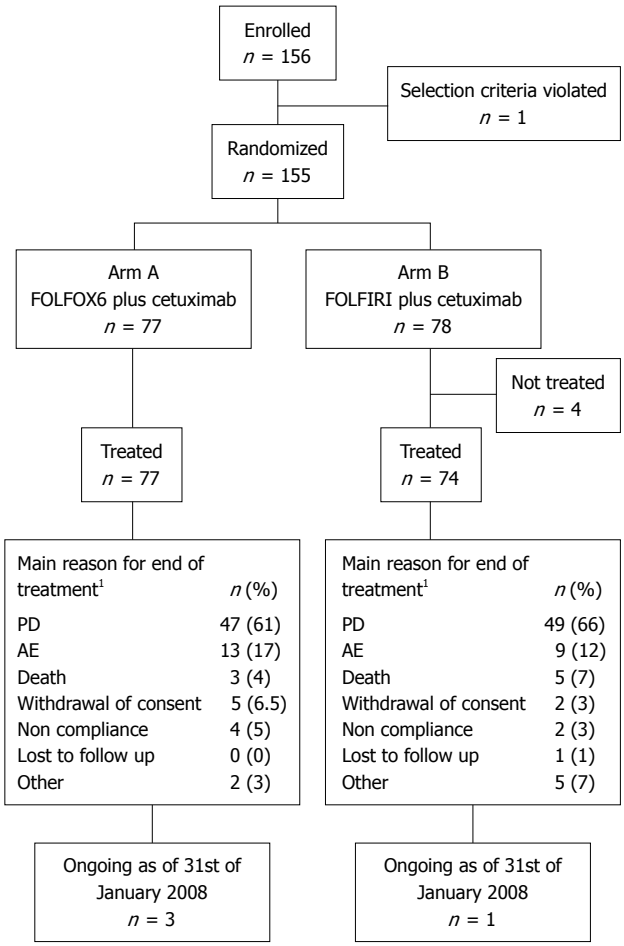

Figure 1 Disposition of patients as of clinical cut-off date January 2008. The intention to treat (ITT) population comprised 77 patients randomized to FOLFOX6 plus cetuximab and 74 randomized to FOLFIRI plus cetuximab. [1]Values based on all treated patients (*n* = 151). FOLFOX: 5-fluorouracil (5-FU) folinic acid (FA) and oxaliplatin; FOLFIRI: 5-FU FA and irinotecan; PD: Progressive disease; AE: Adverse event.

Statistical analysis

The primary objective of the study was to estimate the difference in 9-mo PFS rates between the treatment arms. In accordance with the objective of this phase Ⅱ study, the planned sample size was fixed to 2 × 75 patients to achieve appropriate precision for the estimate of the difference in 9-mo PFS rates. With 75 patients in each treatment arm evaluable for 9-mo PFS, the two-sided 95% confidence intervals (CI) for the difference in PFS rates had a range of not more than ± 16% assuming a low number of censored cases (up to 5%) and PFS rates in both treatment arms being approximately 50%-60%.

Statistical analyses were performed on data accrued up until the clinical cut-off date (January 31, 2008). The efficacy analyses were performed on the intention to treat

Table 1 Patient characteristics at baseline

Characteristic	ITT population		*KRAS* population			
			KRAS wild-type		*KRAS* mutant	
	FOLFOX6 plus cetuximab (*n* = 77)	FOLFIRI plus cetuximab (*n* = 74)	FOLFOX6 plus cetuximab (*n* = 34)	FOLFIRI plus cetuximab (*n* = 28)	FOLFOX6 plus cetuximab (*n* = 23)	FOLFIRI plus cetuximab (*n* = 32)
Gender, *n* (%)						
Male	43 (56)	45 (61)	22 (65)	17 (61)	11 (48)	21 (66)
Female	34 (44)	29 (39)	12 (35)	11 (39)	12 (52)	11 (34)
Age (yr)						
Median (Q1-Q3)	62.0 (54-67)	62.5 (54-68)	62.5 (55-67)	64.0 (56-68)	63.0 (49-68)	62.5 (54-70)
< 65, *n* (%)	46 (60)	46 (62)	19 (56)	17 (61)	13 (57)	19 (59)
> 65, *n* (%)	31 (40)	28 (38)	15 (44)	11 (39)	10 (43)	13 (41)
ECOG PS, *n* (%)						
0	46 (60)	38 (51)	20 (59)	17 (61)	13 (57)	14 (44)
1	31 (40)	36 (49)	14 (41)	11 (39)	10 (43)	18 (56)
Primary tumor location, *n* (%)						
Colon	52 (68)	47 (64)	26 (76)	15 (54)	13 (57)	22 (69)
Rectum	25 (32)	27 (36)	8 (24)	13 (46)	10 (43)	10 (31)
Metastasis[1], *n* (%)	45 (58)[a]	46 (62)	17 (50)	18 (64)	16 (70)	18 (56)
Organs with metastases, *n* (%)						
1-2	59 (77)	56 (76)	28 (82)	23 (82)	17 (74)	26 (81)
> 2	18 (23)	18 (24)	6 (18)	5 (18)	6 (26)	6 (19)
Metastatic sites[2], *n* (%)						
Intestine/bowel	12 (16)	12 (16)	3 (9)	6 (21)	6 (26)	5 (16)
Liver	66 (86)	63 (85)	30 (88)	24 (86)	20 (87)	26 (81)
Lung	27 (35)	28 (38)	11 (32)	10 (36)	8 (35)	10 (31)
Lymph nodes						
Chest	7 (9)	5 (7)	2 (6)	2 (7)	3 (13)	2 (6)
Abdomen	22 (29)	24 (32)	9 (26)	8 (29)	5 (22)	8 (25)
Bone	2 (3)	4 (5)	0 (0)	1 (4)	2 (9)	1 (3)
Other	10 (13)	10 (14)	5 (15)	3 (11)	2 (9)	4 (13)
Duration of disease, mo						
CRC, median (Q1-Q3)	2.1[a] (1-15)	1.9 (1-14)	2.2 (1-18)	1.8 (1-6)	1.8 (1-3)	2.4 (1-18)
mCRC median (Q1-Q3)	1.4 (1-2)	1.2 (1-2)	1.1 (1-2)	1.0 (1-2)	1.3 (1-2)	1.4 (1-2)
EGFR status, *n* (%)						
Detectable	43 (56)	46 (62)	21 (62)	20 (71)	17 (74)	24 (75)
Undetectable	17 (22)	12 (16)	10 (29)	4 (14)	5 (22)	7 (22)
Non evaluable	17 (22)	16 (22)	3 (9)	4 (14)	1 (4)	1 (3)
Prior treatment, *n* (%)						
At least 1 therapy	63 (82)	59 (80)	31 (91)	22 (79)	19 (83)	29 (91)
Adjuvant chemotherapy[3]	14 (18)	10 (14)	9 (26)	2 (7)	2 (9)	6 (19)
Surgery	61 (79)	58 (78)	30 (88)	22 (79)	18 (78)	29 (91)
Other	8 (10)	5 (7)	3 (9)	2 (7)	3 (13)	2 (6)

[1]Metastases detected within 1 mo of tumor diagnosis; [2]Patients with >1 metastasis per organ site, the organ site was counted once only; [3]Three patients included with rectal cancer received neoadjuvant therapy; [a]Value determined from 76 patients. Patients receiving FOLFOX6 plus cetuximab (arm A) and those receiving FOLFIRI plus cetuximab (arm B). ITT: Intention to treat; FOLFOX: 5-fluorouracil (5-FU) folinic acid (FA) and oxaliplatin; FOLFIRI: 5-FU FA and irinotecan; ECOG: Eastern Cooperative Oncology Group; PS: Performance status; Q1-Q3: Interquartile range; mCRC: Metastatic colorectal cancer.

(ITT) population defined as all randomized patients who received at least one dose of study medication, which was the same as the safety population. Time to event data were analyzed using the Kaplan-Meier method[24]. Standard errors were calculated using Greenwoods formula[25] and the hazard ratio (HR) for PFS between both treatment groups and corresponding 95% CIs was calculated using an unadjusted Cox proportional hazard model. Differences in survival were tested using the logrank test. Estimates of ORR in each treatment group, odds ratios and associated 95% CIs were calculated using the Cochran-Mantel-Haenszel procedure.

The study was initiated and patient recruitment finished (2006) before the evidence from a randomized trial that *KRAS* tumor mutation was associated with clinical outcome in patients treated with cetuximab in combination with chemotherapy was first presented[26]. Subsequently a retrospective analysis of efficacy and safety was performed in the subgroup of patients with available tumor material that was evaluable for *KRAS* mutation status (wild-type *vs* mutant). Exploratory Cox proportional hazard models and logistic regression models were used to investigate the impact of *KRAS* mutation status on PFS, overall survival and ORR across the treatment groups adjusted for other significant confounding factors. A significance level of 0.2 was used to enter a factor into the model and a significance level of 0.10 was used for removing a factor from the model. Following an update of survival time, all information available by December 16th 2008 was considered for survival analyses. All calculations

Table 2 Treatment exposure in the safety population

Characteristic	FOLFOX6 plus cetuximab (arm A, *n* = 77)	FOLFIRI plus cetuximab (arm B, *n* = 74)
Exposure to cetuximab (Q1-Q3)		
Median duration, wk	28.0 (17-46)	29.1 (13-46)
Median number of infusions	26.0 (14-40)	26.0 (12-42)
Relative dose intensity, *n* (%)		
Only initial dose	4 (5)	3 (4)
< 60%	2 (3)	3 (4)
60% to < 80%	15 (19)	8 (11)
80% to < 90%	21 (27)	20 (27)
⩾ 90%	35 (45)	40 (54)
Exposure to chemotherapy (Q1-Q3)		
Median duration, wk	25.1 (19-28)	25.5 (14-28)
Median number of cycles	12 (7-12)	12 (6-12)
Relative dose intensity, *n* (%)		
Oxaliplatin		
No dose	1 (1)	74 (100)
< 60%	4 (5)	-
60% to < 80%	24 (31)	-
80% to < 90%	22 (29)	-
⩾ 90%	26 (34)	-
Irinotecan		
No dose	77 (100)	2 (3)
< 60%	-	3 (4)
60% to < 80%	-	18 (24)
80% to < 90%	-	13 (18)
⩾ 90%	-	38 (51)
Bolus 5-FU		
No dose	1 (1)	2 (3)
< 60%	1 (1)	2 (3)
60% to < 80%	28 (36)	19 (26)
80% to < 90%	19 (25)	14 (19)
⩾ 90%	28 (36)	37 (50)
Continuous infusion 5-FU		
No dose	1 (1)	2 (3)
< 60%	1 (1)	3 (4)
60% to < 80%	21 (27)	14 (19)
80% to < 90%	13 (17)	11 (15)
⩾ 90%	41 (53)	44 (59)
Dose reductions[1], *n* (%)		
Cetuximab	9 (12)	5 (7)
Chemotherapy	25 (32)	17 (23)
Treatment delays[1], *n* (%)		
Any cetuximab		
⩾ 3 d	59 (77)	47 (64)
⩾ 16 d	12 (16)	8 (11)
Any chemotherapy		
⩾ 3 d	59 (77)	51 (69)
⩾ 14 d	25 (32)	15 (20)
Treatment discontinuation[1], *n* (%)		
Cetuximab	13 (17)	9 (12)
Chemotherapy	9 (12)	4 (5)

[1]Dose reductions, treatment delays and discontinuations due to adverse events.

were performed with SAS release 8.2 (SAS Institute, Cary, NC USA).

RESULTS

Patient demographics

Patients were enrolled between July 2005 and July 2006; patient disposition is shown in Figure 1. Four patients randomized to receive cetuximab plus FOLFIRI withdrew their consent prior to treatment, with 151 patients subsequently receiving treatment. Reasons for discontinuing the study treatment were similar for both treatment arms.

Patient characteristics at baseline were generally well balanced between treatment groups (Table 1). *KRAS* mutation status was evaluable in 117/151 (77%) patient tumors; of these, *KRAS* mutations were detected in 55/117 (47%) patient tumors. Baseline characteristics of the *KRAS* subpopulation were representative of those of the ITT population (Table 1).

Table 3 Efficacy in the ITT population

Characteristic	FOLFOX6 plus cetuximab (arm A, *n* = 77)	FOLFIRI plus cetuximab (arm B, *n* = 74)
PFS		
Events, *n* (%)	61 (79)	59 (80)
Median[1], mo (95% CI)	8.6 (6.3-9.7)	8.3 (7.4-8.7)
Log rank *P*-value	0.7375	
Hazard ratio (95% CI)	1.06 (0.74-1.52)	
PFS rate[1], % (95% CI)		
3 mo	92 (85-98)	78 (68-88)
6 mo	69 (58-80)	69 (58-80)
9 mo	45 (33-58)	34 (23-46)
12 mo	18 (8-27)	18 (8-27)
Overall survival		
Events, *n* (%)	54 (70)	50 (68)
Median[1], mo (95% CI)	17.4 (14.9-22.6)	18.9 (14.7-23.9)
Logrank *P*-value	0.9230	
Hazard ratio[2] (95% CI)	0.98 (0.67-1.44)	
Survival rate[1], % (95% CI)		
9 mo	79 (70-88)	79 (70-89)
12 mo	70 (60-80)	71 (60-81)
18 mo	46 (35-57)	53 (42-65)
24 mo	33 (22-44)	38 (26-50)
Best overall response, *n* (%)		
CR	2 (3)	6 (8)
PR	31 (40)	27 (36)
SD	31 (40)	24 (32)
PD	6 (8)	9 (12)
NE	7 (9)	8 (11)
Objective response rate, *n* (%)	33 (43)	33 (45)
95% CI	32-55	33-57
Odds ratio (95% CI)	0.93 (0.49-1.77)	

[1]Median time and rates are based on Kaplan-Meier estimates; [2]Hazard ratio and corresponding 95% CI based on unadjusted Cox proportional hazard model: hazard rate FOLFIRI plus cetuximab divided by hazard rate FOLFOX6 plus cetuximab. CR: Complete response; NE: Not evaluable; PD: Progressive disease; PFS: Progression-free survival; PR: Partial response; SD: Stable disease.

Treatment compliance

Patient exposure to cetuximab and chemotherapy was similar for both treatment arms (Table 2). The proportion of dose reductions and treatment delays for cetuximab was slightly higher in arm A than arm B. Treatment delays were more commonly due to diarrhea in the FOLFIRI arm and to neuropathy in the FOLFOX6 arm. In treatment arm A, median exposure to cetuximab in patients with *KRAS* wild-type patients was 35.4 wk and *KRAS* mutant tumors 23.3 wk, compared with 24.5 and 32.4 wk, respectively, in arm B. Exposure to chemotherapy in each treatment arm by *KRAS* mutation status was similar to

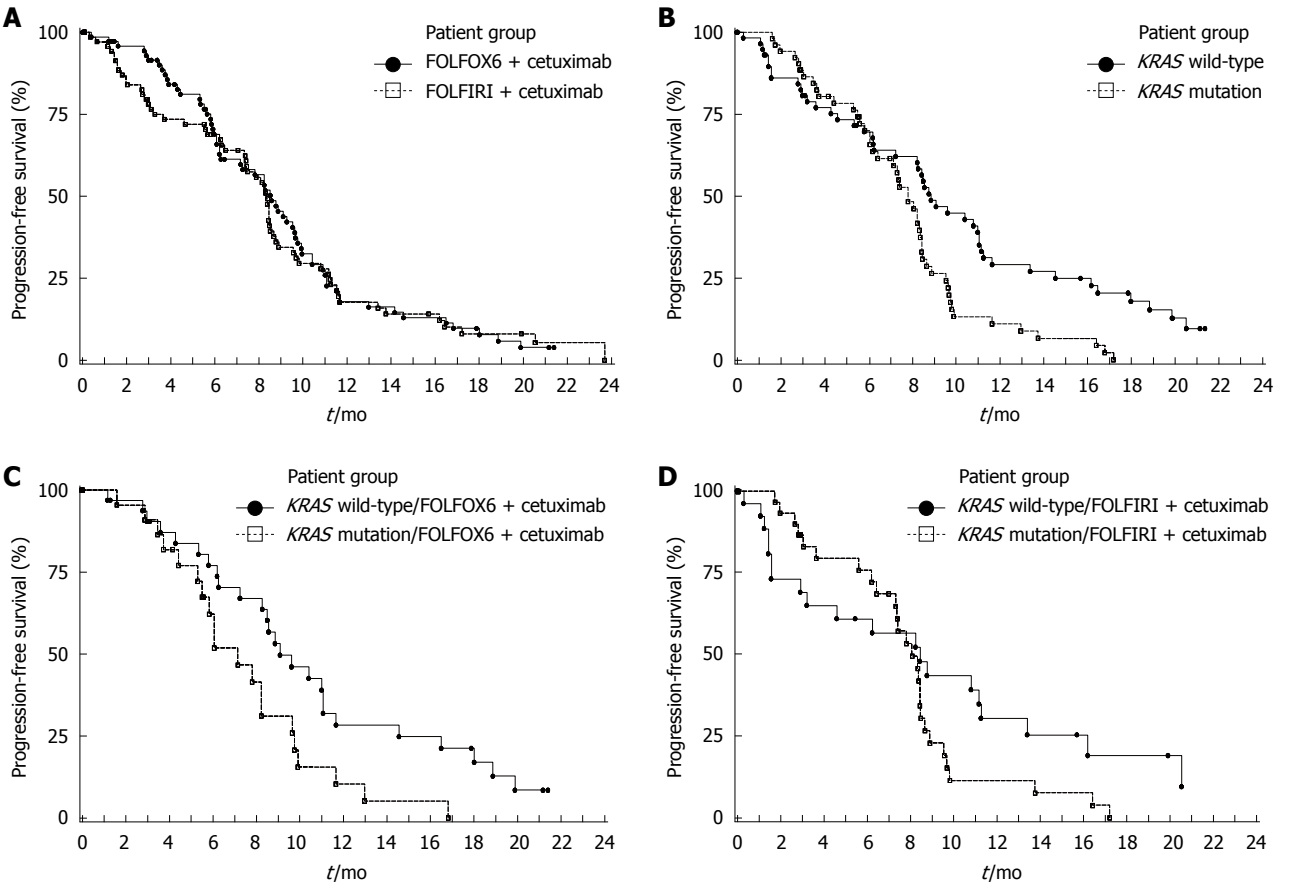

Figure 2 Kaplan Meier estimates for progression-free survival. A: By treatment group in the ITT population, FOLFOX6 plus cetuximab (*n* = 77) *vs* FOLFIRI plus cetuximab (*n* = 74); B: By *KRAS* mutation status in the *KRAS* population, *KRAS* wild-type (*n* = 62) *vs KRAS* mutation (*n* = 55); C: By tumor *KRAS* mutation status in patients receiving FOLFOX6 plus cetuximab, *KRAS* wild-type (*n* = 34) *vs KRAS* mutation (*n* = 23); D: By tumor *KRAS* mutation status in patients receiving FOLFIRI plus cetuximab, *KRAS* wild-type (*n* = 28) *vs KRAS* mutation (*n* = 32).

that for the safety population. The proportion of patients experiencing dose reductions, delays in treatment, and treatment discontinuations for cetuximab or chemotherapy in each treatment arm by *KRAS* mutation status was not markedly different and was comparable with that found in the safety population.

Efficacy

Efficacy data for the ITT population are summarized in Table 3. The 9-mo PFS rate was 11% higher in arm A than arm B (45% *vs* 34%); however, the 95% CI for the difference was -6% to 28%, indicating no significant difference. The risk of disease progression (Figure 2A), death (Figure 3A) and the ORR were also similar between treatment arms.

The influence of tumor *KRAS* mutation status on clinical outcome is summarized in Table 4. The 9-mo PFS rate was higher and the risk of disease progression was significantly reduced in patients with *KRAS* wild-type tumors compared with those with *KRAS* mutations (Figure 2B). A significant improvement in survival (Figure 3B) and an increase in ORR were also demonstrated in patients with *KRAS* wild-type tumors compared with *KRAS* mutated tumors. In multivariate analyses of the *KRAS* evaluable population using Cox proportional hazard models (baseline characteristics and acne-like rash), only *KRAS* tumor mutation status (wild-type *vs* mutant) was identified as a significant prognostic indicator for prolonged PFS (HR = 0.55, $P = 0.006$), while *KRAS* mutation status (wild type *vs* mutant, HR = 0.51, $P = 0.003$), prior adjuvant/neoadjuvant therapy (no *vs* yes, HR = 0.32, $P < 0.001$) and acne-like rash during the first 6 wk (grade 2-3 *vs* grade 0-1, HR = 0.47, $P = 0.004$) were significant independent prognostic indicators for prolonged overall survival.

In treatment arm A, the 9-mo PFS rate in patients with *KRAS* wild-type tumors was higher and the PFS time was significantly longer compared with patients with *KRAS* mutated tumors (Table 4, Figure 2C). In arm B, the 9-mo PFS rate was also higher in *KRAS* wild-type patients, although the PFS time was not significantly different compared with patients with *KRAS* mutated tumors (Figure 2D, Table 4). Similarly, in treatment arm A, survival time was significantly higher in patients with *KRAS* wild-type tumors compared with tumors with *KRAS* mutations (Figure 3C, Table 4). In arm B survival time was not significantly different according to tumor *KRAS* mutation status (Figure 3D, Table 4). In both treatment arms the ORR was higher in patients with *KRAS* wild-type tumors compared with *KRAS* mutated tumors (Table 4).

In treatment arm A *vs* B; median PFS was 8.2 *vs* 8.4 mo

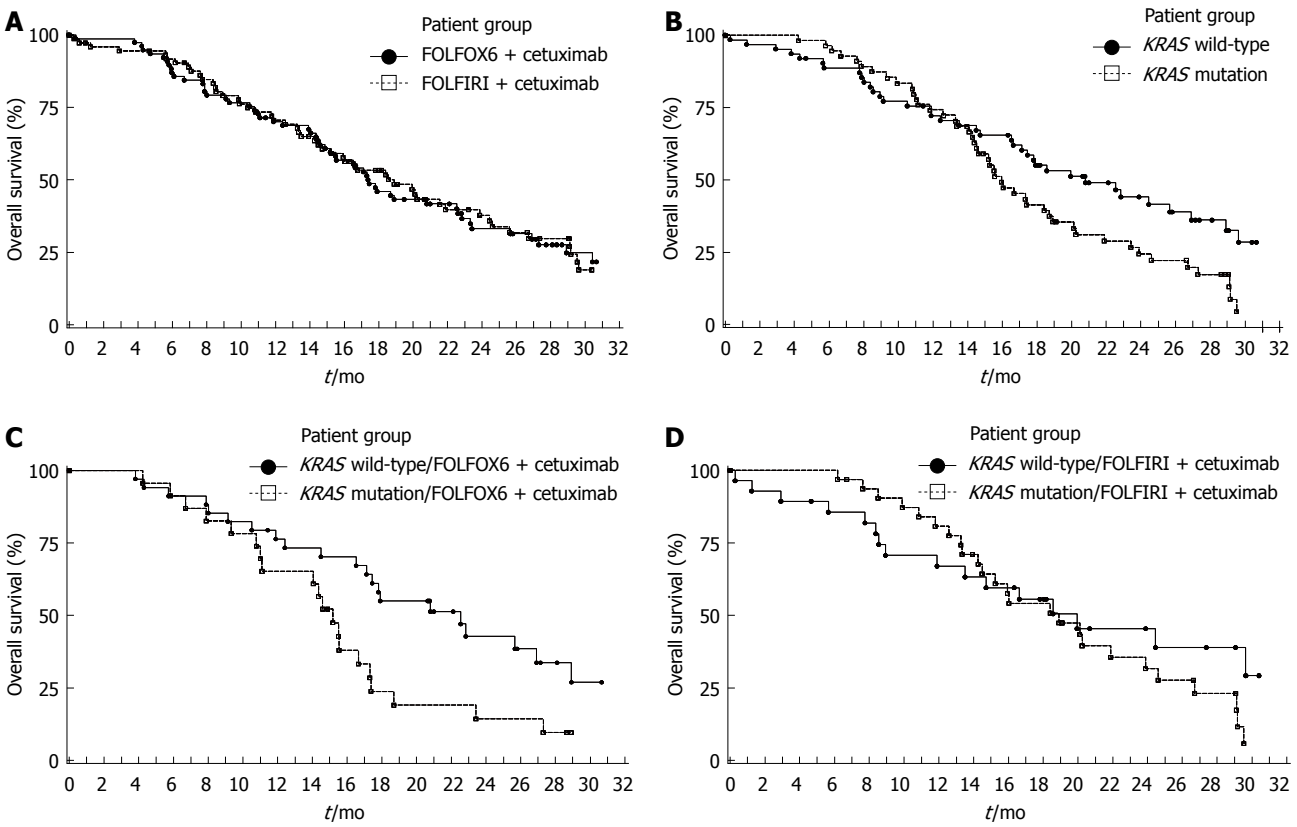

Figure 3 Kaplan Meier estimates for survival. A: By treatment group in the ITT population, FOLFOX6 plus cetuximab (*n* = 77) *vs* FOLFIRI plus cetuximab (*n* = 74); B: By tumor *KRAS* mutation status, KRAS wild-type (*n* = 62) *vs KRAS* mutation (*n* = 55); C: By *KRAS* mutation status in patients receiving FOLFOX6 plus cetuximab, *KRAS* wild-type (*n* = 34) *vs KRAS* mutation (*n* = 23); D: By tumor *KRAS* mutation status in patients receiving FOLFIRI plus cetuximab, *KRAS* wild-type (*n* = 28) *vs KRAS* mutation (*n* = 32).

in patients with EGFR-detectable tumors (*n* = 43 *vs n* = 46), and 11.0 mo *vs* 8.1 mo in patients with EGFR-undetectable tumors (*n* = 17 *vs n* = 12). Median OS was also comparable by EGFR tumor status between the treatment groups. In arm A *vs* arm B; median OS was 15.5 mo *vs* 21.6 mo in patients with EGFR-detectable tumors and 23.3 mo *vs* 17.6 mo in patients with EGFR-undetectable tumors.

Adverse events

The number of patients experiencing serious AEs was balanced between the treatment groups (27% in arm A *vs* 28% in arm B). The frequencies of the most common treatment emergent AEs (TEAE) in arm A *vs* arm B were: neutropenia (47% *vs* 36%); nausea (40% *vs* 26%); diarrhea (44% *vs* 58%); rash (36% *vs* 34%); vomiting, (26% *vs* 23%); stomatitis (22% *vs* 18%); dermatitis acneiform (21% *vs* 23%); anorexia (22% *vs* 20%), pyrexia (22% *vs* 20%). Peripheral neuropathy was reported only in arm A (13%).

Grade 3/4 TEAEs related to study treatment (Table 5) were slightly higher in arm A than in arm B. Grade 4 neutropenia occurred more frequently in patients in arm A than in patients in arm B. The incidence of the special AEs, acne-like rash and infusion-related reactions (composite categories), was not significantly different between the treatment groups (Table 5).

Fifty-four deaths (70%) were reported for patients in arm A and 50 deaths (68%) in arm B. Ten deaths (13%) occurred on-treatment or within 60 d after the last dose in arm A and six (8%) in arm B. None of these deaths were assessed as being due primarily to treatment. Progressive disease and death related to disease complications were the most common reasons for death in both treatment groups.

The frequencies of serious AEs by *KRAS* tumor mutation status and treatment group were similar across the 4 groups (29%-31%). The only noteworthy finding was the relatively low incidence of related grade 3/4 AEs in *KRAS* wild-type patients treated with FOLFIRI plus cetuximab (36%) in comparison to the other 3 groups (56%-71%), mainly due to a lower incidence of neutropenia (Table 5). However, the low sample size in the subgroup analysis should be taken into account, when considering this finding.

DISCUSSION

No significant differences in efficacy were found for cetuximab combined with FOLFOX6 or FOLFIRI in the first-line treatment of mCRC. Efficacy data in the current study are comparable with those reported from the corresponding arms of the CRYSTAL (median PFS of 8.9 mo, median overall survival of 19.9 mo and an ORR of 47%)[13] and OPUS studies (median PFS value of 7.2 mo and an ORR of 46%)[14].

The *KRAS* evaluable population was representative of the ITT population. The *KRAS* tumor mutation

Table 4 Efficacy in the *KRAS* population

Parameter	*KRAS* population		FOLFOX6 plus cetuximab (arm A)		FOLFIRI plus cetuximab (arm B)	
	KRAS wild-type (*n* = 62)	*KRAS* mutation (*n* = 55)	*KRAS* wild-type (*n* = 34)	*KRAS* mutation (*n* = 23)	*KRAS* wild-type (*n* = 28)	*KRAS* mutation (*n* = 32)
PFS						
Events, *n* (%)	46 (74)	47 (85)	26 (76)	20 (87)	20 (71)	27 (84)
Median[1], mo (95% CI)	8.9 (7.3-11.1)	7.8 (6.4-8.4)	9.1 (8.3-11.1)	7.2 (5.5-9.7)	8.4 (3.2-11.3)	8.1 (7.3-8.5)
Logrank *P*-value	0.0051		0.0196		0.1737	
HR[2] (95% CI)	0.55 (0.36-0.84)		0.49 (0.27-0.91)		0.66 (0.36-1.21)	
PFS rate[1], % (95% CI)						
3 mo	81 (70-91)	88 (80-97)	90 (80-100)	91 (79-100)	69 (51-87)	87 (75-99)
6 mo	70 (58-82)	70 (57-83)	77 (62-92)	62 (41-83)	61 (42-80)	76 (60-91)
9 mo	49 (35-62)	26 (14-39)	53 (35-71)	31 (11-52)	43 (24-63)	23 (7-39)
12 mo	29 (17-41)	11 (2-20)	28 (12-45)	10 (0-24)	30 (12-49)	11 (0-24)
Overall survival						
Events, *n* (%)	37 (60)	45 (82)	21 (62)	20 (87)	16 (57)	25 (78)
Median[1], mo (95% CI)	20.8 (16.6-26.9)	15.9 (14.4-18.9)	22.5 (17.1-28.9)	15.2 (11.1-17.3)	19.9 (11.9-na)	18.9 (14.5-23.9)
Logrank *P*-value	0.0296		0.0201		0.3608	
HR[2] (95% CI)	0.62 (0.40-0.96)		0.48 (0.26-0.90)		0.74 (0.39-1.40)	
Survival rate[1] (95% CI)						
9 mo	79 (69-89)	87 (78-96)	85 (73-97)	83 (67-98)	71 (54-88)	90 (80-100)
12 mo	72 (61-83)	74 (63-86)	76 (62-91)	65 (46-85)	67 (49-85)	81 (67-95)
18 mo	55 (42-68)	41 (28-55)	55 (38-72)	24 (6-42)	56 (37-74)	54 (36-72)
24 mo	44 (31-57)	24 (12-36)	43 (25-61)	14 (0-29)	45 (26-65)	32 (14-49)
Best overall response, *n* (%)						
CR	6 (10)	1 (2)	2 (6)	-	4 (14)	1 (3)
PR	27 (44)	19 (35)	17 (50)	7 (30)	10 (36)	12 (38)
SD	14 (23)	26 (47)	9 (26)	12 (52)	5 (18)	14 (44)
PD	8 (13)	6 (11)	3 (9)	3 (13)	5 (18)	3 (9)
NE	7 (11)	3 (5)	3 (9)	1 (4)	4 (14)	2 (6)
ORR, *n* (%)	33 (53)	20 (36)	19 (56)	7 (30)	14 (50)	13 (41)
95% CI	40-66	24-50	38-73	13-53	31-69	24-59
Odds ratio (95% CI)	1.99 (0.95-4.18)		2.90 (0.95-8.84)		1.46 (0.53-4.07)	

[1]Median time and rates are based on Kaplan Meier estimates; [2]Hazard ratio and corresponding 95% CI based on unadjusted Cox proportional hazard model: Hazard rate *KRAS* mutation divided by *KRAS* wild-type. na: Not available; ORR: Objective response rate.

frequency (47%) was similar to that previously reported for mCRC[13,14,27]. Across the treatment groups, PFS and overall survival were significantly improved and there was an increased chance of a tumor response in patients with *KRAS* wild-type tumors compared with *KRAS* mutant tumors; differences in PFS and survival appeared to increase over time. Multivariate analysis also confirmed that tumor *KRAS* mutation status is a prognostic marker for PFS and overall survival after adjustment by other independent predictors such as acne-like rash in the first 6 wk.

Patients with *KRAS* wild-type tumors receiving cetuximab plus FOLFOX6 demonstrated significantly improved PFS and overall survival and an increased chance of tumor response compared with patients with *KRAS* mutated tumors. Similar findings were reported in the OPUS study in patients receiving cetuximab plus FOLFOX4, where patients with *KRAS* wild-type tumors had a reduced risk of disease progression (HR 0.45, $P = 0.0009$) and a higher response rate (61% *vs* 37%) compared with those with tumor mutations[14]. However in the present study for patients receiving FOLFIRI plus cetuximab, no significant benefit was apparent with regard to PFS, survival or ORR according to *KRAS* tumor mutation status. This contrasts somewhat with the CRYSTAL study, where a significant clinical benefit was associated with the addition of cetuximab to FOLFIRI in patients with *KRAS* wild-type tumors, but not in patients with *KRAS* mutant tumors[13]. This non-significance may be due to the comparatively small sample size in the *KRAS* subgroup analysis in the present study compared with the CRYSTAL study[13]. It should also be noted that the difference in the predictive power of *KRAS* tumor mutation status in patients receiving FOLFOX with cetuximab compared with those receiving FOLFIRI with cetuximab described here is consistent with the *KRAS* analysis from CRYSTAL and OPUS studies[13,14,26]. Furthermore, FOLFIRI and cetuximab were given until disease progression in the CRYSTAL study, whereas FOLFIRI was given for 6 mo and cetuximab until progression in the present study. Within this context it is noteworthy that the PFS curves cross after 6 mo in the FOLFIRI subgroup.

In the absence of chemotherapy-alone control arms, the present study was not able to accurately assess the influence of *KRAS* mutation status on clinical outcome for cetuximab or chemotherapy-alone as individual treatment components. The influence of *KRAS* tumor mutation status on patients treated with 5-FU-based chemotherapy remains controversial. The MRC FOCUS study of mCRC patients randomized to receive first-line 5-FU, 5-FU plus irinotecan or 5-FU plus oxaliplatin, reported that patients whose tumors harbored *KRAS* tumor mutations displayed significantly worse survival than those with *KRAS* wild-

Table 5 Grade 3/4 adverse events related to study treatment and special adverse event categories in the safety and *KRAS* populations *n* (%)

Adverse event	FOLFOX6 plus cetuximab (arm A)		FOLFIRI plus cetuximab (arm B)	
	Grade 3/4[a]	Grade 4	Grade 3/4[a]	Grade 4
Safety population[b]				
Any related AE	48 (62)	12 (16)	37 (50)	6 (8)
Neutropenia	22 (29)	9 (12)	15 (20)	4 (5)
Diarrhea	7 (9)	-	9 (12)	-
Rash	5 (6)	-	3 (4)	-
Dermatitis acneiform	4 (5)	-	2 (3)	-
Special AE categories				
Skin reactions[c]	11 (14)	-	6 (8)	-
Acne-like rash[d]	10 (13)	-	6 (8)	-
Infusion-related reactions[e]	5 (6)	2 (3)	1 (1)	1 (1)
Allergy/anaphylaxis	5 (6)	2 (3)	1 (1)	1 (1)
KRAS wild-type population[f]				
Any related AE	24 (71)	5 (15)	10 (36)	1 (4)
Neutropenia	12 (35)	3 (9)	3 (11)	1 (4)
Diarrhea	3 (9)	-	2 (7)	-
Dermatitis acneiform	3 (9)	-	-	-
Mucosal inflammation	3 (9)	-	-	-
Rash	2 (6)	-	-	-
Neuropathy peripheral	2 (6)	-	-	-
Hypersensitivity	2 (6)	1 (3)	-	-
Special AE categories				
Skin reactions[c]	6 (18)	-	1 (4)	-
Acne-like skin rash[d]	5 (15)	-	1 (4)	-
Infusion-related reactions[e]	2 (6)	1 (3)	-	-
Allergy/anaphylaxis	2 (6)	1 (3)	-	-
KRAS mutation population[g]				
Any related AE	14 (61)	4 (17)	18 (56)	4 (13)
Neutropenia	6 (26)	3 (13)	9 (28)	2 (6)
Diarrhea	3 (13)	-	4 (13)	-
Thrombocytopenia	2 (9)	-	-	-
Rash	1 (4)	-	2 (6)	-
Mucosal inflammation	-	-	2 (6)	-
Dehydration	-	-	2 (6)	
Special AE categories				
Skin reactions[c]	2 (9)	-	3 (9)	-
Acne-like rash[d]	2 (9)	-	3 (9)	-
Infusion-related reactions[e]	2 (9)	1 (4)	1 (3)	1 (3)
Allergy/anaphylaxis	2 (9)	1 (4)	1 (3)	1 (3)

[a]Grade 3/4 adverse events occurring in ⩾ 5% of patients in either treatment group in each population are reported; [b]Safety population: 77 patients received FOLFOX6 plus cetuximab in arm A, 74 patients received FOLFIRI plus cetuximab in arm B; [c-e]Composite categories. Skin reactions include the terms: acne*, acne pustular*, cellulitis, dermatitis acneiform*, dry skin*, erysipelas, erythema*, face edema, folliculitis*, hair growth abnormal, hypertrichosis, nail bed infection, nail disorder, nail infection, paronychia, pruritus*, rash*. Of these, all terms marked with an asterisk constituted the acne-like rash subset of the skin reaction category. Infusion-related reactions refer to special adverse-event categories, including the MedDRA preferred terms: acute myocardial infarction, acute respiratory failure, anaphylactic reaction, **anaphylactic shock, **anaphylactoid reaction, **anaphylactoid shock, **angina pectoris, apnea, bronchial obstruction, bronchospasm, cardiac failure, cardiopulmonary failure, chills, clonus, convulsion, cyanosis, drug hypersensitivity, **dyspnea, dyspnea at rest, dyspnea exacerbated, dyspnea exertional, epilepsy, hyperpyrexia, hypersensitivity, **hypotension, **hypoxia, infusion-related reaction, loss of consciousness, myocardial infarction, myocardial ischemia, orthopnea, pyrexia, respiratory distress, respiratory failure, shock, sudden death, syncope. A double asterisk refers to those included regardless of when they occurred, all other terms were included only if the onset of the adverse event occurred on the same day as the first administration of cetuximab; [g]Patients with *KRAS* wild-type tumors: 34 in arm A, 28 in arm B; [f]Patients whose tumors harbored *KRAS* mutations: 23 in arm A, 32 in arm B. AE: Adverse event.

type tumors (HR = 1.24, P = 0.08)[28]. In contrast, in patients treated in the FOLFIRI-alone arm in the CRYSTAL trail, *KRAS* mutation status was not associated with clinical outcome. Furthermore, in the large PETACC-3 trial of stage Ⅱ and Ⅲ colon cancer patients treated with adjuvant chemotherapy, *KRAS* tumor mutation status was found not to be of prognostic value[29]. The data from the present study lends support to the findings from retrospective analyses of randomized trials that demonstrated a lack of efficacy of cetuximab (either in combination with chemotherapy or best supportive care) in the treatment of mCRC patients with *KRAS* mutant tumors[13,14,19], adding to the view that *KRAS* tumor mutation status is predictive of resistance to EGFR-targeted antibodies.

No marked difference in efficacy between the treatment groups for patients with EGFR-undetectable and EGFR-detectable tumors was found. Whilst this result should be treated with caution given the low numbers of EGFR-undetectable patients, the efficacy of cetuximab in combination with chemotherapy in EGFR-undetectable tumors has been reported previously[30,31].

The combination of FOLFIRI with cetuximab was generally better tolerated than FOLFOX6 plus cetuximab with regard to grade 3/4 related AEs, and the frequency of study withdrawal being slightly higher in the latter group. The observed chemotherapy toxicity profiles are similar to those previously reported[32]. AEs associated with cetuximab were typically acne-like skin rash, which was observed with both chemotherapy combinations. *KRAS* tumor mutation status did not appear to markedly influence the toxicity profiles of either treatment regimen as would be expected and as previously reported[26].

In summary, this CECOG study shows that combinations of cetuximab with either FOLFOX6 or FOLFIRI have similar efficacy and acceptable toxicity profiles, in the first-line treatment of patients with unresectable mCRC. Analyses of tumor *KRAS* mutational status demonstrated cetuximab in combination with chemotherapy to have an increased treatment effect on tumor response, overall survival and PFS in patients with *KRAS*-wild-type tumors compared with those with *KRAS* mutated tumors. Whether there is a stronger predictive effect of *KRAS* mutation status in patients treated with cetuximab plus FOLFOX6 compared with cetuximab plus FOLFIRI requires further investigation.

ACKNOWLEDGMENTS

We would like to thank all patients participating in this trial and their families. We also acknowledge the following colleagues from centers in the participating countries for enrolling patients: Austria: Professor Felix Keil, LKH-Leoben, Abt. f. Innere Med., Leoben; Czech Republic: Dr. Lubos Petruzelka, Charles University, Department of Oncology Prague; Dr. Igor Kiss, Department of Clinical Oncology, University Hospital Brno; Israel: Professor Moshe Inbar, Department of Oncology, Tel-Aviv Medical Center, Tel-Aviv; Dr. Ayahla Hubert, Hadassah Medical Center, Jerusalem; Portugal: Dr. Sergio Barroso, Hospital de Beja,

Servico de Oncologia, Beja; Dr. Maria Amélia Monteiro, Hospital San Teotóno Viseu; Dr. Carlos Carvalho, Hospital Amadora Sintra, Servico de Oncologia, Amadora; Dr. Moreira Pinto, Hospital Geral de Sto. António, Porto; Dr. Irene Furtado, Hospital Distrital de Faro, Faro; Romania: Professor Dr. Rodica Anghel, Institutul Oncologic Bucuresti, Bucuresti.

COMMENTS

Background

The standard first-line treatment for patients with metastatic colorectal cancer (mCRC) is a combination of 5-fluorouracil (5-FU) and folinic acid (FA) with either irinotecan or oxaliplatin. The addition of cetuximab, one of a new class of drug known as biological therapeutics, to both these regimens has led to an increase in efficacy in some studies. This is even more pronounced in patients who do not carry a mutation in the *KRAS* gene (some 60% of the CRC population). No direct comparison has been made between the efficacy of these two regimens and the current study was undertaken to address this.

Research frontiers

Cetuximab is one of a number of biological therapies which have the potential to improve the outcome of patients with mCRC. However, as with all treatments, some patients respond well to this therapy while others do not. The current research hotspot is to identify key biomarkers which will predict the treatment to which patients are more likely to respond. The *KRAS* gene is one such biomarker and many others are under investigation.

Innovations and breakthroughs

A number of studies have produced encouraging results for combinations of cetuximab with various regimens containing 5-FU, FA and irinotecan or 5-FU, FA and oxaliplatin for the first-line treatment of mCRC. The current CECOG study was important in that it directly compared cetuximab combined with 5-FU FA and irinotecan (FOLFIRI) and 5-FU FA and oxaliplatin (FOLFOX) and showed that there was no statistical difference in efficacy between the two regimens in this setting. Efficacy data in the current study are also comparable with those reported from the corresponding arms of the CRYSTAL (cetuximab plus FOLFIRI) and OPUS studies (cetuximab plus FOLFOX). Of further interest is the retrospective analysis of *KRAS* data, which demonstrated that the 9-mo PFS rate was higher and the risk of disease progression was significantly reduced in patients receiving cetuximab in combination with chemotherapy who had *KRAS* wild-type tumors compared with those with *KRAS* mutations.

Applications

The results of this study suggest that cetuximab plus FOLFIRI and cetuximab plus FOLFOX are equally effective in treating patients with mCRC. The data consolidate the view that cetuximab in combination with standard chemotherapy should be tailored to patients with *KRAS* wild-type mCRC. Whether there is a stronger predictive effect of *KRAS* mutation status in patients treated with cetuximab plus FOLFOX6 compared with cetuximab plus FOLFIRI requires further investigation.

Peer review

This is a well written manuscript which describes the effect of cetuximab combined with FOLFOX6 or FOLFIRI. The authors report that in general, in patients with *KRAS* wild-type tumors the treatment was more effective compared with patients with *KRAS* mutated tumors.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3144

World J Gastroenterol 2010 July 7; 16(25): 3144-3152
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Celiac disease serology in patients with different pretest probabilities: Is biopsy avoidable?

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Supported by (in part) A Grant from the Consejo de Investigación en Salud del Ministerio de Salud del Gobierno Autónomo de la Ciudad de Buenos Aires, Argentina

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Received: February 4, 2010 Revised: March 22, 2010
Accepted: March 29, 2010
Published online: July 7, 2010

Abstract

AIM: To establish the diagnostic performance of several serological tests, individually and in combination, for diagnosing celiac disease (CD) in patients with different pretest probabilities, and to explore potential serological algorithms to reduce the necessity for biopsy.

METHODS: We prospectively performed duodenal biopsy and serology in 679 adults who had either high risk (n = 161) or low risk (n = 518) for CD. Blood samples were tested using six assays (enzyme-linked immunosorbent assay) that detected antibodies to tissue transglutaminase (tTG) and deamidated gliadin peptide (DGP).

RESULTS: CD prevalence was 39.1% in the high-risk population and 3.3% in the low-risk group. In high-risk patients, all individual assays had a high diagnostic efficacy [area under receiving operator characteristic curves (AU ROC): 0.968 to 0.999]. In contrast, assays had a lower diagnostic efficacy (AU ROC: 0.835 to 0.972) in the low-risk group. Using assay combinations, it would be possible to reach or rule out diagnosis of CD without biopsy in 92% of cases in both pretest populations. We observed that the new DGP/tTG Screen assay resulted in a surplus compared to more conventional assays in any clinical situation.

CONCLUSION: The DGP/tTG Screen assay could be considered as the best initial test for CD. Combinations of two tests, including a DGP/tTG Screen, might be able to diagnose CD accurately in different clinical scenarios making biopsy avoidable in a high proportion of subjects.

Key words: Celiac disease; Serology; Gliadin peptide antibodies; Tissue transglutaminase; Antigliadin antibodies; Small bowel biopsy; Diagnostic accuracy

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INTRODUCTION

The current criterion for diagnosing celiac disease (CD) is mainly based on the presence of a characteristic enteropathy in an intestinal biopsy and evidence that these changes are gluten-triggered[1,2]. While intestinal biopsy is still considered necessary for diagnosing CD, the presence of positive CD-specific serology tests associated with enteropathy are the most commonly used surrogate markers for gluten dependency of damage[3,4]. The assessment of intestinal biopsies requires a certain level of expertise and skill, and the diagnostic accuracy can be affected by variability in sample quality and subjective interpretation[5]. Thus, while pathologists specialized in gastroenterology might have difficulties interpreting mild forms of damage; general pathologists might be susceptible to misdiagnosis, especially if a severe villous atrophy is not present[6,7].

Serological tests have been used for more than 20 years as valuable markers for screening candidates for the need of a duodenal biopsy. Clinical research has demonstrated very high sensitivity and specificity in the detection of IgA antibodies to human tissue transglutaminase [anti-tissue transglutaminase (a-tTG) and anti-endomysial antibodies (EmA)], which are now widely used for identifying patients who might require biopsy, or as a non-invasive confirmation of the CD enteropathy[6-10]. More recently, a new generation of promising assays detecting the presence of deamidated synthetic peptides of gliadin (a-DGP) have shown very high diagnostic performance equivalent to conventional tests[11,12].

As the difficulties in histological diagnosis of CD in clinical practice have been well documented[4,13,14], the appropriate use of simpler and more accurate tools would add reliability to the diagnosis of a condition with high comorbidity and mortality[15]. A new diagnostic standard based on serology alone, which could accurately detect patients in any clinical setting, has been previously suggested[16]. Thus, based on the very high positive predictive values (PPVs) of reliable serological tests, some authors have proposed that intestinal biopsy could no longer be mandatory for the diagnosis of CD in some patients[16-19].

The utility of serological tests finding the search for a new reliable and accurate diagnostic standard for CD requires additional analysis[20]. To obtain the best serological algorithm, studies need to evaluate as many tests and combinations of tests as possible. Furthermore, they should assess a diversity of patient populations, taking in consideration different relevant aspects, such as the disease prevalence and the magnitude of intestinal mucosal damage[10,21-23]. Our aims in this prospective study were threefold: (1) to determine the diagnostic effectiveness of a complete panel of CD-specific serological tests as compared against CD enteropathy in two groups of patients with different risk characteristics; (2) to analyze the performance of individual tests and two-test combinations; and (3) to explore the performance of serology-based algorithms that can potentially obviate the need for intestinal biopsies for diagnosis. The gold standard used in the study to confirm the CD diagnosis was the presence of enteropathy in duodenal biopsies, as assessed by expert gastrointestinal (GI) pathologist.

MATERIALS AND METHODS

Study design

This is a prospective, cross-sectional study on the predictive performance of a set of CD-related antibodies in the diagnosis of CD in two patient populations with different pretest probabilities for having the disorder. All study patients underwent an intestinal biopsy, regardless of the clinical, laboratory and endoscopic findings. For the serological tests, all serum samples were obtained from study subjects before the endoscopy. Endoscopy and biopsies were performed at the endoscopy units of two tertiary care institutions: the "Dr. Carlos Bonorino Udaondo" Gastroenterology Hospital in Buenos Aires and the Endoscopy Service at the HIGA "San Martín" Hospital of La Plata, Buenos Aires Province, Argentina.

Subjects

Between December 2004 and December 2006, we enrolled and performed intestinal biopsies in 679 adult subjects who underwent upper GI endoscopy. Based on their pretest probability of having CD, subjects were categorized as having either high or low risk for the disorder.

High-risk group: During the study period, 161 consecutive adults with suspected but undiagnosed intestinal disorders were enrolled in the study upon their first visit to the Small Bowel Diseases clinic at "Dr. Carlos Bonorino Udaondo" Gastroenterology Hospital. Inclusion criteria required that patients (1) were referred to the clinic because of a suspected small bowel disorder (diarrhea, weight loss, chronic iron-deficiency anemia, malabsorption signs, *etc.*), (2) had no previously known diagnosis of a GI disorder and (3) signed the informed consent. Patients with CD serology performed before the endoscopy, a previous diagnosis of CD, prior treatment with a gluten-free diet, or a former diagnosis of dermatitis herpetiformis, were

excluded from the study. Some data from this population was reported in a prior study[12]. Here, we report the results of a new test in the same population and the analysis of a combination of tests addressing our aims.

Low-risk group: We randomly selected 518 subjects from patients who had been referred for routine upper GI endoscopy because of nonspecific symptoms not primarily related to CD (heartburn, regurgitation, epigastric pain, non-ulcer dyspepsia, *etc*.). Exclusion criteria were the same as those for the high risk group. The first two patients appointed daily for the Endoscopy Unit who fulfilled the inclusion/exclusion criteria were invited to enroll in the study.

Study endpoints

The CD enteropathy was diagnosed based on currently accepted histological criterion: the presence of a type Ⅲ a or more severe enteropathy according to the Marsh's modified classification[24,25]. The final diagnosis of CD was supported by additional presence of positive anti-tTG antibodies or EmA. In addition, the clinical and/or histological response to a gluten-free diet was assessed when possible, and the typical CD-related HLA DQ was tested in some seronegative patients with enteropathy.

Endoscopic procedure and small bowel histology

All endoscopic procedures were performed by experienced endoscopists who were blinded to the clinical and laboratory data. At least three biopsy samples were obtained from a subject's descendent duodenum at different levels distal to the papilla. Morphological and quantitative assessments (intraepithelial lymphocyte -IEL-density) were performed by one experienced pathologist from one center (A.C.). Morphology was categorized according to the modified Marsh classification[25].

Celiac disease-specific serology

Serum samples were kept frozen at -20℃ until the assay was performed in only one center. The CD-related tests and cut-offs were: (1) a-tTG IgA by enzyme-linked immunosorbent assay (QUANTA Lite™, h-tTG IgA, Inova Diagnostic Inc., San Diego, CA, USA); cut-off at 20 units (U)/mL; (2) IgA and (3) IgG antibodies reacting with deamidated gliadin-derived peptides (IgA and IgG a-DGP), (QUANTA Lite™ DGP IgA or IgA. Inova Diagnostic Inc., San Diego, CA); cut-off: 20 U/mL; (4) IgA + IgG isotypes of a-DGP in a single assay (DGP Dual) (cut-off at 20 U/mL); (5) detection of IgA + IgG isotypes of both a-DGP and a-tTG in a single assay (DGP/tTG Screen) (QUANTA Lite™, h-DGP/tTG Screen. Inova Diagnostic. Cut-off: 20 U/mL); (6) IgA antiactin antibodies (AAA) (QUANTA Lite™ F-Actin IgA. Inova Diagnostic Inc., San Diego, CA; cut-off at 25 U/mL); and (7) total serum IgA by radial immunodiffusion (Diffu-Plate, Biocientífica S.A., BA, Argentina) only for cases with enteropathy but negative IgA serology tests. IgA endomysial antibody (IgA EmA) by immunofluorescence on primate esophagus substrates (INOVA Diagnostics Inc., San Diego, CA) was used only in cases with discrepancies between histology and serology. The characteristics of the tests have been reported in previous studies[11,12]. Positive tests were checked in duplicate assays.

Statistical analysis

Based on data distribution, descriptive data are reported as either mean and standard deviation (SD) or median and range. The diagnostic performance of individual serological tests was determined by calculating the sensitivity, specificity, 95% confidence intervals (95% CI), positive and negative predictive values (PPVs and NPVs), and likelihood ratios using cut-offs provided by the manufacturer and at cut-off values that would give a PPV of 100%. Data were analyzed using MedCalc® version 9.3.8.0 (MedCalc Software; Broekstraat, Mariakerke, Belgium). The area under the receiving operator characteristic curve (AU ROC) and corresponding 95% CIs were determined using MedCalc. When assessing the performance of different assay combinations, a particular combination was considered positive if both tests produced concentrations above the cut-off. Comparisons were performed using the Student's *t* test, Mann-Whitney *U* test, χ^2 test, or Fisher's exact test, as appropriate.

To explore the effectiveness of serology for predicting CD in a theoretical context of reducing the necessity of intestinal biopsy, we compared different algorithms for individual assays and two-assay combinations, in both the high-risk and low-risk groups. For individual assays, we devised an algorithm in which only patients with positive serology results would receive biopsy. For combinations of two tests, we explored an algorithm where a patient would receive a biopsy if only one of the assays was positive while the other was negative. For each algorithm, we estimated the number of true positives, false positives, false negatives, and the proportion (%) of biopsies correctly avoided.

RESULTS

Subject characteristics and CD diagnosis

The demographics and some clinical and histological features of the subjects in both groups are presented in Table 1. Compared to those with a high probability of having CD, subjects with low-risk for the disease had a significantly higher mean body mass index (BMI) ($P < 0.0001$). The prevalence of CD correlated with the pretest probability of the disease. Sixty-three (39.1%) of the 161 patients in the high-risk group were diagnosed with CD. In contrast, 17 (3.3%) of the 518 subjects undergoing routine upper GI endoscopy at the two endoscopic units (low-risk group) had a diagnosis of CD. As expected, newly diagnosed CD patients in the high-risk group had a significantly more severe clinical picture and greater degree of histological damage (*P* values between < 0.001 to < 0.0001) compared to those diagnosed in the low-risk group (Table 1).

Table 1 Demographic, clinical, and histological characteristics of subjects categorized by their pretest probability of having CD and from newly diagnosed CD patients from each subgroup

Characteristic	High pretest	Low pretest	*P* value
No. of subjects enrolled (F/M)	161 (131/30)	518 (351/167)	
Mean age (range), yr	40 (16-80)	46 (16-87)	
No. of CD patients (%)	63 (39.1)	17 (3.3)	
Body mass index			
mean ± SE (kg/m^2)	20.6 ± 3.9	25.2 ± 5.0	< 0.0001
Histological characterization of duodenal biopsy samples (Marsh's modified)[25]			
Type 0 (No. of patients)	97	495	
Type Ⅰ	0	5	
Type Ⅱ	1	1	
Type Ⅲa	4	6	
Type Ⅲb	12	4	
Type Ⅲc	47	7	< 0.0001
Newly diagnosed CD patients			
No. of patients	63	17	
Mean age (range), yr	37 (24-74)	37 (19-72)	
Body mass index			
mean ± SE (kg/m^2)	19.6 ± 3.1	23.6 ± 5.0	< 0.0001
Clinical categorization at diagnosis			
No. of patients (%)			
Classical CD	52	1	< 0.0001
Atypical CD	11	10	< 0.0010
Silent CD	0	6	< 0.0001

CD: Celiac disease.

Performance of serological tests

Individual assays in high-risk group: Some of the data collected from patients in the high-risk group in this study were reported in a previous publication[12]. Data reported here, however, includes results of the newly developed assay (DGP/tTG Screen), explores the value of combinations of two assays, and analyzes the performance of tests using theoretical cut-offs with an absolute (100%) PPV. All newly diagnosed CD patients in the high-risk group had at least one positive serology result.

Table 2 shows that sensitivities for the different assays ranged from 95.2% (for IgA a-tTG) to 100% (for the DGP/tTG Screen), except for the AAA assay (87.3%), which had the worst performance. The IgG a-DGP test had an optimal specificity and PPV (100%). Very high values of AU ROC curves were seen for all individual assays (0.968 to 0.999). Table 2 also shows the performance of assays if the cut-off values had been set to values that would give a PPV of 100%. In most assays, a slight deterioration of the sensitivity was associated with almost absolute specificity. Two assays require further consideration: the IgG a-DGP tests, which had a 100% PPV at the manufacturer's cut-off level (20 U/mL); and the AAA assay, which reached the optimal PPV at a 64 U/mL cut-off, resulting in poor sensitivity (52.4%).

Individual assays in the low-risk group: Table 2 shows the statistical performance of individual assays in the low-risk population. Using the cut-offs provided by the manufacturer, all assays had lower diagnostic efficacy compared with their performance in the high-risk group. (The AU ROC ranged from 0.835 for AAA to 0.972 for the DGP/tTG Screen). The IgA a-DGP and DGP/tTG Screen tests had high sensitivity (82.3%), and all assays had very high specificity (ranging from 88.2% to 99.0%). The PPVs were overall quite low, ranging from 17.6% for AAA to 70.6% for IgG a-DGP. The PPVs for both, the widely used a-tTG (at a cut-off value of 20 U/mL) (50.0%) and the sensitive DGP/tTG Screen (19.2%) were frustratingly low. Once again, the performance of the assays was also determined using cut-off values that would produce a 100% PPV (Table 2). The sensitivity at a 100% PPV cut-off was also disappointing for all assays. For example, the cut-off for the IgA a-tTG would be 139 U/mL, consistent with a sensitivity of 35.3%. The DGP/tTG Screen was the most sensitive test (64.7%) at this cut-off value.

Two-assay combinations in both groups

Table 3 shows the performance of some of the assay combinations in the high-risk group. As expected, the combination tests were more specific, but less sensitive, than individual tests. Furthermore, combinations had excellent AU ROC curves (0.962 to 0.984). Most of these combinations had PPVs of 100% when both assays were positive. Considering the association of two assays in the low-risk group (Table 3), the best sensitivity (82.3%) was achieved by the a-tTG plus the DGP/tTG Screen with an almost absolute specificity (99.0%) and the best NPV and AU ROC curve.

Seronegative patients with mild enteropathy and type Ⅰ damage

In the low-risk group, three patients had mild enteropathy (both with type Ⅲa villous atrophy) but a negative CD-specific serology (one case was positive for AAA and negative for the haplotype DQ2 or DQ8). She had a positive clinical response to the gluten-free diet. The remaining two cases died during the follow-up period: one from an acute myocardial infarction and the other as result of an esophageal malignancy discovered at the time of the endoscopy.

Five other patients had histological features characterized as type I of Marsh's modified classification (IELs count > 30%) and, based on the inclusion criteria, were not categorized as having a CD diagnosis. Two of these patients had a positive serology (IgA a-DGP, IgA a-tTG).

Exploring the value of tests aiming to reduce the necessity of intestinal biopsy

Based on the serology findings, we explored the diagnostic algorithms that might reduce the number of patients required to undergo the invasive diagnostic duodenal biopsy. The algorithm for the use of individual assays would avoid biopsies for patients with negative serology and require biopsy only for those with positive serology. For the theoretical algorithm using combination assays, patients

Table 2 Statistical performance of individual CD serologic tests for high- and low-risk populations at cut-offs provided by the manufacturer and when the cut-off is set for a 100% PPV

Test	% (95% CI)			(%)	
	Sensitivity	Specificity	AU ROC	PPV	NPV
High-risk population					
IgA a-tTG					
(cut-off 20 U/mL)	95.2 (86.7-99.0)	97.9 (92.8-99.7)	0.997 (0.971-0.998)	96.9	96.8
(cut-off 34 U/mL)	93.6 (84.5-98.2)	100.0 (96.3-100.0)	0.968 (0.928-0.989)	100.0	96.0
IgA a-DGP					
(cut-off 20 U/mL)	98.4 (91.4-99.7)	92.7 (85.5-97.1)	0.995 (0.968-0.999)	90.0	98.9
(cut-off 77 U/mL)	87.3 (76.5-94.3)	100.0 (96.3-100.0)	0.995 (0.968-0.999)	100.0	92.5
IgG a-DGP					
(cut-off 20 U/mL)	95.2 (86.7-99.0)	100.0 (96.2-100.0)	0.989 (0.958-0.998)	100.0	97.0
(cut-off 20 U/mL)					
DGP Dual					
(cut-off 20 U/mL)	96.8 (89.0-99.5)	99.0 (94.4-99.8)	0.995 (0.967-0.999)	98.4	97.9
(cut-off 22 U/mL)	96.8 (89.0-99.5)	100.0 (96.3-100.0)	0.984 (0.951-0.997)	100.0	98.0
DGP/tTG Screen					
(cut-off 20 U/mL)	100.0 (94.3-100.0)	92.8 (85.8-97.1)	0.999 (0.976-1.000)	90.3	100.0
(cut-off 54 U/mL)	98.4 (91.4-99.7)	100.0 (96.3-100)	0.992 (0.963-0.999)	100.0	99.0
IgA AAA					
(cut-off 25 U/mL)	87.3 (76.5-94.3)	94.9 (88.5-98.3)	0.968 (0.927-0.989)	91.9	91.8
(cut-off 64 U/mL)	52.4 (39.4-65.0)	100.0 (96.3-100.0)	0.770 (0.697-0.832)	100.0	76.6
Low-risk population					
IgA a-tTG					
(cut-off 20 U/mL)	76.5 (50.1-93.0)	97.4 (95.6-98.6)	0.921 (0.894-0.942)	50.0	99.2
(cut-off 139 U/mL)	35.3 (14.3-61.6)	100.0 (98.9-100.0)	0.706 (0.665-0.745)	100.0	97.8
IgA a-DGP					
(cut-off 20 U/mL)	82.3 (56.6-96.0)	96.2 (94.1-97.7)	0.932 (0.907-0.952)	42.4	99.4
(cut-off 313 U/mL)	35.3 (14.3-61.6)	100.0 (99.3-100.0)	0.706 (0.655-0.745)	100.0	97.9
IgG a-DGP					
(cut-off 20 U/mL)	70.6 (44.1-89.6)	99.0 (97.7-99.7)	0.926 (0.900-0.947)	70.6	99.0
(cut-off 109 U/mL)	29.4 (10.4-55.9)	100.0 (99.3-100.0)	0.676 (0.634-0.717)	100.0	97.7
DGP Dual					
(cut-off 20 U/ml)	76.5 (50.1-93.0)	95.8 (93.7-97.4)	0.963 (0.943-0.978)	38.2	99.2
(cut-off 77 U/mL)	47.1 (23.0-72.1)	100.0 (99.3-100.0)	0.735 (0.695-0.773)	100.0	98.2
DGP/tTG Screen					
(cut-off 20 U/mL)	82.3 (56.6-96.0)	88.2 (85.1-90.9)	0.972 (0.954-0.984)	19.2	99.3
(cut-off 128 U/mL)	64.7 (38.4-85.7)	100.0 (98.9-100.0)	0.824 (0.788-0.855)	100.0	97.8
IgA AAA					
(cut-off 25 U/mL)	52.9 (27.9-77.0)	91.6 (88.8-93.9)	0.835 (0.800-0.866)	17.6	98.3
(cut-off 106 U/mL)	29.4 (10.4-55.9)	100.0 (99.3-100.0)	0.647 (0.604-0.688)	100.0	97.7

The statistical performance of tests if concentrations are above the cutoff. PPV: Positive predictive value; NPV: Negative predictive value; tTG: Tissue transglutaminase; DGP: Deamidated gliadin peptide; AAA: IgA antiactin antibodies; AU ROC: Area under the ROC curve.

would not have a biopsy if both assays were congruent (either both positive or both negatives), but would have a biopsy only if the two serology results disagree (one positive and the other negative).

Table 4 shows the performance of the algorithm for single assays. In the high-risk group, the percentage of cases that would not require biopsy ranged from 56.5% to 62.7%, and the single use of DGP/tTG Screen would not miss any CD case. In the low-risk group, the use of single assays would avoid biopsy in most cases (90.3% to 96.7%). However, a substantial number of CD diagnoses (three to eight cases) would be missed, mainly because three CD patients were negative for all serology tests.

The simultaneous combination of two assays for the high-risk group would allow significant reduction in the percentage of intestinal biopsies (92.0% to 98.7%) with no case missed for any of the following four pairs of tests (a-tTG + IgG a-DGP, a-tTG + IgA a-DGP, a-tTG + DGP Dual and a-tTG + DGP/tTG Screen) (Table 5). The use of assay combinations in the low-risk group would result in a similarly high proportion of biopsies avoided (92.1% to 99.0%), but three to five CD cases would be missed.

DISCUSSION

Small bowel histology is still considered the gold standard for diagnosing CD, notwithstanding the fact that the morphological features are not specific, and that other conditions can produce similar findings[1,2]. The possibility of a noninvasive diagnostic algorithm for CD has been explored before[16-19], but no definitive standard has been established yet. Our first aim was to assess the diagnostic performance of serological tests in two patient groups

Table 3 Statistical performance of combinations of two tests in the high- and low-risk populations at the cut-off provided by the manufacturer

Test	% (95% CI)			(%)	
	Sensitivity	Specificity	AU ROC	PPV	NPV
High-risk					
IgA a-DGP + IgA a-tTG	93.6 (84.5-98.2)	99.0 (94.4-99.8)	0.963 (0.921-0.986)	98.4	95.9
IgG a-DGP + IgA a-tTG	90.5 (80.4-96.4)	100.0 (96.3-100)	0.952 (0.907-0.980)	100.0	94.0
DGP Dual + IgA a-tTG	92.0 (82.4-97.3)	100.0 (96.3-100)	0.960 (0.917-0.985)	100.0	95.0
DGP/tTG Screen + IgA a-tTG	95.2 (86.7-99.0)	100.0 (96.3-100.0)	0.976 (0.939-0.994)	100.0	96.9
IgA a-DGP + DGP/tTG Screen	98.4 (91.4-99.7)	96.9 (91.3-99.3)	0.977 (0.940-0.994)	95.4	99.0
IgG a-DGP + IgA a-DGP	95.2 (86.7-99.0)	100.0 (96.3-100.0)	0.976 (0.939-0.994)	100.0	97.0
IgA a-DGP + DGP Dual	96.8 (89.0-99.5)	100.0 (96.3-100.0)	0.984 (0.951-0.997)	100.0	98.0
IgG a-DGP + DGP Dual	95.2 (86.7-99.0)	100.0 (96.3-100.0)	0.976 (0.939-0.994)	100.0	97.0
IgG a-DGP + DGP/tTG Screen	95.2 (86.7-99.0)	100.0 (96.3-100.0)	0.976 (0.939-0.994)	100.0	97.0
DGP Dual + DGP/tTG Screen	96.8 (89.0-99.5)	100.0 (96.3-100.0)	0.984 (0.951-0.997)	100.0	98.0
Low-risk					
IgA a-DGP + IgA a-tTG	72.2 (46.5-90.2)	99.8 (98.9-100.0)	0.860 (0.827-0.889)	92.9	99.0
IgG a-DGP + IgA a-tTG	66.7 (41.0-86.6)	100.0 (99.3-100.0)	0.833 (0.798-0.864)	100.0	98.8
DGP Dual + IgA a-tTG	72.2 (46.5-90.2)	99.8 (99.3-100.0)	0.861 (0.828-0.890)	92.8	99.0
DGP/tTG Screen + IgA a-tTG	72.2 (46.5-90.2)	98.8 (97.4-99.6)	0.855 (0.822-0.884)	68.4	99.0
IgA a-DGP + DGP/tTG Screen	82.3 (56.6-96.0)	99.0 (97.7-99.7)	0.907 (0.878-0.930)	73.6	99.4
IgG a-DGP + IgA a-DGP	70.6 (44.1-89.6)	100.0 (99.3-100.0)	0.853 (0.819-0.882)	100.0	99.0
IgA a-DGP + DGP Dual	76.5 (50.1-93.0)	99.6 (98.6-99.9)	0.880 (0.849-0.907)	86.6	99.2
IgG a-DGP + DGP Dual	70.6 (44.1-89.6)	99.0 (97.7-99.7)	0.848 (0.814-0.878)	70.5	99.0
IgG a-DGP + DGP/tTG Screen	70.6 (44.1-89.6)	99.4 (98.3-99.9)	0.850 (0.816-0.880)	80.0	99.0
DGP Dual + DGP/tTG Screen	76.5 (89.0-99.5)	99.0 (96.3-100.0)	0.877 (0.846-0.904)	72.2	99.2

The statistical performance of the 10 combinations assessed is reported, considering a result to be positive if both tests of the combination have concentrations above the cutoff.

Table 4 Performance of individual assays in both risk populations in the theoretical analysis aiming to avoid duodenal biopsy when serology is negative

Individual serology tests	High-risk		Low-risk	
	Biopsy avoided (%)	Missed CD cases (*n*)	Biopsy avoided (%)	Missed CD cases (*n*)
IgA a-tTG	61.5	3	95.4	4
IgA a-DGP	57.1	1	93.6	3
IgG a-DGP	62.7	3	96.7	5
DGP Dual	61.5	2	96.0	4
DGP/tTG Screen	56.5	0	91.3	3
AAA	62.7	8	90.3	8

with different risk levels of having CD. In this context, we postulated that different assays might perform differently in populations with low pre-test risk for the disease compared to those with high-risk. We hypothesized that this could change the selection of the best serological algorithm to be used in case finding among disorders with increased prevalence of CD (e.g. chronic anemia, osteoporosis, irritable bowel syndrome, *etc.*) or for screening the general population. Notably, the use of serological tests for the selection process of cases in clinical situations with low pretest probabilities has been mostly based on the performance of assays assessed in cohort studies with post-test probabilities greater than 95%.

Considering the high-risk group, we confirmed that all the individual serological assays had very high diagnostic efficacy. Interestingly, our present study shows the DGP/tTG Screen test is the only assay with optimal sensitivity, and only the IgG a-DGP test had 100% specificity and PPV at the cut-off provided by the manufacturer. If the cut-off values were set to obtain 100% PPV, the sensitivity would be minimally reduced for the IgA a-DGP and the DGP/tTG Screen, but more profoundly affected for IgA a-tTG, IgG a-DGP and DGP Dual. The sensitivity for AAA would be reduced from 87.3% to 52.4% with the 100% PPV cut-off, making its use non recommendable in the diagnostic work-up. Therefore, our results highlight the value of the new DGP/tTG Screen, which was the most sensitive assay in detecting CD among subjects with high-risk for the disorder at both cut-offs: the value set by the manufacturers and at a 100% PPV. As far as we know, this is the second study showing the efficacy of the newly developed DGP/tTG Screen test for CD[26].

As we expected, the serological tests did not perform as well in the low-risk group. The sensitivity of individual tests varied between 52.9% and 82.3%, and the highest were again the DGP/tTG Screen and the IgA a-DGP assays. The specificity was high, ranging from 88.2% to 99.0%. As expected, the NPVs were excellent (98.3% to 99.4%) and the PPVs were disappointingly low for all the assays, ranging from 17.6% for the IgA AAA test to 70.6% for the IgG a-DGP. The commonly used IgA a-tTG assay had an unacceptable PPV of 50.0%. Once again, we assessed the performance of individual assays at cut-off values that would result in a 100% PPV. Sensitivity dropped

Table 5 Performance of combinations of two assays in both risk populations exploring the potential avoidance of duodenal biopsy if the procedure is only performed in +/- cases

Combination of two tests	High risk		Low risk	
	Biopsy avoided (%)	Missed CD cases (*n*)	Biopsy avoided (%)	Missed CD cases (*n*)
IgA a-tTG + IgA a-DGP	93.2	0	96.7	4
IgA a-tTG + IgG a-DGP	95.6	0	94.4	3
IgA a-tTG + DGP Dual	95.0	0	96.1	4
IgA-tTG + DGP/tTG Screen	92.0	0	94.4	3
IgA aDGP + DGP/tTG Screen	95.0	2	92.1	3
IgG a-DGP + IgA a-DGP	94.4	1	95.0	3
IgA a-DGP + DGP Dual	94.4	1	95.2	3
IgG a-DGP + DGP Dual	98.7	2	99.0	4
IgG a-DGP + DGP/tTG Screen	93.8	0	93.6	3
DGP Dual + DGP/tTG Screen	93.8	0	93.8	5

significantly to unacceptable values, with the highest being the DGP/tTG Screen at 64.7%. Notably, the sensitivity of the commonly used IgA a-tTG was 35.3%. Overall, these observations for patients with a low-risk for CD (with a prevalence that is intermediate between those disorders at risk and that of the general population) suggest the possibility of underdiagnosis using the most commonly employed serological algorithms. Interestingly, the sensitivity of the assays in the low-risk group was affected by the fact that three of the 17 new patients with villous atrophy (17%) had a minor damage (type IIIa) and were negative for all tests. These cases were considered as having a CD-like enteropathy. We confirmed former observations that a minor degree of damage is frequently detected in patients diagnosed from populations with low pretest probability[27]. Furthermore, our observations on the behavior of serological tests in this group are consistent with former findings showing that CD patients with a minor histological damage might have seronegative results[21,22,25]. However, confirmation of a definitive diagnosis of CD in seronegative cases (and even more in cases with lesser degree of histological damage) requires additional features indicative of gluten dependency that often are difficult to meet. This was the case with the small group identified in this study. Interestingly, one patient had a positive clinical response to the GFD, but was negative for the HLA-DQ2 and DQ8 investigated in the β1 chain. The other two cases died some time after the biopsy without having performed a GFD, one due to a myocardial infarct, and the other due to an esophageal malignancy diagnosed at the time of endoscopy. We estimate that the lack of the specific HLA alleles in the first case minimized the possibility of the patient having a gluten-triggered enteropathy[28]. However, although the inclusion of these three not well defined cases has a negative impact on the performance of serology in the low-risk population, the fact of all patients in our study have had a biopsy evaluation, makes our study a reflection of real clinical practice. We consider that although the diagnosis of these cases is uncertain, they should be included in the analysis, unlike those cases with mild inflammation (Marsh's type I) with a positive serology, which were excluded on the bases of our strict protocol.

To determine if a combination of assays could improve diagnostic accuracy, we explored the performance of all possible combinations of two serological tests, with the condition that a given combination was considered positive or negative if both assays were concordantly above or below the cut-off values, respectively. We observed that the performance of all combinations for the high-risk group was slightly lower than that of single assays, as evidenced by the AU ROC (> 0.960). However, the specificity and PPVs increased to 100% with acceptable sensitivity (above 90.5%) for all possible combinations excluding from this analysis AAA. Furthermore, we observed that combinations of the DGP/tTG Screen with either IgA a-tTG or IgA a-DGP add accuracy to the diagnosis or exclusion of CD.

In the low-risk group, all combinations had poorer performances than the single assays due to a lower sensitivity with minimally increased specificity. However, PPVs improved significantly (with most combinations approaching 100.0%) as expected. Once again, as it was for the high-risk population, the DGP/tTG Screen assay used in combination with the IgA a-DGP exhibited the best performance with acceptable sensitivity (82.3%), optimal specificity, and predictive values. Our observations are in agreement with a recent study from our group that clearly showed that the use of the DGP/tTG Screen assay enhances the sensitivity of detecting gluten sensitivity among a-tTG seronegative patients with CD-like enteropathy (including cases with dermatitis herpetiformis)[29]. Interestingly, it is well-established in clinical practice to use the IgA a-tTG test to select patients for biopsy, in both case-finding processes and in population screening studies. The performance of this popular assay in the low pretest population suggests that its use alone does not seem to be a wise strategy, because it would miss up to 23% of potential new cases.

Based on the present findings, we finally analyzed the number of cases missed or biopsy procedures avoided in the theoretical situation where serology could be used as a single non-invasive tool for diagnosing CD. With this aim, we assessed the effectiveness of two algorithms for the use of a single assay or two-assay combinations. The algorithm for single assays was devised such that biopsy should only be performed for patients with a positive test, as the pretest risk was below 50% in most clinical situations and the number of biopsies avoided would be greater. The second algorithm was designed for combinations of two serological assays, in which biopsy would be omitted if a patients had two positive or negative assays. Biopsy would be reserved for patients with conflicting results.

For the high-risk group, the DGP/tTG Screen assay was the only single test that did not miss any CD cases, and would avoid duodenal biopsy in 56.5% of subjects. In contrast, the algorithm exploring combinations of two assays was highly effective, and could avoid intestinal biopsy in more than 92% of subjects. The higher performance was seen in the combinations using DGP/tTG Screen with other assays. As expected, the serological algorithms

did less well in the low-risk group, as three cases were negatives in all assays. In this population, the two diagnostic algorithms did not differ significantly in terms of false negatives or biopsies avoided. The use of the DGP/tTG Screen and the IgA anti-DGP assays alone, in combination with each other, or in combination with other assays, would miss the three mentioned cases and avoid biopsy in 91.3% to 95.4% of subjects.

In conclusion, we suggest that appropriate use of CD serology might accurately identify the vast majority of CD patients in populations with different pretest probabilities. Furthermore, a negative serology might still miss underlying CD, but the clinical importance of the disease in such patients is probably minimal. The combination of two assays makes diagnostic accuracy higher. However, a proportion of patients (17%) in the low-risk group would be missed by all serology tests or their combinations. Definitive confirmation of CD in these seronegative cases is often difficult and doubtful, sometimes requiring long-term observation. We also confirm the additional value of the new DGP/tTG Screen assay, which resulted in a surplus to more conventional assays and should be considered as the best initial test in investigation for CD. Our study also suggests that this assay in combination with IgA a-tTG or IgA a-DGP could be used to obviate the need for duodenal biopsy in more than 92% of individuals in the high- and low-risk populations. Future validation of the algorithms is required to confirm our findings before new diagnostic guidelines are proposed.

ACKNOWLEDGMENTS

The authors thank Dr. Walter Binder for his generous cooperation.

COMMENTS

Background

Diagnosis of celiac disease is based on a characteristic enteropathy in an intestinal biopsy and evidence that these changes are gluten-triggered. The appropriate use of simpler and more accurate tools would add reliability to the diagnosis of celiac disease. Thus, the celiac disease-related serology might have a key role in defining new diagnostic standards for celiac disease.

Research frontiers

A new diagnostic standard based on serology alone could make intestinal biopsy no longer mandatory for diagnosis of celiac disease. With this purpose in mind, the authors aimed to establish the diagnostic performance of several serological tests, individually and in combination, for diagnosing celiac disease in patients with different pretest probabilities and to explore potential serological algorithms to reduce the necessity for biopsy.

Innovations and breakthroughs

This study demonstrates that the deamidated gliadin peptide (DGP)/tissue transglutaminase (tTG) Screen assay could be considered as the best initial test for suspected CD. The authors also show that combinations of two serology tests, including the DGP/tTG Screen and IgA a-tTG or IgA a-DGP, might be able to diagnose celiac disease accurately in different clinical scenarios, and that they could diagnose or rule out the disorder, avoiding intestinal biopsy in almost 92% of subjects under study.

Applications

This study confirms the diagnostic value of the DGP/tTG Screen assay and proposes that it should be considered as the first line test in the screening algorithm for celiac disease. The combination of two tests, which include the DGP/tTG Screen and either IgA a-tTG or IgA a-DGP, strengthen the serological diagnosis of celiac disease, rendering biopsy unnecessary if both tests are congruent.

Terminology

The DGP/tTG Screen assay is a single kit that assess simultaneously the presence of both antibody isotypes (IgA and IgG), the fully synthetic selectively deamidated gliadin peptide, and the human recombinant tTG.

Peer review

Although the study seems to be an "add-on" to their original work, the methodology is adequate and it merits publication for discussion amongst the wider scientific community and for debate into the merits of saving unnecessary biopsies or in patients where gastroscopies are contraindicated/declined.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3153

World J Gastroenterol 2010 July 7; 16(25): 3153-3160
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Promoter hypermethylation and loss of *CD133* gene expression in colorectal cancers

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Supported by (in part) The Korea Science and Engineering Foundation (KOSEF) funded by the Korean government (MEST R01-2008-000-20108-0)
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Received: March 17, 2010 Revised: April 21, 2010
Accepted: April 28, 2010
Published online: July 7, 2010

Abstract

AIM: To understand CD133 promoter hypermethylation and expression in 32 colorectal cancer cell lines.

METHODS: Nucleic acid was isolated from 32 colorectal cancer cell lines and CD133 expression levels were measured by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. Promoter methylation status of the *CD133* gene was analyzed with a methylation-specific PCR after sodium-bisulfite modification and by clonal sequencing analysis. The correlation between expression and promoter methylation of *CD133* gene was confirmed with treatment of 5-aza-2′-deoxycytidine.

RESULTS: We measured CD133 expression levels in 32 colorectal cancer cell lines. RT-PCR analysis showed undetectable or low levels of CD133 expression in 34.4% of cell lines. To verify the relation between CD133 expression and methylation status of the *CD133* gene promoter in colorectal carcinogenesis, *CD133* gene promoter hypermethylation was analyzed in 32 cancer cell lines. Promoter hypermethylation was detected in 13 (40.6%) of the cell lines using methylation specific-PCR and confirmed by bisulfite sequencing analysis. Treatment of 11 of the cell lines with the demethylation agent 5-aza-2′-deoxycytidine recovered CD133 expression in most of them.

CONCLUSION: Transcriptional repression of CD133 is caused by promoter hypermethylation of the CD133 CpG islands in some of colorectal cancer cell lines. The study may contribute to the understanding of the role of CD133 inactivation in the progression of colorectal cancers.

Key words: CD133; Promoter; Hypermethylation; Colorectal cancer; Sodium bisulfite modification

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Jeon YK, Kim SH, Choi SH, Kim KH, Yoo BC, Ku JL, Park JG. Promoter hypermethylation and loss of *CD133* gene expression in colorectal cancers. *World J Gastroenterol* 2010; 16(25): 3153-3160 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3153.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3153

INTRODUCTION

The cell surface antigen CD133 is a glycoprotein of 858-865 amino acids with a total molecular weight of 97-120 kDa and five transmembrane domains. Originally identified in neuroepithelial stem cells, CD133 has been

detected in hematopoietic stem cells, endothelial progenitor cells, glioblastomas, neuronal and glial stem cells, and some other cell types[1,2]. As it has also been identified in cancer-initiating cells in several solid malignancies, it is regarded as one of the cancer stem cell (CSC) markers in colorectal carcinoma[3,4]. CD133 was the first identified member of a pentaspan membrane protein family in both humans and mice[1]. The glycoprotein specifically localizes in microvilli and protruding plasma membrane[5].

The CSC theory is a newly emerged concept of cancer initiation and development. According to this theory, only a small population of cells is clonogenic and contains tumor initiating potency, whereas the majority of the tumor cells have undergone differentiation and lost this potency. In colorectal cancer, these cells have been reported to express CD133 and a CD133-positive population of colon cancer cells was recently demonstrated to be highly enriched in tumor-initiating colon CSCs that have the ability to self-renew and to recapitulate the bulk tumor population[3,6-9].

The *CD133* gene transcriptional regulation is rather complicated and poorly understood. A possible involvement of five alternative TATA-less promoters has been suggested to explain the pattern of transcripts differing in the lengths and sequences of 5' untranslated regions (UTRs). Two of these promoters, P1 and P2 (Figure 1A), are active in *in vitro* tests with a reporter gene. A common transcriptional initiation site was assigned to exons 1A and 1B (Figure 1A), and an mRNA transcribed from exon 1A or 1B was found to be the major transcript, with the choice between the transcription start sites depending on tissues. In particular, colon-expressed transcripts contain both exons 1A and 1B[10-12].

Changes in DNA methylation patterns are an important hallmark of tumor development and progression. Methylation of the C^5 position of cytosine residues in DNA is one of the most fundamental epigenetic characteristics. This methylation is performed by DNA methyl-transferases (DNMTs), which have been implicated in many processes including transcriptional regulation, genomic stability, X chromosome inactivation and silencing of parasitic DNA transposable elements[13]. The importance of DNA methylation is highlighted by the finding that many human diseases result from its abnormal control[14]. Moreover, the aberrant methylation of CpG islands is characteristic of many human cancers and is detected during early carcinogenesis[15]. Hypermethylation of promoter CpG islands is the signature of transcriptional silencing of tumor suppressor genes in various human cancers, and this is as effective as inactivation by gene mutation or deletion[16,17].

To examine whether CD133 expression is related to promoter methylation of the gene, we assessed the expression of the *CD133* gene in 32 colorectal cancer cell lines and determined the methylation status of the CD133 promoter in each cell line.

MATERIALS AND METHODS

Cell culture

A total of 32 colorectal cancer cell lines were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea). Sixteen SNU-colorectal cancer cell lines were established as previously reported by our laboratory[18]. All the cell lines were maintained in RPMI1640 medium except for two cell lines; Caco-2 and WiDr were maintained in Minimum Essential Medium and Dulbecco's modified Eagle's medium, respectively. The media were supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Cultures were maintained in humidified incubators at 37℃ in a 5% CO_2 and 95% air atmosphere. All cell lines were absent of mycoplasma (e-myco mycoplasma PCR detection kit, Intron Biotechnology, Gyeonggi, Korea) and bacteria contamination and genetic heterogeneity by DNA fingerprinting analysis (AmpFlSTR Identifiler PCR amplification kit, Applied Biosystems, Foster City, CA, USA).

Nucleic acid isolation and cDNA synthesis

Genomic DNA and total RNA were isolated from washed cell pellets. Genomic DNA was extracted using a G-DEX™ kit (Intron Biotechnology) according to the manufacturer's instructions. Total RNA was isolated according to the manufacturer's instructions using easy-BLUE™ kits (Intron Biotechnology). For cDNA synthesis, 2 μg of total RNA was reversely transcribed using a random primer, dNTP, and 1 μL (200 units) of Superscript™ Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μL for 80 min at 42℃ after a 15-min denaturation at 80℃. Eighty microliters of distilled water was then added to the reverse-transcription reaction.

Reverse transcription-polymerase chain reaction

For CD133 expression analysis, the cDNA was amplified in 25 μL of a PCR reaction mixed with 1 μL of the reverse-transcription reaction, primers and 1 unit of Taq DNA polymerase. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using RT specific primers (located +343 to +679 from the translation start site in the mRNA sequence); CD133 RT sense (5'-CTGGGGCTGCTGT TTATTATTCTG-3'), and CD133 RT antisense (5'-ACGCCTTGTCCTTGGTAGTGTTG-3'). PCR conditions consisted of 5 min at 94℃ for initial denaturation, followed by 35 cycles of 94℃ (30 s), 55℃ (30 s), and 72℃ (60 s) and a final elongation of 7 min at 72℃. PCR amplification was performed in a programmable thermal cycler (PCR System 9700; Applied Biosystems; Foster City, CA, USA). Primers for β-actin were used to confirm RNA integrity. Both CD133 and β-actin RT-PCR reactions used the same cDNA synthesis. The amplified DNA fragments were fractionated in 2% agarose gel and stained with ethidium bromide.

Quantitative real-time PCR

Real-time PCR was performed in 386 well PCR plates containing the 2X FastStart Universal SYBR Green Master (ROX) mix (Roche, Basel, Switzerland), 10 ng of cDNA template, 300 nmol/L of CD133 RT sense primer and 600 nmol/L of CD133 RT antisense primer in a final

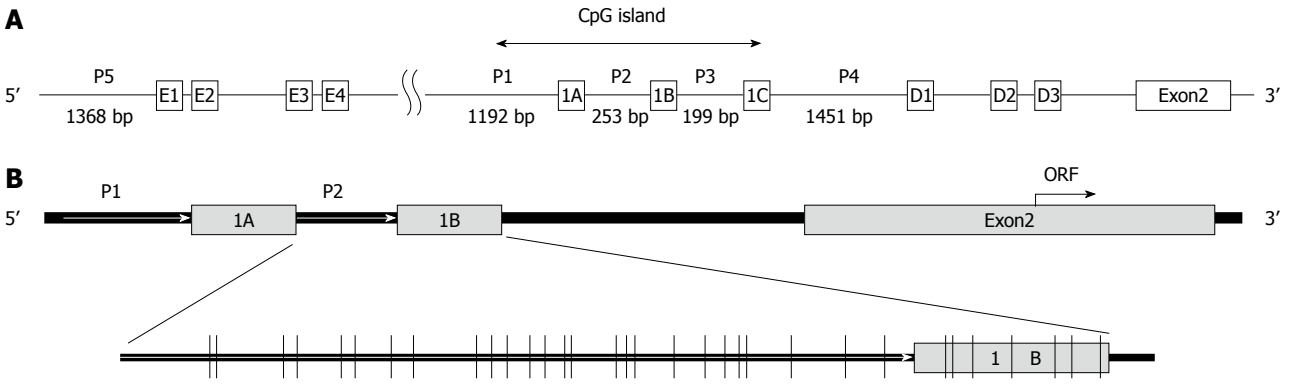

Figure 1 Schemes of the promoter region of CD133. A: Schemes of the *CD133* gene 5' terminus; B: Distribution of CpG dinucleotides in a fragment of the *CD133* gene harboring full P2 (250 bp) promoter and 5'-UTR exon 1B (The citation from Tabu *et al*[5] and Pleshkan *et al*[11]).

volume of 10 μL. Each primer/cDNA set was set up in triplicate. Real-time PCR reactions in a 7900HT Fast Real-Time PCR System (Applied Biosystems) were initiated by heating to 50℃ for 2 min and then to 95℃ for 10 min, followed by 40 cycles of 95℃ (15 s), and 60℃ (60 s). The relative quantification of gene expression was performed using the standard curve method. The standard curve for CD133 expression level was constructed using serial dilutions of SNU-407 cDNA, which in preliminary experiments displayed strong RT-PCR expression of the *CD133* gene. Samples normalized to β-actin served as an internal control.

Methylation specific-PCR

For methylation analysis, 2 μg of genomic DNA obtained from colorectal cancer cell lines was modified using EZ DNA Methylation™ Kit (Zymo Research, Orange, CA, USA). The primers specific for bisulfite modified DNA were designed using MethPrimer software (http://www.urogene.org/methprimer/index1.html). The used primers (located -8061 to -7782 from transcription start site) were as follows; CD133 M primer sense (5'-TTCGGGATAGAGGAAGTCGTAA-3'), CD133 M primer antisense (5'-CTCCCGCCCTAATCACCGCT-3'), CD133 U primer sense (5'-TTTGGGATAGAGGAAGTTGTAA-3') and CD133 U primer antisense (5'-CTCCCACCCTAATCACCACT-3'). The PCR conditions were as follows : 94℃ for 5 min, and then 43 cycles of 94℃ for 30 s, 60℃(methylation specific PCR, MSP) or 62℃ (unmethylation specific PCR, USP) for 30 s, and 72℃ for 60 s, and finally 72℃ for 7 min. The amplified DNA fragments were fractionated in a 2% agarose gel and stained with ethidium bromide.

Bisulfite sequencing analysis

Sequencing primers recognizing both methylated and unmethylated sites (MU), CD133 MU sense (5'-TATTTGGTTATGTTTTTAGTTTTTT-3') and CD133 MU antisense (5'-CCTAATCAACAAATACCTCTCTC-3') primers also were designed using the MethPrimer software. These primers were located approximately -8103 to -7708 from transcription start site. The PCR conditions used were 5 min at 94℃ for initial denaturation, 40 amplification cycles of 94℃ (30 s), 59℃ (30 s) and 72℃ (60 s) and a final elongation of 7 min at 72℃. The PCR products obtained with bisulfite sequencing primers were inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA) for cloning. Sequences of five individual colonies for each analyzed cell line were determined using universal pUC/M13 primers and each sequence was analyzed using a Taq dideoxy terminator cycle sequencing kit on an ABI 3730 DNA sequencer (Applied Biosystems).

5-aza-2'-deoxycytidine treatment to cell lines

For treatment with 5-aza-2'-deoxycytidine, 2×10^5 cells were seeded in two 75 cm^2 culture flasks on day 0. The cells were untreated or treated with 5 μmol/L of 5-aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h on day 2. The culture was re-dosed every 48 h (days 4 and 6) and medium was changed 24 h after adding 5-aza-2'-deoxycytidine. The cells were harvested on day 8 for RNA isolation. The RNA was used for cDNA synthesis and analysis of the CD133 expression as described above.

RESULTS

Expression of CD133 in colorectal cancer cell lines

We analyzed expression of CD133 in 32 colorectal cancer cell lines by both RT-PCR and quantitative real-time PCR. In RT-PCR analysis, CD133 expression was observed in 21 of the 32 cell lines (65.6%) (Figure 2A). On the other hand, in 11 cell lines (34.4%), CD133 expression was either undetectable (SNU-81 and SW480) or low (SNU-61, SNU-503, SNU-769A, SNU-C4, Colo320, HCT-8, LS174T, NCI-H716 and SW1116). To verity the RT-PCR results, we performed real-time PCR and quantified CD133 expression against β-actin expression. All 11 cell lines showed low relative expression (< 3.5%) (Figure 2B). Real-time PCR also demonstrated that 16 of 20 cell lines having strong expression of the *CD133* gene displayed high relative expression (> 6%); the four exceptions were SNU-1197, SNU-C1, SNU-C5 and DLD-1.

Analysis of CD133 methylation status by methylation specific-PCR

To assess if *CD133* gene expression was influenced

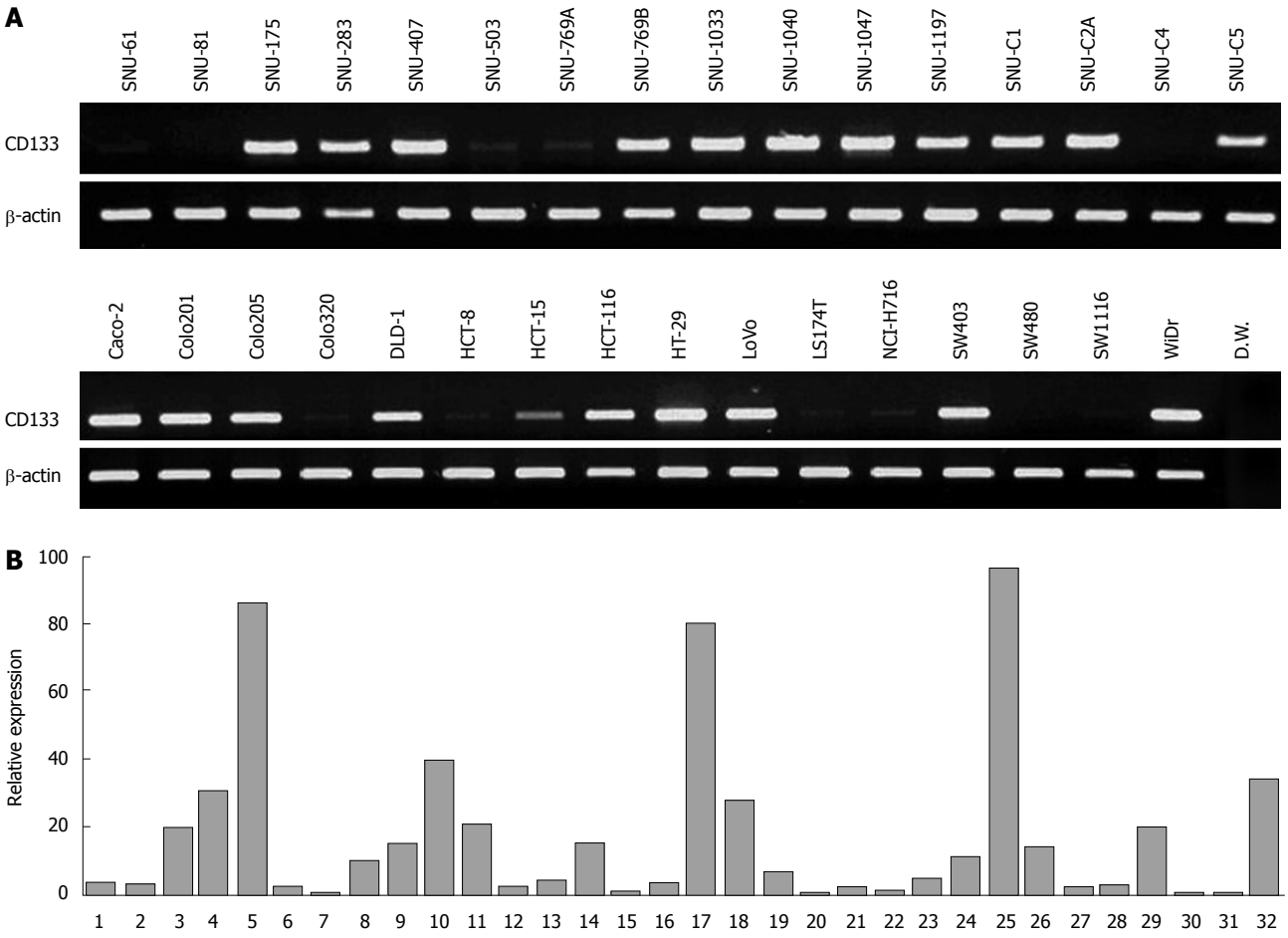

Figure 2 Expression analysis of *CD133* gene in 32 colorectal cancer cell lines. A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the *CD133* gene in 32 colorectal cell lines; B: Quantitative differences of CD133 expression by real-time PCR analysis.

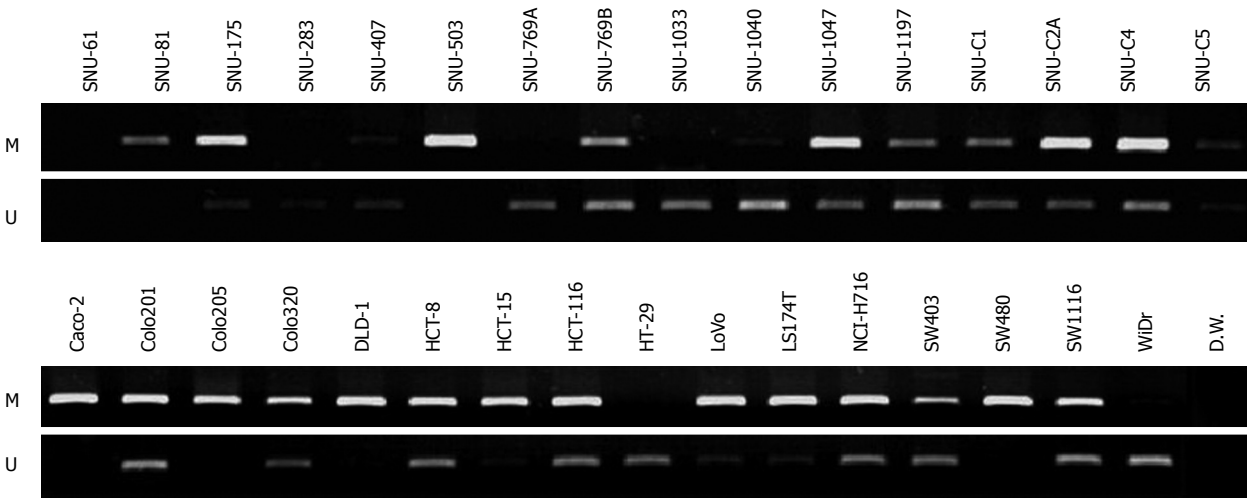

Figure 3 Methylation analysis of *CD133* gene in 32 colorectal cancer cell lines by methylation specific-PCR (MS-PCR). Lanes M and U denote that the product amplified by primer recognizing a methylated sequence and the product amplified by primer recognizing an unmethylated sequence, respectively.

by methylation of its promoter, we checked whether promoter CpG islands of the gene were methylated or unmethylated in the 32 colorectal cancer cell lines by performing methylation specific-PCR (MS-PCR) with designed methylation and unmethylation primers (Figure 3). Methylated DNAs were amplified in 26 cell lines (SNU-81, SNU-175, SNU-407, SNU-503, SNU-769B, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Caco-2, Colo201, Colo205, Colo320, DLD-1, HCT-8, HCT-15, HCT116, LoVo, LS174T, NCI-H716, SW403, SW480 and SW1116) and unmethylated DNAs were amplified in 24 cell lines (SNU-175, SNU-283,

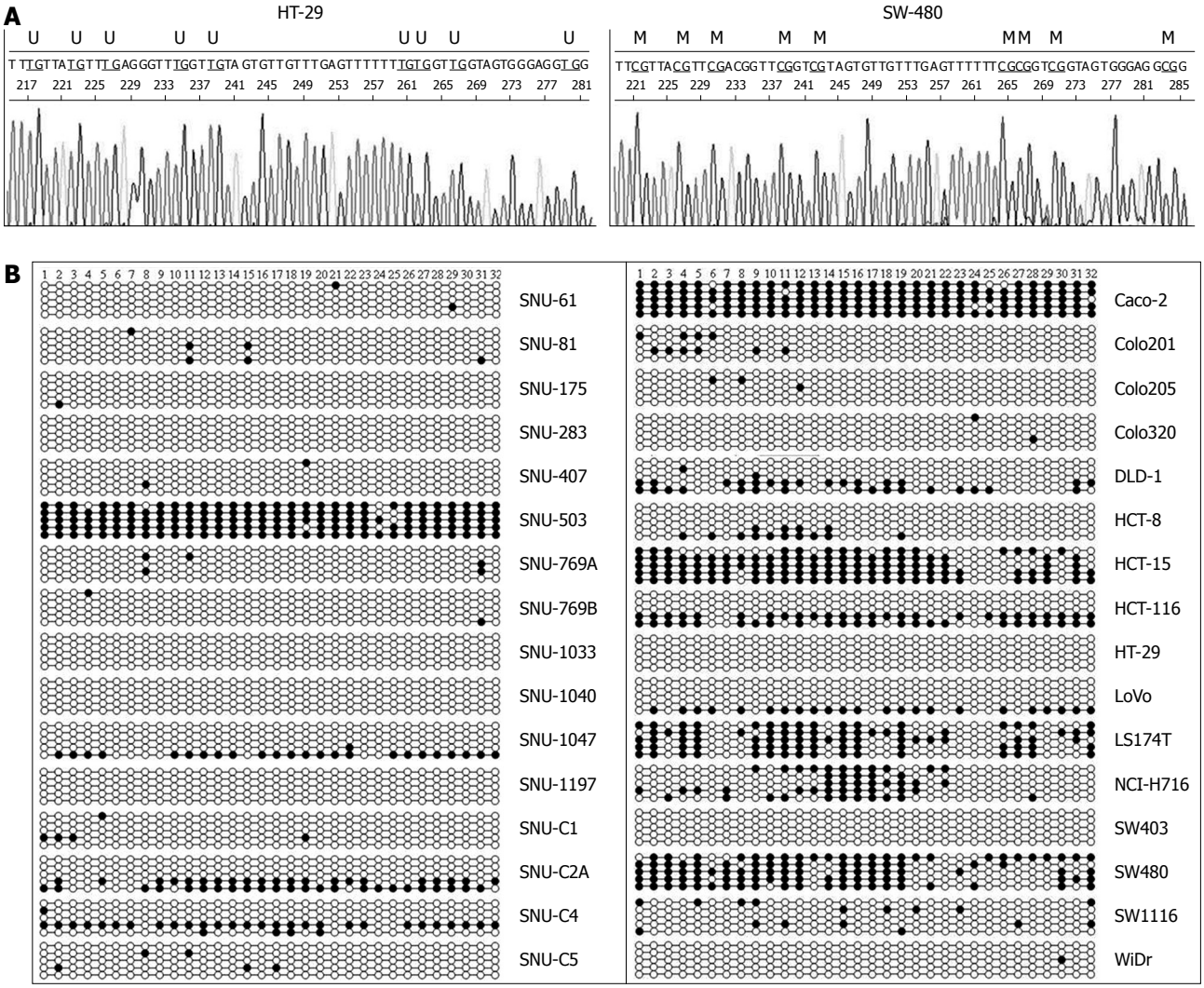

Figure 4 Analysis of methylation status of promoter CpG islands of CD133 by clonal sequencing. A: Representative sequence diagrams of DNA sequencing analysis in (a) HT-29 (unmethylated) and (b) SW480 (methylated) cell lines. M: Methylated site; U: Unmethylated site; B: DNA sequencing analysis of 32 CpG dinucleotides. The numbers at the top show the CpG sites of amplified DNA by bisulfite sequencing primers. Circle represents CpG dinucleotides; closed circle: Methylation; open circle: Unmethylation.

SNU-407, SNU-769A, SNU-769B, SNU-1033, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Colo201, Colo320, HCT-8, HCT-15, HCT116, HT-29, LoVo, LS174T, NCI-H716, SW403, SW1116 and WiDr). Only methylated DNA bands were evident in SNU-81 and SW480, with undetectable CD133 expression, and in the SNU-503 cell line, with weak *CD133* gene expression. In contrast, only unmethylated DNA bands were evident in SNU-283, SNU-1033, HT-29, and WiDr, in which *CD133* gene expression was strong. Both methylated and unmethylated DNA bands were detected in 13 cell lines (SNU-175, SNU-407, SNU-769B, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C5, Colo201, HCT116, LoVo and SW403), which also showed strong expression of the *CD133* gene. Three cell lines (Caco-2, Colo205 and DLD-1) contained only methylated DNA bands, even though these cell lines expressed CD133. Methylated or unmethylated DNA bands were not evident in SNU-61 cell line.

Analysis of promoter methylation status of CD133 gene by bisulfite sequencing analysis

A study reported that methylation of promoter P1 and exon A1 did not correlate with the *CD133* gene transcription level because promoter P1 and exon 1A were hypermethylated with a high (90%-94%) content of methylated CpG dinucleotides in all cell lines examined[11]. Appropriately, we investigated the methylation status of 32 CpG sites in promoter P2 and exon 1B (Figure 1B) relative to the transcription initiation site of *CD133* gene by clonal bisulfite sequencing analysis. Representative sequence diagrams of two cell lines are shown in Figure 4A. The methylation status of promoter CpG dinucleotides (Figure 4B) was correlated with CD133 expression (Table 1). The promoter CpG dinucleotides of CD133 of 13 cell lines (SNU-175, SNU-283, SNU-407, SNU-769B, SNU-1033, SNU-1040, SNU-1197, SNU-C1, SNU-C5, Colo201, Colo205, HT-29, SW403 and WiDr) were mostly unmethylated. Hypermethylation of CpG islands was 64% in the

Table 1 Correlation between promoter methylation status and CD133 expression

Cell lines (32 in total)	M	U	% methylation	% expression
SNU-61	-	-	1	3.1
SNU-81	+	+	4	2.8
SNU-175	++	+	1	19.9
SNU-283	-	+	0	30.7
SNU-407	+	+	1	86.2
SNU-503	++	-	95	2.4
SNU-769A	-	+	3	0.7
SNU-769B	+	++	1	10.2
SNU-1033	-	+	0	14.7
SNU-1040	+	++	0	40.2
SNU-1047	++	+	16	20.3
SNU-1197	+	++	0	2.4
SNU-C1	+	+	3	4.0
SNU-C2A	++	+	29	14.7
SNU-C4	++	++	21	0.5
SNU-C5	+	+	3	3.7
Caco-2	++	-	95	79.6
Colo201	++	++	6	27.4
Colo205	++	-	2	6.8
Colo320	++	+	1	0.7
DLD-1	++	-	21	2.4
HCT-8	++	++	9	1.4
HCT-15	++	+	73	4.7
HCT-116	++	++	31	11.2
HT-29	-	++	0	96.0
LoVo	++	+	14	13.9
LS174T	++	+	53	2.4
NCI-H716	++	++	24	2.6
SW403	+	++	0	19.7
SW480	++	-	64	0.6
SW1116	++	++	10	0.7
WiDr	+	++	0	33.3

M: Methylated CpG site; U: Unmethylated CpG site; -: Not detected; +: Barely detected; ++: Detected; +++: Greatly detected; % methylation: Methylation level of CpGs, (# methylated CpG/# total CpG) × 100; % expression: Relative expression level of CD133 as the ratio to the β-actin expression level, (CD133 level/β-actin level) × 100.

SW480 cell line (undetectable *CD133* gene expression), 64%-95% in SNU-503, HCT-15 and LS174T cell lines (low expression of *CD133* gene) and 9%-24% in SNU-C4, HCT-8, NCI-H716 and SW1116 cell lines (also low expression of *CD133* gene). The distribution of methylated CpG dinucleotides in different clones was not uniform. For example, in SNU-C2A cells the methylation level of CpG in promoter was markedly increased through hypermethylation in two clones, whereas the CpG dinucleotides in other clones were fully unmethylated (Figure 4B). Similar variations were observed in SNU-1047, DLD-1, HCT116, and LoVo cell lines. The variance was suggestive of the origin of different clones from different alleles of the gene. However, further studies are needed to confirm this possibility.

Re-expression of CD133 after treatment with 5-aza-2'-deoxycytidine

To confirm whether the differential CD133 expression was related to DNA methylation, 11 cell lines with undetectable or low expression of CD133 were treated with 5-aza-2'-deoxycytidine. The treatment recovered CD133 expression in four of the weakly CD133 expressing cell lines (SNU-61, SNU-769A, SNU-C4 and Colo320) and two cell lines without detectable CD133 expression (Figure 5A). However, 5-aza-2'-deoxycytidine treatment did not significantly influence *CD133* gene expression in SNU-503, HCT-8, LS174T, NCI-H716 and SW1116 cell lines. Examination of CD133 expression by real- time PCR revealed that CD133 expression was increased after 5-aza-2'-deoxycytidine treatment in SNU-503, HCT-8, LS174T and SW1116 cell lines (Figure 5B), and decreased in the NCI-H716 cell line.

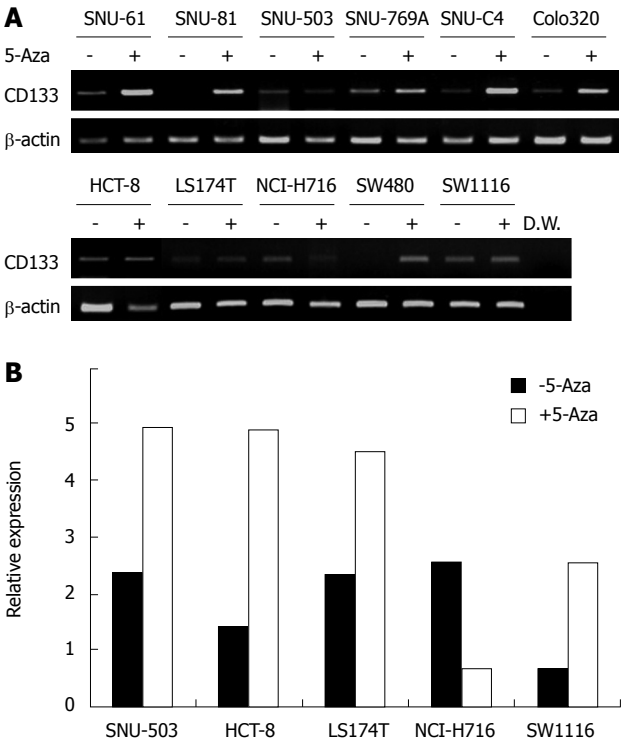

Figure 5 Expression analysis after treatment with 5-aza-2'-deoxycytidine (5-Aza). A: RT-PCR analysis of CD133 expression in 11 cell lines with or without 5-aza-2'-deoxycytidine treatment; B: Representative results of real-time PCR analysis revealing CD133 expression in 5 cell lines with or without 5-aza-2'-deoxycytidine treatment.

DISCUSSION

It has been postulated that CSCs are able to maintain tumor bulk due to the abilities of self-renewal and differentiation into cells with low potential. CD133 is one of CSC markers in colorectal carcinoma and CD133-positive cells in colon cancer exhibit a high tumorigenic ability *in vivo*. In a previous study, the CD133-positive cells separated from tumors were able to form tumor bulk in the immunocompromised mouse models with less numbers than the CD133-negative cells and these cells showed a long-term tumorigenic potential[4]. Furthermore, CD133-positive cells in colon cancer cell lines showed higher levels of proliferation, colony formation and invasive ability *in vitro* than CD133-negative cells[19].

Presently, CD133 was down-regulated in 11 of the 32 colorectal cancer cell lines. Since abnormal DNA meth-

ylation of *CD133* gene is related to CD133 expression in colorectal tumors[20], we hypothesize that CD133 expression is regulated by hypermethylation of the promoter region in this gene, and analyzed promoter methylation status of the *CD133* gene with a methylation-specific PCR after sodium-bisulfite modification and by clonal sequencing analysis. A methylation analysis of promoters P1 and P2 in cell lines demonstrated tissue specificity of the promoter P2 methylation and practically no specificity in the methylation of the P1 promoter[11]. Therefore, we designed the primers with promoter P2 sequences for MS-PCR and bisulfite sequencing analysis and sequencing primers containing 32 of CpG dinucleotides (Figure 1B).

Methylation of the *CD133* gene promoter was observed in 13 cell lines (SNU-503, SNU-1047, SNU-C2A, SNU-C4, Caco-2, DLD-1, HCT-15, HCT116, LoVo, LS174T, NCI-H716, SW403 and SW1116). These cells exhibited significant methylation bands in MS-PCR analysis, and bisulfite sequencing analysis revealed that > 10% of the total CpG islands were hypermethylated. DNA methylation in the promoter region of a gene is associated with a loss of gene expression and plays an important role in gene silencing. The inactivation of tumor-suppressor genes by aberrant methylation in the promoter region is well-recognized in carcinogenesis[20].

However, loss of CD133 expression in early colorectal cancer is different from expression loss of tumor suppressor genes. Acquisition of CD133 promoter methylation of cells without CD133 expression resulted from CD133 positive cell division. The inverse correlation between CD133 transcription and methylation provides a mechanistic explanation for the loss of cell surface CD133 expression in differentiated cells. This is consistent with the notion that cell differentiation is accompanied by epigenetic changes that are responsible for guiding the future phenotypic profile of the progeny[21]. This phenomenon is not only unique to normal stem cells but also presents aberrantly in CSCs, which may initiate carcinogenesis[22,23]. In advanced colorectal carcinoma, the *CD133* gene was more frequently demethylated[24]. The carcinomas with demethylation of *CD133* gene showed a bigger maximal tumor size and a trend toward the development of a lymph node metastasis.

In our results of bisulfite sequencing analysis, there are differences of methylation status among the colonies of the same cell line. The variance was suggestive of the origin of different clones from different alleles of the gene. Definitely, heterogeneity of DNA methylation for several genes has been observed in total cell populations from cultured and primary cancers. The present observations for CD133 promoter methylation are unique in showing striking heterogeneity between isolated cell populations in single-tumor culture lines. This seems to be a more uniform heterogeneity involving cells of the tumor and manifesting as quantitative differences between alleles of a given gene. These quantitative differences of abnormal promoter DNA methylation can be quantitatively altered by changes in environmental surrounding for cultured tumor cells[25].

CD133 has been re-expressed by demethylation with 5-aza-2'-deoxycytidine in some cell lines. This agent reactivates gene expression when gene expression is reduced by methylation of CpG islands. Our results confirm that inactivation of CD133 expression is related to epigenetic modification, which, in colorectal cancer cell lines, is promoter methylation. The function of CD133 is currently unknown, but it was reported that CD133 expression is repressed by DNA methylation in CD133-negative progeny of CD133-positive cells[26], supporting a role for CD133 in CD133-positive cells. It has been found that the expression pattern of several genes was changed in neurosphere cells by treatment of chromatin-modifying agents, 5-aza-2' -deoxycytidine and trichostatin A, and these cells induced hematopoietic activity *in vivo*[27]. Therefore, re-expression of CD133 by treatment of demethylation agent is expected to discover the functions of *CD133* gene in cancer.

The failure to detect methylated or unmethylated DNA bands in MS-PCR SNU-61 cells warrants comment. We believe that there are some problems with the quality of the modified DNA. This remains to be confirmed. The strong expression of CD133 by Caco-2 cells, in which promoter hypermethylation was detected, supports the suggestion that regulation of CD133 expression might be caused by another mechanism.

In conclusion, we observed hypermethylation in the promoter region of the *CD133* gene in 13 of 32 colorectal cancer cell lines. We confirmed the methylation status by MS-PCR, bisulfite sequencing analysis and treatment of 5-aza-2'-deoxycytidine. The expression status of the CD133, one of CSC markers, was correlated with methylation status of CpG islands in the CD133 promoter. These results may contribute to the understanding of the role of CD133 inactivation in the pathogenesis of colorectal cancers.

COMMENTS

Background

The caner stem cell theory is a newly emerged concept of cancer initiation and development. These cells have the ability to self-renew and to recapitulate the bulk tumor population. In colorectal cancer, a CD133-positive population of colon cancer cells was recently demonstrated to be highly enriched in tumor-initiating colon cancer stem cells.

Research frontiers

It is reported that tumor initiating cells in colorectal cancer cells express CD133, the cell surface glycoprotein. However, the *CD133* gene transcriptional regulation is rather complicated and poorly understood. This study demonstrates that CD133 expression could be regulated by methylation status of promoter of the gene.

Innovations and breakthroughs

Recently, a CD133 has become a matter of common interest in the research of colorectal cancer stem cells. This study suggests that transcriptional repression of CD133 is caused by promoter hypermethylation of the CD133 in some of colorectal cancer cell lines. Moreover, each colorectal cancer cell line represented different methylation status of promoter and each colony of cell lines exhibited different levels of methylation.

Applications

Cancer stem cells lost their potential during differentiation by loss of expression of stem cell-specific expressed genes. Therefore, this study may contribute to the understanding of the role of CD133 inactivation in the progression of colorectal cancers.

Terminology

Promoter methylation is one of the most essential epigenetic characteristics.

The importance of DNA methylation is highlighted by the finding that many human diseases result from its abnormal control. Moreover, the aberrant methylation of CpG islands is characteristic of many human cancers and is detected during early carcinogenesis.

Peer review

This manuscript investigates the promoter hypermethylation and expression status of CD133, which is one of several putative cancer stem cell markers in colon cancer. The overall findings indicate that there is a correlation between this epigenetic change in some cancer cell lines, and that demethylation is capable of restoring CD133 expression in some cell lines. Furthermore, there is a surprising finding that different colonies of a particular cell line exhibited different levels of methylation, indicating as the authors point out, that other influences (e.g. tumor environment) may be involved.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3161

World J Gastroenterol 2010 July 7; 16(25): 3161-3167
ISSN 1007-9327 (print)

BRIEF ARTICLE

Diffusion-weighted magnetic resonance imaging to predict response of hepatocellular carcinoma to chemoembolization

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Supported by National Institutes of Health R01 CA126809
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Received: February 17, 2010 Revised: March 27, 2010
Accepted: April 3, 2010
Published online: July 7, 2010

Abstract

AIM: To investigate whether intra-procedural diffusion-weighted magnetic resonance imaging can predict response of hepatocellular carcinoma (HCC) during transcatheter arterial chemoembolization (TACE).

METHODS: Sixteen patients (15 male), aged 59 ± 11 years (range: 42-81 years) underwent a total of 21 separate treatments for unresectable HCC in a hybrid magnetic resonance/interventional radiology suite. Anatomical imaging and diffusion-weighted imaging (b = 0, 500 s/mm^2) were performed on a 1.5-T unit. Tumor enhancement and apparent diffusion coefficient (ADC, mm^2/s) values were assessed immediately before and at 1 and 3 mo after TACE. We calculated the percent change (PC) in ADC values at all time points. We compared follow-up ADC values to baseline values using a paired t test (α = 0.05).

RESULTS: The intra-procedural sensitivity, specificity, and positive and negative predictive values (%) for detecting a complete or partial 1-mo tumor response using ADC PC thresholds of ±5%, ±10%, and ±15% were 77, 67, 91, and 40; 54, 67, 88, and 25; and 46, 100, 100, and 30, respectively. There was no clear predictive value for the 3-mo follow-up. Compared to baseline, the immediate post-procedure and 1-mo mean ADC values both increased; the latter obtaining statistical significance (1.48 ± 0.29 mm^2/s *vs* 1.65 ± 0.35 × 10^{-3} mm^2/s, P < 0.014).

CONCLUSION: Intra-procedural ADC changes of > 15% predicted 1-mo anatomical HCC response with the greatest accuracy, and can provide valuable feedback at the time of TACE.

Key words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Diffusion-weighted imaging; Apparent diffusion coefficient; Functional imaging biomarker

Peer reviewer: Dr. Andreas G Schreyer, Professor, Department of Radiology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Chung JC, Naik NK, Lewandowski RJ, Deng J, Mulcahy MF, Kulik LM, Sato KT, Ryu RK, Salem R, Larson AC, Omary

RA. Diffusion-weighted magnetic resonance imaging to predict response of hepatocellular carcinoma to chemoembolization. *World J Gastroenterol* 2010; 16(25): 3161-3167 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3161.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3161

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer worldwide[1] and the main cause of death among cirrhotic patients[2]. Transcatheter arterial chemoembolization (TACE) is commonly employed to treat HCC patients who are not candidates for curative surgical resection or transplantation. To date, two separate randomized controlled trials have demonstrated survival benefit in patients undergoing TACE compared to best supportive care[3,4], and TACE is currently recommended as first-line non-curative therapy for non-surgical patients with intermediate-stage HCC[5,6].

Monitoring tumor response is paramount to determine the time interval of repeat treatment or to identify treatment failure. An imaging modality that can serve as an early surrogate marker of tumor necrosis, and objectively predict future tumor response at the time of treatment, would be immensely beneficial, especially because treatment response has been identified as an independent predictor of survival[3].

Conventionally, response is evaluated by magnetic resonance imaging (MRI) at 1-3 mo after TACE. Assessment of anatomical response in the early post-treatment period can be misleading, however, because a reduction in tumor size often does not correlate with the degree of tumor necrosis[7,8]. Furthermore, conventional gadolinium-enhanced MRI might not discern therapy-induced inflammation and granulation tissue from viable tumor[8,9]. Given these limitations, alternative MRI techniques are desired to reflect tumor response more accurately.

Diffusion-weighted imaging (DWI), a functional MRI technique, detects MR signal changes in tissues due to water proton motion that varies based upon the degree of cell membrane integrity. The intact membranes of viable tumor cells restrict water diffusion, whereas necrotic tumor cells with disrupted cell membranes exhibit increased water diffusion. This mobility of water is quantified by a constant known as the apparent diffusion coefficient (ADC). Previous preclinical[10,11] and clinical[9,12] studies have shown that tumor necrosis is associated with an increase in ADC value, thereby allowing differentiation between viable and necrotic portions of tumor. Additionally, DWI can determine treatment response several weeks earlier than anatomical imaging[9,12]. However, it remains unclear if these changes in water mobility occur immediately during TACE. If so, it is possible that such changes could provide interventional radiologists with a functional parameter to determine the endpoint of TACE and to predict future response to therapy. Therefore, we tested the hypotheses that ADC changes are present at the time of TACE, and that they can predict conventional 1.5-T MR anatomical image changes at 1 and 3 mo post-TACE.

MATERIALS AND METHODS

Clinical setting and patients

Our local Institutional Review Board approved this Health Insurance Portability and Accountability Act compliant study, which was performed at a single urban, academic tertiary care center. All patients provided informed consent. We enrolled 16 consecutive patients with a total of 21 separate treatment sessions for 21 primary HCC lesions from March 2006 to September 2009. Candidates for TACE were evaluated and treated for unresectable HCC after discussion at a multidisciplinary tumor board conference. Inclusion and exclusion criteria were modified from the report of Brown *et al*[13]. We included patients who met the following criteria: (1) age > 18 years; (2) ability to provide informed consent; (3) Eastern Cooperative Oncology Group (ECOG) performance status no greater than 2; (4) Child-Pugh class A or B disease; (5) focal or multifocal hepatic malignancy; (6) no contraindications to MRI; and (7) TACE performed in an MR/interventional radiology (IR) suite. Patients were excluded if they met any of the following criteria: (1) life expectancy < 6 mo; (2) Eastern Cooperative Oncology Group performance status ≥ 3; (3) Child-Pugh class C disease; (4) uncorrectable abnormal laboratory values (international normalized ratio > 1.5; platelet count < $5.0 \times 10^4/\mu L$; total serum bilirubin > 4.0 mg/dL; serum creatinine > 2.0 mg/dL); or (5) contraindications to MRI.

Treatment protocol

Certificate of Added Qualification (CAQ)-certified interventional radiologists who specialize in interventional oncology performed all TACE procedures in a sterile MR/IR suite (Miyabi; Siemens, Erlangen, Germany). This suite contains a 1.5-T Espree MR imager connected to an Artis dTA digital subtraction angiography (DSA) unit *via* a sliding patient table (Figure 1).

Patients first underwent DSA for superselective hepatic arterial catheter placement. Next, patients were transferred to the adjacent MR unit for pre-TACE tumor imaging. After baseline tumor imaging, patients were transferred back to the DSA unit and underwent TACE. Subsequently, patients were transferred back to the MR suite to obtain a set of post-TACE images. Lastly, patients underwent a final transfer to the IR suite to remove the vascular sheath and to compress the arterial puncture site manually to achieve hemostasis. Patients were admitted to the hospital for monitoring and discharged 1-2 d later after verification of adequate pain control, ambulation, and oral intake.

DSA and TACE protocols

DSA was performed with a 5.5-F visceral catheter and a 2.8-F microcatheter (Renegade Hi-Flo, Boston Scientific, Natick, MA, USA) that were coaxially inserted over a 0.016-inch diameter guide wire (Headliner, Terumo, Tokyo, Japan) to superselect the hepatic lobar or segmental hepatic artery that supplied the tumor. DSA was performed with iohexol injections (Omnipaque 350; Amersham Health, Princeton, NJ, USA).

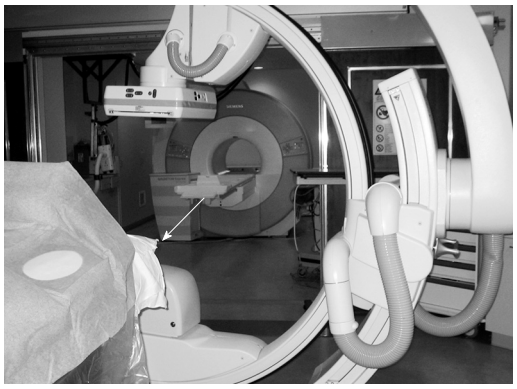

Figure 1 Hybrid MR/IR suite at investigators' institution. Moving table (arrow) allows the rapid transfer of patients between radiographic DSA and MRI during TACE. MRI: Magnetic resonance imaging; IR: Interventional radiology; DSA: Digital subtraction angiography; TACE: Transcatheter arterial chemoembolization.

We performed TACE using a 1:1 solution of emulsifying oily contrast agent and chemotherapeutic agents: 10 mL Ethiodol (Savage Laboratories, Melville, NY, USA) combined with a 10-mL mixture of 100 mg cisplatin, 30 mg doxorubicin, and 30 mg mitomycin C. Using fluoroscopic monitoring, we infused the solution until initial slowing of antegrade blood flow was noted. TACE was then completed by injecting 500-700-μm diameter Embospheres (Biosphere Medical, Rockland, MA, USA) mixed with iohexol. Angiographic endpoint was selected at the discretion of the treating interventional radiologist and ranged from subjective angiographic chemoembolization endpoint (SACE) levels Ⅱ-Ⅳ[14].

MRI protocol

All patients underwent anatomical and functional MRI prior to and immediately following TACE therapy. A subset of these patients also received imaging at the 1- and 3-mo follow-up time periods. All MRI was performed using a flexible six-channel phased-array abdominal imaging coil on a 1.5-T MRI scanner (Espree; Siemens Medical Solutions, Erlangen, Germany). Anatomical MRI included T2-weighted (T2W) half-Fourier acquisition single-shot turbo spin-echo and T1-weighted (T1W) gradient-recalled-echo imaging sequences, with and without contrast, with fat suppression in the arterial and delayed venous phases. Functional DWI was performed using single-shot spin-echo echo-planar imaging during one or more breath holds (repetition time/echo time = 2500/82 ms; slice thickness/gap = 8/4 mm; bandwidth 1.5 kHz/pixel; partial Fourier factor 6/8; non-selective fat saturation; twice refocused spin-echo diffusion weighting to reduce eddy-current induced distortion with *b* values of 0 and 500 s/mm^2). ADC parametric maps were reconstructed from each set of DW images acquired at each slice position.

Image analysis

A Siemens Argus image workstation was used to process all MR images. CAQ-certified interventional radiologists assessed anatomical tumor response on T1W contrast-material-enhanced MRI. Areas of persistent tumor enhancement after treatment were used as an indication of residual tumor, as proposed by the European Association for the Study of the Liver (EASL)[7]. Radiographic tumor enhancement was assessed at baseline and at 1 and 3 mo after therapy. Relative change in tumor enhancement was divided into four categories: (1) complete disappearance of tumor enhancement after treatment denoted a complete response (CR); (2) ⩾ 50% decrease in area of tumor enhancement corresponded to a partial response (PR); (3) progressive disease (PD) was indicated by a ⩾ 25% increase in area of tumor enhancement; and (4) tumor enhancement changes that did not fit any of the previous three categories were designated as stable disease (SD). These percentages were chosen to correspond to the World Health Organization guidelines regarding bi-dimensional tumor size measurements[15]. An objective EASL response included CR and PR alone.

DWI and ADC maps were used to determine functional tumor response. A region of interest (ROI) was drawn around each entire tumor on DWI using the T1W images as a reference. For accuracy, the ROI measurements for mean ADC values were then obtained by transferring the DWI ROI to the ADC maps at the corresponding slice positions. ADC values were measured for each tumor at baseline, immediately after treatment, and at 1 and 3 mo. Functional tumor response was reported in ADC units of mm^2/s.

To determine if the ADC values changed immediately after TACE and whether they predicted anatomical tumor response at long-term follow-up, we calculated the percent change (PC) in ADC values at all time points according to the formula PC = [(ADC_{post}-ADC_{pre})/ADC_{pre}] × 100, where ADC_{pre} was the baseline measurement and ADC_{post} was the measurement obtained at each follow-up time period. Using the anatomical response at 1 and 3 mo as the gold standard, we determined the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PC in ADC immediately after TACE. These values were calculated using ADC PC absolute thresholds; an increase or decrease in ADC value of more than 5%, 10%, or 15% was considered to be a positive outcome.

Figures were prepared using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

We collected and reviewed all patient demographic data, including α-fetoprotein (AFP), total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and alkaline phosphatase levels, as well as tumor characteristics, including HCC staging and radiographic evaluations before and after TACE.

The mean ± SD was calculated for patient age, time elapsed from treatment to follow-up, liver function laboratory values, and MR measurements of ADC values. A paired *t* test with α = 0.05 was used to compare differences between ADC_{pre} and ADC_{post} in all patients. In cases in which a single patient underwent multiple treatments to target separate tumors, ADC values and EASL responses

Table 1 Patient demographic data (*n* = 16)

Demographics	*n*
Sex	
Male	15
Female	1
Age	
> 65 yr	7
≤ 65 yr	9
Ethnicity	
Caucasian	9
Asian	3
Hispanic	2
African American	1
Other	1
Etiology of liver disease[1]	
Alcoholic hepatitis	4
Hepatitis B	4
Hepatitis C	8
Hemochromatosis	1
Auto-immune hepatitis	1
Cryptogenic cirrhosis	1
ECOG class	
0	7
1	9
Tumor distribution	
Solitary	3
Multifocal	13
Extrahepatic metastases	
Yes	2
No	14
Infiltrative tumor	
Yes	0
No	16
Portal vein thrombosis	
Yes	1
No	15
Tumor burden	
> 50% of liver volume	0
< 50% of liver volume	16

[1]Two patients had both hepatitis C and alcoholic hepatitis; one patient had both hepatitis B and alcoholic hepatitis. ECOG: Eastern Cooperative Oncology Group.

were tabulated according to each individual treatment session and used as separate data points. All statistical tests were performed using Minitab 14 software (Minitab, State College, PA, USA).

RESULTS

Patient demographics

Sixteen patients (15 male) successfully completed both baseline pre-chemoembolization and immediate post-chemoembolization anatomical MRI and DWI for a total of 21 treatment sessions. From the initial 21 treatments, 15 (71%) and eight (38%) of these cases included the 1-mo (37 ± 11 d) and 3-mo (94 ± 16 d) follow-up imaging, respectively. Mean age was 59 ± 11 years. Table 1 lists the demographic data for this 16-patient cohort. The majority of patients had multi-nodular, non-invasive HCC with moderately preserved underlying liver function (i.e. Child-Pugh class B disease). At the time of treatment, no patients exhibited advanced disease (Okuda stage Ⅲ) or a cancer of the liver

Table 2 HCC staging (*n* = 16)

Staging system	*n*
Child-Pugh	
A	7
B	9
C	0
CLIP	
0	1
1	5
2	10
Okuda	
Ⅰ	7
Ⅱ	9
Ⅲ	0

HCC: Hepatocellular carcinoma; CLIP: Cancer of the liver italian program.

Table 3 Liver function tests (mean ± SD)

Serum test	Baseline	1 mo	3 mo
AFP (ng/mL)	1633 ± 5997	5599 ± 21080	1264 ± 2684
AST (U/L)	62.6 ± 23.9	66.2 ± 32.1	80.7 ± 64.5
ALT (U/L)	52.6 ± 31.0	54.8 ± 39.1	53.1 ± 49.4
Alkaline phosphatase (U/L)	160 ± 75.0	203 ± 107	173 ± 68.8
Total bilirubin (mg/dL)	1.38 ± 0.644	1.28 ± 0.75	2.23 ± 2.22

AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

italian program (CLIP) score greater than 2 (Table 2). Liver function tests are summarized in Table 3.

Anatomical tumor response after TACE

On the anatomical contrast-material-enhanced T1W MR images, the 1-mo follow-up demonstrated CR, PR, SD, and PD rates of 6%, 75%, 13%, and 6%, respectively, whereas the 3-mo follow-up showed rates of 38%, 0%, 25%, and 38%. Thus, a favorable EASL response (CR or PR) was achieved in 81% of patients at 1 mo and 38% of patients at 3 mo.

Functional MRI changes and prediction of response after TACE

Mean baseline tumor ADC ($\times 10^{-3}$ mm^2/s) was 1.48 ± 0.30, which increased to 1.51 ± 0.36 immediately after TACE, 1.65 ± 0.35 at 1 mo, and then declined to 1.56 ± 0.48 at 3 mo. Compared to baseline, the increase in mean ADC value at 1 mo was statistically significant ($P < 0.014$); however, the immediate post-procedure ADC change was not significant.

Overall mean ADC PC was 3.00% ± 16.4% immediately after TACE, 13.4% ± 14.8% at 1 mo, and 4.25% ± 17.9% at 3 mo. Figure 2 is a scatter-plot graph of 16 different treatment sessions showing individual ADC PC during TACE and corresponding 1-mo EASL status. Shaded regions of the graph represent the ADC PC absolute threshold values of 5%, 10%, and 15%. The sensitivity, specificity, PPV, and NPV of the immediate post-TACE ADC PC in predicting an objective EASL response (CR

Table 4 Utility of intra-procedural ADC %Δ to predict 1-mo HCC response *n* (%)

ADC %Δ threshold	Sensitivity	Specificity	PPV	NPV
±5	77	67	91	40
±10	54	67	88	25
±15	46	100	100	30

ADC: Apparent diffusion coefficient; PPV: Positive predictive value; NPV: Negative predictive value.

or PR) at 1 mo are listed in Table 4. There was no discernable predictive value related to the 3-mo anatomical tumor response. Figure 3 is composed of representative MR images from a single patient showing HCC diffusion changes during TACE and corresponding tumor anatomy 1 mo later.

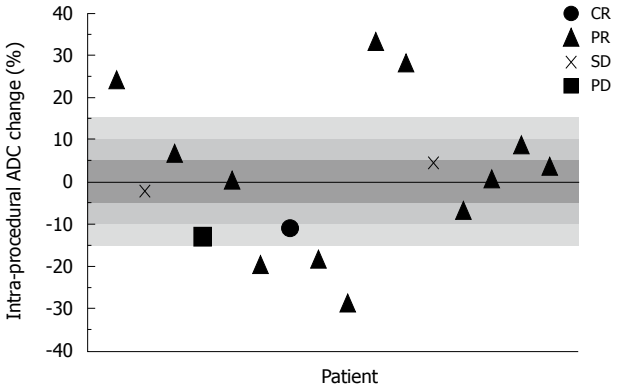

Figure 2 Scatter-plot graph of 16 separately treated HCC lesions showing intra-procedural ADC percent change and their corresponding 1-mo EASL status. ADC: Apparent diffusion coefficient; HCC: Hepatocellular carcinoma; EASL: European Association for the Study of the Liver; CR: Complete response; PR: Partial response; PD: Progressive disease; SD: Stable disease.

DISCUSSION

In this prospective study of 16 patients undergoing 21 separate TACE sessions, we showed that intra-procedural ADC changes in tumor function could help predict future anatomical response with increasing certainty at greater absolute ADC PC cut-off values. Specifically, we showed that patients whose ADC increased or decreased from baseline by > 15% immediately after TACE had a 100% rate in predicting a positive EASL response at 1 mo. Our findings also confirm that ADC increases occur within days to weeks following TACE, but indicate that they do not change significantly at the time of therapy.

Several previous clinical studies have shown the ability of DWI to map water distribution within HCC tumors and quantify tumor necrosis after transcatheter liver-directed therapy. In patients with HCC undergoing TACE, Kamel *et al*[12,16] have confirmed the feasibility of DWI to measure tumor response, which shows an increase in ADC at 4-6 wk after TACE. In their studies, regions of increased ADC corresponded to non-enhancing regions of presumed coagulative necrosis on contrast-material-enhanced MRI. More recently, they have demonstrated ADC changes as early as 1 wk post-TACE with no significant changes occurring at 1 d of follow-up[17]. Chen *et al*[9] have further demonstrated on a 3.0-T MR scanner an increase in HCC ADC values as early as 2-3 d after therapy. Statistically significant ADC increases have also been observed following yttrium-90 radioembolization, and in a feasibility study by Rhee *et al*[18], the 1-mo ADC changes were able to predict the 3-mo anatomical tumor response. Cui *et al*[19] have also looked at the predictive potential of ADC, albeit in cases of systemic chemotherapy for hepatic metastases.

None of the aforementioned studies[9,12,16-19], however, were able to address the question of whether ADC changes at the time of chemoembolization could be used to predict future anatomical response. We were able to address this gap in knowledge through the use of an integrated MR/IR suite. Such a suite allowed us to image patients with DWI immediately before and after TACE. Using this setup, we were able to show that DWI could be used intra-procedurally to predict a future anatomical response 1 mo after TACE. A positive result (ADC PC > 15%) would likely suggest adequate HCC treatment; however, a negative result would not be useful feedback and could not be interpreted as insufficient treatment.

One explanation for the immediate post-TACE increase in ADC is the ischemic effects of embolization itself[20], as ischemia-induced cellular swelling has been shown to increase ADC values[21]. Disruption of cell membrane integrity by the cytotoxic effects of TACE would be expected to require more time to evolve, most likely within the first week after therapy[9,17,20]. Notably, large decreases in immediate ADC_{post} also predicted tumor response. Limiting perfusion could conceivably lead to a measurable ADC reduction because a significant component of ADC values can be due to micro-capillary circulation[22], as opposed to water mobility alone. The relative decrease in mean ADC value at the longer 3-mo follow-up could be due to clearance of apoptotic bodies by scavenger cells, followed by fibrosis and tissue regeneration.

This study had several important limitations. First, the cohort used in our study was limited in size, and not all patients received follow-up imaging due to morbidity, mortality, repeat TACE before 3 mo, liver transplant, or being lost to follow-up. Second, assessments of functional and anatomical tumor response depended upon the selected *b* values and the anatomical response criteria chosen. Third, while an inverse relationship was noted between an increase in ADC value and a qualitative decrease in viable tumor enhancement, a correlation of magnitude could not be determined. Finally, the results of this study need to be validated by future studies with larger cohorts to determine if intra-procedural ADC changes reliably predict patient prognosis or affect outcome.

We concluded that intra-procedural ADC change of > 15% could serve as a useful prognostic indicator of tumor response to TACE. This preliminary result is encouraging because early knowledge of HCC response after initial therapy is essential to revise prognosis and guide

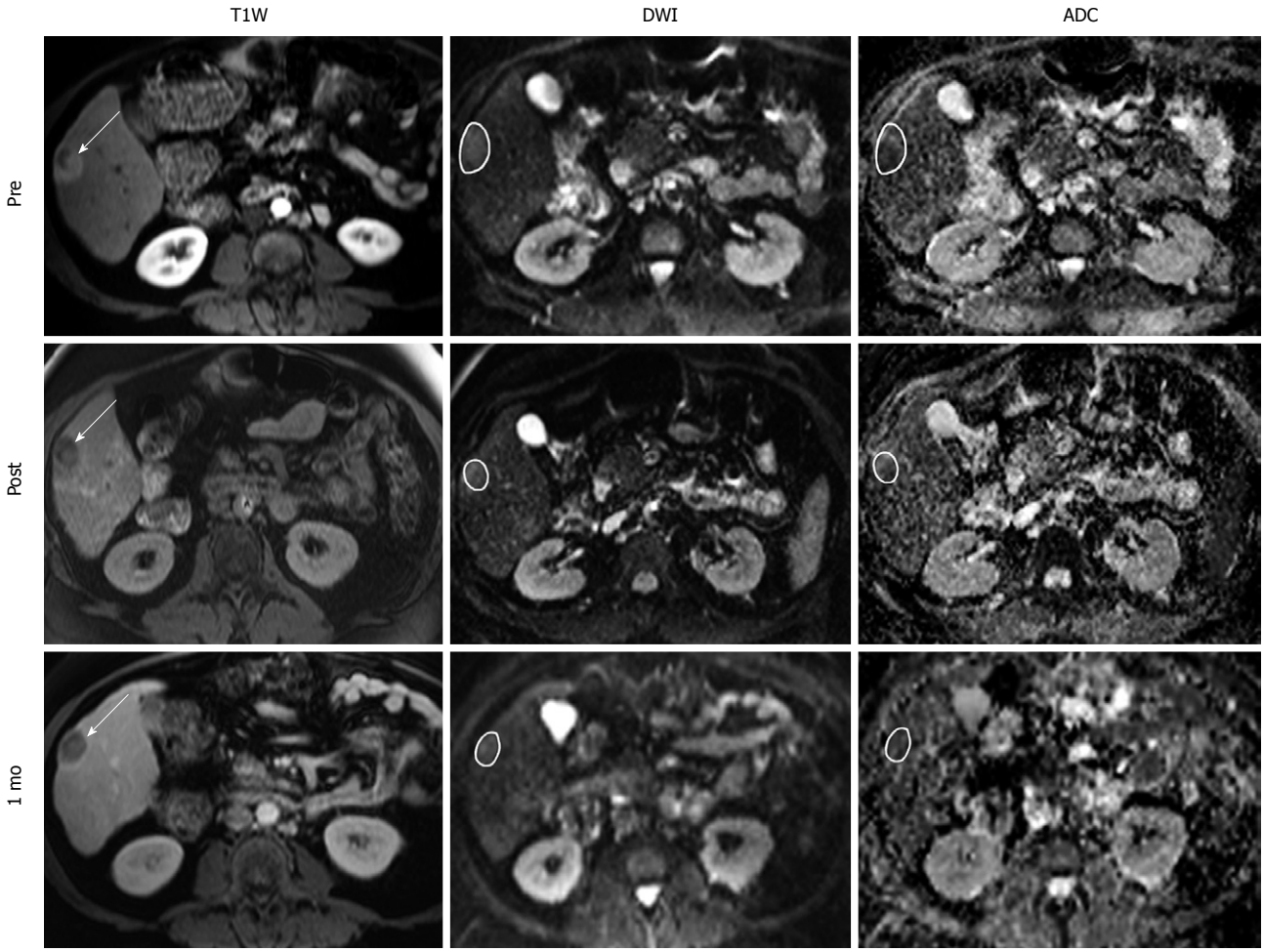

Figure 3 Representative T1W, DW, and ADC MR images from a 70 year-old man with right-lobe HCC secondary to autoimmune hepatitis. Images were acquired before, immediately after, and 1 mo after TACE. Arrows on the anatomical images indicate tumor location with sample ROIs drawn on the functional MR images. Intra-procedural ADC changed by 28.6%, which predicted a favorable EASL response at 1 mo. T1W: T1-weighted; DW: Diffusion-weighted.

future therapy. Use of DWI and ADC mapping in conjunction with traditional anatomical imaging evaluation could further improve tumor response interpretation and subsequent treatment planning. At present, MR/IR suites permit the acquisition of immediate quantitative functional imaging changes, in both tumor perfusion[23,24] and now diffusion. Which of these two functional parameters is more effective as an intra-procedural biomarker to tailor HCC therapy awaits verification by future studies.

ACKNOWLEDGMENTS

We thank Charles Fasanati and Elizabeth Gonda for their expert assistance with patient care during all procedures.

COMMENTS

Background

Early knowledge of hepatocellular carcinoma (HCC) response to transcatheter arterial chemoembolization (TACE) is crucial for determining treatment success, timing of repeat treatment, and patient prognosis. Currently, magnetic resonance imaging (MRI) is used 1-3 mo after treatment to evaluate anatomical tumor response, based upon changes in tumor size and contrast-agent enhancement. Alternatively, diffusion-weighted imaging (DWI) can be used as a functional imaging technique to depict thermally induced motion of water molecules. The extent of water mobility within biological tissues can be quantified by a parameter called the apparent diffusion coefficient (ADC). Recently, ADC values have been shown to change within days to weeks after therapy, which is earlier than changes seen by conventional HCC anatomical size assessment. However, no studies to date have reported the intra-procedural characteristics of ADC and whether these values can predict future tumor response at the time of chemoembolization.

Research frontiers

A non-invasive and non-ionizing imaging modality that could not only detect, but also predict, early HCC response to treatment would be ideal. DWI has demonstrated the capability to monitor and quantify functional tumor changes. Consequently, the ADC value obtained immediately post-treatment is of much interest because it has the potential to serve as an imaging biomarker of current and possibly future tumor response.

Innovations and breakthroughs

Previous studies have described how ADC values change over time during the follow-up period after TACE. Specifically, ADC values of HCC tend to increase after therapy. This initial increase in tumor water mobility is detected as early as 2 d post-treatment, with significant increases during several weeks thereafter. DWI has demonstrated the ability to identify and distinguish between viable and necrotic portions of tumor, and significant increases in ADC often correspond with tumor cell death. Earlier HCC evaluation, confirmation of successful treatment, or discovery of treatment failure could improve therapeutic decision making and patient prognosis. To elucidate further the role of DWI in characterizing tumor response to therapy, the authors investigated whether ADC changes could be detected immediately during intervention, and whether they could pre-

dict future HCC response at the time of treatment. They addressed this question by using a hybrid MR/interventional radiology suite, which permitted intra-procedural imaging immediately before and after chemoembolization.

Applications

This preliminary results suggest that patients whose intra-procedural ADC values increase or decrease by > 15% are more likely to have a favorable anatomical tumor response 1 mo later.

Terminology

TACE involves the minimally invasive delivery of chemotherapy directly to the site of the tumor, *via* an arterial catheter selectively placed during X-ray fluoroscopy (i.e. under real-time image guidance). This targeted method of drug administration decreases systemic drug toxicity and improves local drug efficacy. TACE is a palliative (i.e. non-curative) treatment that has been shown to improve survival for patients with intermediate-stage unresectable HCC.

Peer review

This is an excellent paper. It is a very interesting topic, especially for the radiological gastrointestinal community.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3168

World J Gastroenterol 2010 July 7; 16(25): 3168-3177
ISSN 1007-9327 (print)

BRIEF ARTICLE

Impact of pain on health-related quality of life in patients with inflammatory bowel disease

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Received: January 16, 2010 Revised: March 29, 2010
Accepted: April 5, 2010
Published online: July 7, 2010

Abstract

AIM: To evaluate intensity, localization and cofactors of pain in Crohn's disease and ulcerative colitis patients in connection with health-related quality of life (HRQOL) and disease activity.

METHODS: We reviewed and analyzed the responses of 334 patients to a specifically designed questionnaire based on the short inflammatory bowel disease questionnaire (SIBDQ) and the German pain questionnaire. Pain intensity, HRQOL, Crohn's disease activity index (CDAI) and colitis activity index (CAI) were correlated and verified on a visual analog scale (VAS).

RESULTS: 87.9% of patients reported pain. Females and males reported comparable pain intensities and HRQOL. Surgery reduced pain in both genders (P = 0.023), whereas HRQOL only improved in females. Interestingly, patients on analgesics reported more pain (P = 0.003) and lower HRQOL (P = 0.039) than patients not on analgesics. A significant correlation was found in UC patients between pain intensity and HRQOL (P = 0.023) and CAI (P = 0.027), and in CD patients between HRQOL and CDAI (P = 0.0001), but not between pain intensity and CDAI (P = 0.35). No correlation was found between patients with low CDAI scores and pain intensity.

CONCLUSION: Most IBD patients suffer from pain and have decreased HRQOL. Our study reinforces the need for effective individualized pain therapy in IBD patients.

Key words: Disease activity index; Health-related quality of life; Inflammatory bowel disease; Pain

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Schirbel A, Reichert A, Roll S, Baumgart DC, Büning C, Wittig B, Wiedenmann B, Dignass A, Sturm A. Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J Gastroenterol* 2010; 16(25): 3168-3177 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3168.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3168

INTRODUCTION

Inflammatory bowel disease (IBD) causes inflammation of the small and large intestines. The most common symptoms are persistent bloody diarrhea, pain, weight loss and persistent fatigue. Furthermore, irritable bowel syndrome (IBS)-like symptoms and depression are common in IBD[1,2]. Most patients go through periods during which symptoms flare up followed by periods of remission when symptoms subside.

In recent years, therapy of Crohn's disease (CD) and ulcerative colitis (UC) has improved with the application of new drugs. Biologicals such as TNF-α inhibitors are among a new generation of therapeutic options that control symptoms and inflammation in IBD patients[3,4]. However, medical management of IBD remains challenging and cannot always control all aspects of the disease. Even with therapy, patients frequently suffer not only from diarrhea and rectal bleeding but also from abdominal pain, cramps and arthralgia. Although the established disease activity indices only include abdominal pain as one variable, pain occurs throughout the body with pain attacks severely diminishing the patient's health-related quality of life (HRQOL) and interfering with their social and working habits[5-7]. A strong association between pain and HRQOL is well known for other diseases including neuromuscular disorders[8], rheumatoid arthritis (reviewed in[9]) and cancer[10]. Moreover, our own experience with IBD patients suffering from pain leads us to assume that in this chronic disease HRQOL is also diminished. The degree of impairment of HRQOL due to pain in IBD remains unspecified and is currently underestimated. In addition, the correlation between pain intensity, HRQOL and disease severity is poorly defined. Clearly, in order to offer individualized therapies there is a need for new tools to identify subgroups of patients who continue to suffer from pain despite anti-inflammatory and pain treatment.

The aim of this study was to evaluate pain intensity and localization in IBD patients, to correlate pain levels with HRQOL and disease severity, and to find possible associated factors on the basis of the information provided by the respondents.

We used a questionnaire based on both the standardized short inflammatory bowel disease questionnaire (SIBDQ), one of the most commonly used tools for evaluating HRQOL in IBD patients[11-13], and the well-established and validated German pain questionnaire[13].

In the current study we present a cross sectional analysis evaluating pain intensity, localization and cofactors and examine the relationship to HRQOL in patients with IBD. Furthermore, we correlate pain intensity levels and HRQOL to the crohn's disease activity index (CDAI) and the colitis activity index (CAI), both of which are well-established scores for evaluating current disease activity[14,15].

MATERIALS AND METHODS

Patients

This cross sectional study investigated the relationship between pain experience and HRQOL as well as disease activity indices in patients with IBD. Our multicenter survey was performed during the period 2005-2007 on patients attending the outpatient clinics at three independent campuses of Charité Medical School, Campus Virchow (45%), Campus Mitte (28%) and Campus Benjamin Franklin (27%), where all patients were asked to participate. Inclusion criteria were: (1) patients (aged 18-80 years) who signed informed consent; (2) patients with an endoscopically and histologically confirmed diagnosis of CD or UC of at least 6 mo, independent of clinical activity and extent of the disease; and (3) to analyze pain and HRQOL in patients who had undergone abdominal surgery, we included patients with small or large intestinal resection due to IBD.

Exclusion criteria were: (1) patients with indeterminate colitis or microscopic colitis; and (2) patients with only surgically drained cutaneous abscesses or fistula draining were excluded from subgroup analysis for surgery.

Patient medical records were checked for disease phenotype, disease duration, medication, and concomitant diseases. The survey was approved by the local ethics committee of the Charité Berlin. Since mechanisms of pain in CD and UC may differ, we analyzed most results for each IBD type separately. As a control, a group of 100 age-matched healthy individuals without complaints or medical history were asked to complete the SIBDQ. Aiming to provide a control group with comparable data, we also used the disease-specific questionnaire for the healthy control group.

Questionnaire

To cover all aspects of pain, HRQOL, and the possible factors influencing patients' perception of pain, we developed a new questionnaire, based on the SIBDQ and the validated German pain questionnaire[13,16,17]. Out of the 25 items in the German pain questionnaire, 12 were taken for the current questionnaire (we removed items 6, 8, parts of 10 and 11; 12, 13, 16, 17, 19, 20, 23, 24, 25, module D, parts of module S, and module V). The first part of our questionnaire collected 50 single variables based on the German pain questionnaire and extended these to include demographic parameters (age, gender, working conditions), medical history (time of first diagnosis, course of disease with indication of fistula or stenosis, active disease or clinical remission), IBD-related symptoms (stool frequency, bloody stools, fever), pain characteristics (localization, duration, intensity, impairment due to pain), pain therapy and the use of other drugs (including antidepressants), lifestyle (eating habits, smoking, use of complementary medicine) and quality of life. Pain intensity was evaluated on the basis of a visual analog scale (VAS)[18]. Patients indicated their scores on a 100 mm horizontal line, where the left end point of the line was marked "no pain" (= 0 mm) and the right end point was marked "strongest imaginable pain" (= 100 mm). Patients were asked to document their symptoms over a sustained period, in this case the previous week. The patients' state-

ments were matched with information from the medical records.

The second part of the questionnaire comprised the SIBDQ, a widely accepted and standardized disease-specific health-related quality of life questionnaire for patients with CD and UC, which is composed of 10 questions each with 7 possible responses[11,12]. We used the SIBDQ because it yields results comparable to those obtained with the full 32-item IBDQ for measuring HRQOL in patients with IBD[19]. A maximum of 7 points could be scored on each question. A score of 70 indicated the highest HRQOL, whereas the lower scores indicated lower HRQOL. The questionnaire is recognized as a reliable and sensitive tool for determining health-related quality of life[7]. Both the German pain questionnaire and the SIBDQ are validated[13,16,17].

To analyze the correlation between pain intensity, HRQOL and disease activity, we collected the CDAI and CAI (according to Rachmilewitz)[20] and matched them with pain intensity and HRQOL. The CDAI consists of eight variables (stool frequency, abdominal pain, general well-being, complications, hematocrit, use of loperamide, body weight, presence of abdominal mass), each summed after adjustment with a weighting factor. CD remission is defined as a CDAI score of less than 150. Severe disease is defined as a value greater than 450[21]. Most major research studies on the use of medications in CD use the CDAI as the gold standard[22-24].

For UC the patient's overall evaluation of symptoms was assessed according to the Rachmilewitz's Clinical Activity Index (CAI)-a well established index with good validity and reliability[20,25]. The score considers the number of bloody stools, abdominal pain and cramping, frequency of incontinence and the need for anti-diarrheal drugs, and an assessment of general well-being. Clinical remission was defined as a CAI < 3. Increased mild to moderate disease activity was defined as CAI = 4-9 and relapse was defined as CAI ⩾ 10[26,27].

Statistical analysis

All statistical analyses were conducted using SPSS (version 13.0) and SAS version 9.2 (SAS Institute, Cary, NC, USA). These included means and standard deviations. The χ^2 test was used to analyze discrete variables. The Mann-Whitney *U* test and the Kruskal-Wallis test were used to compare quantitative results between groups. For correlation analysis, the bivariate Pearson correlation was used. The accepted level of statistical significance was 5% ($P < 0.05$). The effect of several factors on HRQOL was examined by analysis of covariance (ANCOVA). Results are presented as adjusted means with 95% confidence intervals for categorical variables, and regression coefficient estimates for continuous variables.

RESULTS

Patients

Four hundred patients were asked to participate in the study, 387 (96.8%) filled out the questionnaire. Of these, 53 questionnaires were incomplete and were thus excluded. 334 (86.3%) questionnaires were included in the further study. CD had been diagnosed in 179 (53.6%) and UC in 155 (46.4%) patients. Table 1 summarizes the demographic characteristics of all the study participants.

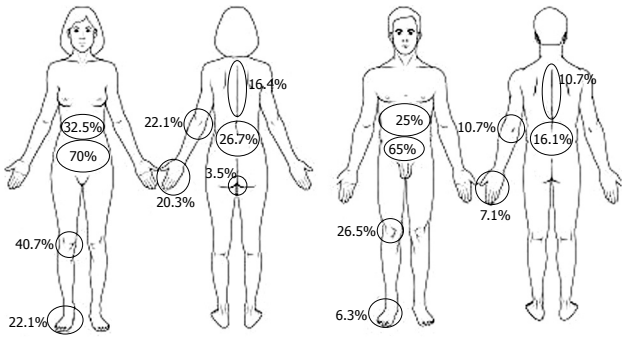

Figure 1 Distribution of pain in female and male patients. The degree of abdominal pain is quite similar in males and females, although females complain more often of arthralgia.

Description, intensity, duration, and localization of pain

In our survey 12.1% of patients reported no pain, 39.7% only had pain during flare-ups, and 48.2% mentioned persistent pain. Patients reported different durations of pain attacks ranging from seconds (17.4%), minutes (44.8%), or hours (27.4%) to days (10.4%). When asked to specify what time of day the pain occurred, 66.8% of patients reported pain unrelated to the time of day, 14.5% had pain only before noon, 14.9% during daylight hours, and 16.5% only at night. The latter group was associated with significantly lower HRQOL ($P = 0.016$).

A comparison of pain intensities and HRQOL between males and females revealed no difference ($P = 0.073$ and $P = 0.6$, respectively).

All indicated pain localizations are depicted in Figure 1 and were significantly different in males and females, with females complaining more often of arthralgia. Most patients indicated more than one pain site: 2 pain sites (18.6%), 3 pain sites (11.5%), 4 pain sites (13.6%), 5 pain sites (9.1%), and > 5 pain sites (17.7%). 39% of the patients described the pain as superficial, 61% as "deep insight". Multivariate analysis showed that pain intensity significantly reduced HRQOL ($P < 0.0001$), independently of sex, pain localization or disease activity.

Although we did not evaluate present disease location, a comparison of pain localization in CD and UC patients revealed higher pain frequency in the right upper abdomen in CD than in UC (39.2% *vs* 18.9%), although for abdominal pain in general there was no statistically significant difference between CD and UC. In contrast, in UC patients, lower left abdominal pain was statistically more frequent (76.4% *vs* 55.6%) than in CD patients. The lower left abdomen was the pain site that significantly influenced ($P = 0.0002$) HRQOL, independent of other factors (Table 2). Interestingly, although arthralgia was not different between CD and UC, CD patients complained more often about pain in hips, knees, and hands.

Table 1 Demographic data of IBD patients and healthy controls, separated into CD and UC groups

Demographics	CD	UC
Total	179	155
Age, mean, (SD), [range], yr	38.9 (± 11.6) [18-71.6]	39.8 (± 13.7) [18.6-75.5]
Age at disease onset, mean, (median), [interquartile range], yr	28 (24.5) [14]	31.6 (29.2) [16.2]
Age in males	28.9 (± 11.7) [12.6-70.7]	32.6 (± 12.2) [10.3-66.7]
Age in females	27.5 (± 12.0) [6.2-72.9]	30.6 (± 12.6) [11.8-70.6]
Gender, *n* (%)		
Male	71 (39.6%)	71 (45.8%)
Female	108 (60.3%)	84 (54.2%)
IBD		
Disease duration mean, (median), [inter quartile range], yr	10.9 (9.2) [12.8]	8.4 (6.7) [9.9]
Extraintestinal manifestations		
Arthralgia	102	79
Uveitis	6	2
Primary sclerosing cholangitis	0	5
Pyoderma gangraenosum	2	3
Erythema nodosum	3	0
Autoimmune hepatitis	0	1
Autoimmune hemolytic anemia	0	1
CD-associated polyneuropathy	1	-
Pulmonary manifestations	1	0
Current medication (% of users)		
Prednisolone		
Total	37.7	41.3
Systemic	25.7	32.7
Local	13.7	11.3
Local and systemic	1.7	2.7
5-Aminosalicylates		
Total	26.3	70.7
Local	1.1	3.3
Immunosuppressants		
Total	54.9	30.0
Azathioprine/6-mercaptopurine	46.9	26.4
6-MP	2.9	4.0
Methotrexate	2.3	1.3
Tacrolimus	0.6	4.0
TNF-α-inhibitors	9.1	0.7
Analgesics		
Total	29.1	20.7
No information	25.1	32.7
Mean IBD severity, mean, (median), [interquartile range]		
CDAI	173 (166) [186]	
CAI		5.08 (4.5) [5]
SIBDQ, mean, (median), [interquartile range]	48.3 (49) [21]	46.7 (47) [21]
Males	49.6 (51) [25]	48.9 (48) [22]
Females	47.4 (48) [19]	44.5 (45) [19]
SIBDQ healthy controls	58.5 (59) [10]	

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; CDAI: Crohn's disease activity index; CAI: Colitis activity index; SIBDQ: Short inflammatory bowel disease questionnaire.

Association between IBD type, pain levels and HRQOL

Pain levels in CD and UC patients were not significantly different ($P = 0.056$) and HRQOL scores were comparable ($P = 0.302$) (Figure 2A and B). Compared to healthy controls, HRQOL was significantly reduced in IBD patients (SIBDQ of healthy controls ($P < 0.0001$), regardless of whether they had CD or UC (Figure 2C).

A separate sub-analysis of CD and UC patients with abdominal pain or joint pain showed that HRQOL in UC patients was not significantly impaired by abdominal or joint pain ($P = 0.17$ and $P = 0.52$, respectively). In contrast, HRQOL in CD patients was significantly reduced by abdominal pain ($P = 0.01$) but not by joint pain ($P = 0.09$).

Cofactors of pain and reduced HRQOL

Having assessed pain intensity and HRQOL in IBD patients in general, we next assessed cofactors affecting pain and HRQOL. Pain was intensified in 38.3% of patients by mental stress, in 28.1% by ingestion of food, in 18.9% by physical activity, in 12.0% by the consumption of coffee, tobacco, or alcohol, in 9.9% by weather change, and in 7.2% by resting. In 22.9% of female patients, increased pain was associated with menses. Only 47.3% of patients declared their smoking status and remaining data were not statistically significantly different. Only 12 patients (3.6%, 7 UC, 5 CD, 9 female and 3 male) had depression.

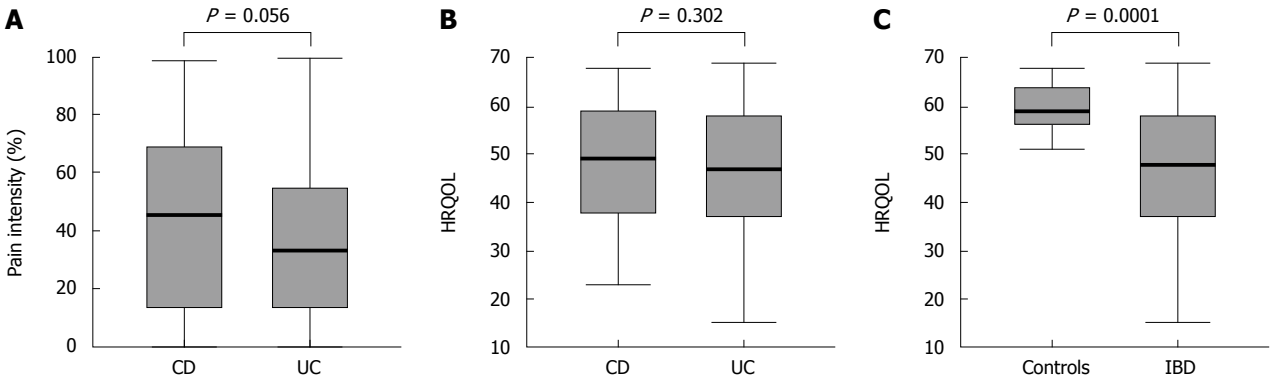

Figure 2 Differences in pain intensity (A) and health-related quality of life (HRQOL) (B) in Crohn's disease (CD) and ulcerative colitis (UC) patients. CD and UC patients have similar pain levels and HRQOL scores. Compared to healthy controls IBD patients have decreased HRQOL (C).

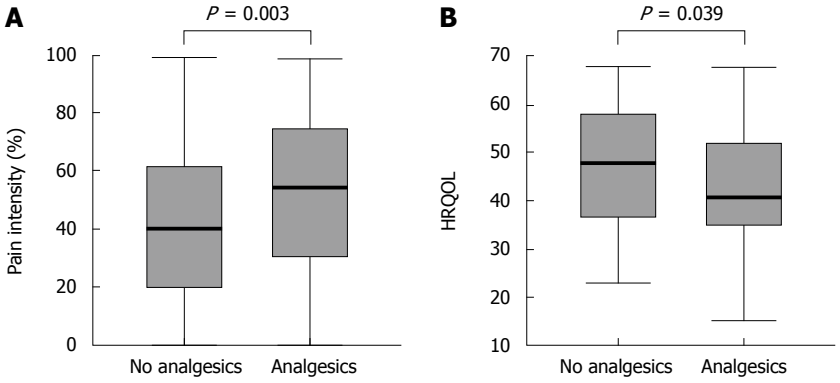

Figure 3 Comparison of (A) pain intensity and (B) HRQOL in patients with and without analgesic treatment. Pain intensity is even higher in patients receiving analgesic treatment.

Extraintestinal manifestations

In our study, 44.9% of patients had extraintestinal manifestations (EIM), including cutaneous manifestations, uveitis, primary sclerosing cholangitis, arthralgia or autoimmune hemolytic anemia (Table 1). Eighty-seven percent of patients with EIM complained of arthralgia. Patients indicated whether they had ever had or currently had EIM. Data were matched with medical records. As expected, patients with EIM had significantly more pain than patients without EIM ($P = 0.001$), irrespective of whether they had CD ($P = 0.001$) or UC ($P = 0.001$). Patients with EIM also had significantly lower HRQOL than patients without EIM ($P = 0.001$), again regardless of whether they had CD ($P = 0.005$) or UC ($P = 0.038$). Multivariate analysis revealed that EIM significantly ($P = 0.0052$) reduced HRQOL, independent of other variables (Table 2).

Association between analgesics, pain and HRQOL

In our study, 25.1% of all patients and 29.0% of patients reporting pain used analgesics. A difference between females and males was identified: 30.3% of females but only 18.0% of males took analgesics ($P = 0.033$). In CD, 29.1% of patients used analgesics, whereas in UC only 20.7% used analgesics ($P = 0.013$) (35.8% of females with CD, 18.2% of males with CD, 22.8% of females with UC, 18.3% of males with UC). Within the group of patients taking analgesics, 47.7% used morphine derivatives for no longer than 4 wk, 44.2% used metamizole (a pyrazolone derivative), and 20.9% took non-steroidal anti-inflammatory drugs (NSAID). Additionally, 5.8% of patients received antidepressants, some for pain modification and some for depression.

To evaluate the efficacy of analgesic drugs in IBD, we only included patients reporting pain for further investigation. As depicted in Figure 3A, patients taking analgesics still had higher pain intensities than patients not receiving analgesic treatment ($P = 0.003$). Accordingly, patients on analgesics reported decreased HRQOL ($P = 0.039$) (Figure 3B).

Modification of pain and HRQOL by surgery

In our analysis, 24% of patients had undergone surgery due to IBD (41.5% of CD and 8.0% of UC patients). To obtain information on whether abdominal surgery modified pain intensity or HRQOL in IBD, we included all patients who had undergone abdominal surgery in the subsequent analysis, irrespective of pain status. Patients without prior surgery were excluded from this sub-analysis.

Patients who had undergone IBD-related abdominal surgery had significantly lower pain intensity than patients who had not had surgery ($P = 0.023$), whereas HRQOL was not significantly different in patients with or without surgery ($P = 0.73$) (Table 2).

Interestingly, when analyzing IBD, regardless of whether CD or UC, we identified significant gender differences in the correlation between pain and HRQOL. Pain intensity in females was lower ($P = 0.001$) and HRQOL significantly higher ($P = 0.03$) after surgery, whereas this

Table 2 Multivariate analysis of variables affecting HRQOL

Variable	Mean (95% CI)[1]	*P* value
Sex		
Female	45.3 (43.0-47.6)	0.607
Male	46.1 (43.4-48.8)	
EIM		
Yes	48.1 (46.0-47.7)	0.004
No	43.3 (40.2-46.4)	
Pain LUQ		
Yes	44.6 (41.1-48.2)	0.398
No	46.8 (43.9-50.0)	
Pain LLQ		
Yes	43.0 (41.0-45.6)	0.001
No	48.3 (45.6-51.1)	
Pain RUQ		
Yes	46.5 (43.2-49.8)	0.511
No	44.8 (41.6-48.1)	
Pain RLQ		
Yes	45.5 (43.1-47.8)	0.778
No	45.9 (43.0-48.8)	
GI-related surgeries		
Yes	46.0 (42.7-49.3)	0.738
No	45.4 (43.1-47.6)	
Variable	Regression coefficient estimate[2]	*P* value
Age (yr)	0.075	0.244
IBD duration (mo)	0.004	0.635
Pain intensity (0-100 mm)[3]	-1.734	< 0.0001

Although sex does not affect HRQOL ($P = 0.6$), EIM ($P = 0.0043$) and pain in the lower left abdomen ($P = 0.0014$) do have a significant effect on HRQOL. In addition, pain intensity itself affects HRQOL, whereas sex and disease duration do not influence HRQOL. [1]Adjusted means from analysis of covariance (ANCOVA); [2]Change in HRQOL per variable unit increase; [3]Visual analog scale (VAS). LUQ: Left upper quadrant; LLQ: Left lower quadrant; RUQ: Right upper quadrant; RLQ: Right lower quadrant.

correlation was not observed for males.

Since CD and UC require different types of surgery, we next analyzed the correlation between surgery for CD and UC separately. CD patients benefited from surgery as pain intensity was significantly reduced ($P = 0.036$), however, their HRQOL remained unchanged ($P = 0.466$). In UC patients who had undergone surgery, pain levels ($P = 0.095$) and HRQOL ($P = 0.305$) did not differ from those of UC patients who had not had surgery. Considering all abdominal surgeries including non IBD-related surgeries like appendectomy (36), hysterectomy (9), ovariectomy (8), C-section (4), cholecystectomy (11), inguinal hernia revision (5), sterilization (7) and liver transplantation (1) there was no difference in pain intensity or HRQOL.

Correlation between pain intensity, HRQOL and CAI/CDAI

To assess whether disease activity indices correlate with pain intensity or HRQOL, we examined the relationship between CAI, CDAI, pain intensity and HRQOL. When separating UC patients according to their disease activity index (CAI < 4 = remission; CAI 4-9 = increased disease activity; CAI ⩾ 10 = flare-up), pain intensity increased and the HRQOL dropped with increased disease activity (Figure 4A and B).

In contrast to UC patients, in the overall CD population the disease activity did not correlate with pain intensity ($r = 0.23$). When analyzing subgroups according to the CDAI level, this lack of correlation was due to the relative high pain levels in the patient group despite remission (based on a CDAI ⩽ 150). However, in CD patients with a CDAI > 150, CDAI and pain levels correlated significantly ($r = 0.55$, $P = 0.002$). As in UC, in CD patients the CDAI correlated well with the HRQOL ($r = 0.53$, $P < 0.0001$), and increased pain intensities correlated with decreased HRQOL ($r = 0.58$, $P < 0.0001$) (Figure 4C and D). Interestingly, in both CD and UC higher disease activity levels were associated with the presence of EIM ($P = 0.04$ for both).

DISCUSSION

To optimize patient management in IBD, health care personnel not only need to know the type, localization and intensity of CD or UC to offer the best possible therapy, they also need to acknowledge that pain and impaired HRQOL is a great burden in the lives of IBD patients. Furthermore, we have to consider that anti-inflammatory therapy is not equal to pain management or improvement of HRQOL. However, to date, a systematic analysis of duration, localization and associated factors of pain and a subsequent correlation with major variables in the disease course and HRQOL is still lacking. To address this situation our study aimed to: (1) evaluate pain intensity and localization in IBD; (2) compare the results with HRQOL; and (3) explore whether pain levels and HRQOL are related to disease severity or other factors. This kind of study is timely and of special importance since treatment of patients should not only involve mucosal healing but should consider the patient as a whole.

In our study, the number of patients suffering from pain was very high (87.9%). Since we are a reference center with mostly pre-selected patients with more complex and severe disease courses, these high pain levels might be explained by referral bias. On stratifying pain levels for CD and UC separately, our data revealed that pain levels in patients with CD and UC were not significantly different, confirming the results of Heikenen and coworkers, who found similar results in children with IBD[28].

Although multivariate analysis revealed no gender differences in pain intensity or HRQOL, an analysis of a subgroup of patients who had undergone surgery showed that HRQOL was significantly lower in females, verifying that pain levels and HRQOL do not necessarily correlate[29,30]. In addition, females more often took analgesics indicating that they may be more affected by pain than males. Another explanation for the lower HRQOL in females could be a difference in pain perception with more attention to pain in females or different pain-coping mechanisms. Since IBS-like symptoms, depression and anxiety are more frequent in female than in male IBD patients[31,32], this could be an additional explanation for the gender difference in HRQOL.

Underlining the differences between male and female IBD patients, pain localization also differed between

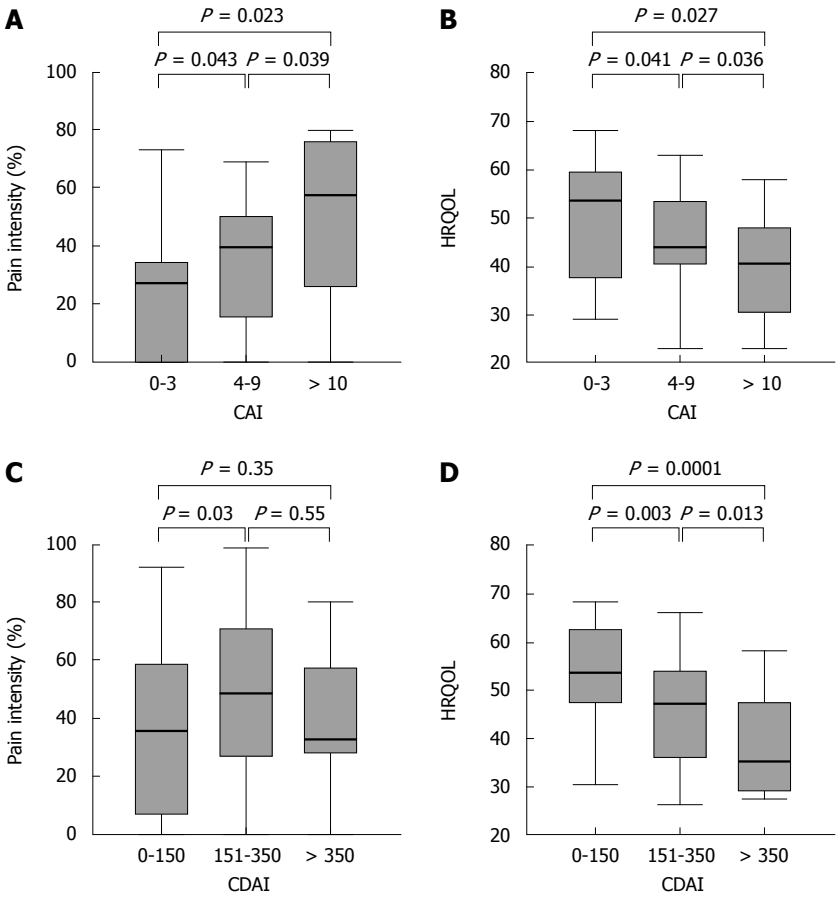

Figure 4 Distribution of disease activity index and pain intensity (A) and disease activity index and HRQOL (B) in UC patients and CD patients (C, D), respectively. UC patients with high disease activity have significantly higher pain levels and lower HRQOL. Although pain intensity does not correlate with CDAI, HRQOL correlates well with CDAI.

genders, with females complaining of significantly more arthralgia. As severe joint pain impacts the mobility of patients, the increased projection of pain in the joints in females might contribute to explaining their decreased HRQOL compared to male IBD patients. Interestingly, Palm and coworkers also found impaired HRQOL in IBD patients with non-inflammatory joint pain compared to IBD patients without joint pain, but they did not find a difference between females and males[33].

Sub-analysis of IBD type revealed differences in pain localization and corresponding HRQOL. Interestingly, in UC patients HRQOL was not significantly impaired by abdominal pain or arthralgia, whereas in CD patients, abdominal pain reduced HRQOL significantly.

Localization of pain is a matter of great interest because of the different treatment options for abdominal pain and arthralgia. While the treatment of arthralgia could be composed of physical therapy, steroids or sulfasalazine, abdominal pain requires a more effective anti-inflammatory therapy. In cases of persistent pain despite remission, IBS-like symptoms, depression or drug-related side effects must also be considered.

A multivariate analysis revealed that HRQOL is not only impaired by pain intensity but also pain localization, the presence of EIM and disease activity influences HRQOL independently of each other and other variables.

Pain can be perceived very differently and its duration varies individually. Given the chronic nature of IBD, nearly half of the patients reporting pain suffered pain irrespective of current disease activity. Accordingly, and in contrast to the pain character reported by patients with IBS, most IBD patients' pain was unrelated to the time of day. However, clearly correlating with disturbed nighttime sleep, patients suffering from pain mostly at night had significantly lower HRQOL, indicating the necessity for increased pain relief therapy in the evening in this group.

More than 40% of IBD patients have EIM of their disease[34,35]. This was confirmed by our study; 44.9% of our patients had EIM, manifesting most frequently as joint pain. Females had significantly more EIM than males ($P = 0.001$). Spondyloarthropathy is the most frequent extraintestinal complication of IBD and is associated with pain and stiffness[36]. Therefore, it was not surprising that patients with EIM had significantly more pain ($P = 0.001$) and significantly lower HRQOL than patients without EIM. EIM in IBD are frequent and play an important role in disease management[37]. The overall incidence of EIM in IBD ranges from 25% to 36%, with more than 60 different manifestations. Rheumatoid extraintestinal manifestations are frequent in patients with CD (38%) and UC (30%)[38,39].

In our study, less than one third of the patients reporting pain took analgesics. Interestingly, although their pain levels were comparable, females more frequently used analgesics than males. Whether or not this suggests a gender-specific approach to pain or pain killers, or a greater fear of side effects, this significant difference

indicates the need to individualize pain therapy. Current pain therapy seems to be ineffective and, in addition, pain perception differs in each individual, which is illustrated by the fact that patients taking pain killers still complained of more pain than patients who reported pain but were not receiving pain treatment.

The use of analgesics and their dosage is difficult to gauge and should be well evaluated. Pain killers have a variety of known effects and side effects. Given the gastrointestinal side effects and the possibility of aggravating mucosal inflammation, NSAR should be avoided in IBD. However, opioids might also worsen pain in IBD patients because long-term use of opioids may also be associated with the development of abnormal sensitivity to pain and a progressive decline in plasma cortisol levels[40]. Consequently, Smith and coworkers recently showed that treatment with naltrexone improved disease course and HRQOL of CD patients[41]. An alternative explanation for the discrepancy between use of analgesics and perception of pain might be the high prevalence of functional somatic symptoms (IBS-like symptoms) among IBD patients[2].

Surgery is an important therapeutic component in the course of IBD. During their lifetime more than 55% of IBD patients undergo abdominal surgery[42-45]. Surgery is often used as a last resort in severe cases with disease complications or in patients who do not respond adequately to therapy. In our study, particularly female CD patients who had undergone IBD-related surgery had significantly lower pain levels and higher HRQOL compared to patients who had not had surgery. Although this phenomenon might be a reflection of the selected patient population treated at university clinics, the observed improvement in HRQOL was confirmed in other studies on CD[46-48] as well as on UC[49] where the disease-related symptoms improved after surgery. In addition, performance of non IBD-related surgeries did not alter pain intensity or HRQOL.

Not surprisingly, we confirmed that compared to healthy individuals HRQOL was significantly decreased in IBD patients[13,50] and correlated with the respective disease activity scores[51]. Most previous clinical studies determined a positive effect of different substances in the treatment of IBD on disease activity and therefore HRQOL[52]. Contrastingly, in our study population disease activity correlated with pain levels in UC patients, but not in CD patients. CD patients, in particular, indicated pain despite low or moderate CDAI scores. This new perception may be due to the dominance of other factors that influence the CDAI, such as EIM or fistulizing disease course, and the underrepresentation of pain in this disease activity index, whereas in the CAI indication of pain is weightier for the complete score. In addition, as mentioned before, IBS-like symptoms, depression or drug-related side effects could cause pain without increased disease activity. Therefore, it is very important to have this new questionnaire in addition to the commonly used disease activity indices to measure pain as a strong impact factor for HRQOL in patients with IBD. The new combined questionnaire contains some questions that have not been validated before, which might affect interpretation of the results.

The strength of our study is its combined analysis of physiological complaints and parameters relevant to HRQOL, but it also has limitations. First, our study population consisted of referred patients who may have more complex and severe disease courses and higher pain levels compared to other IBD patient cohorts with more moderate disease courses.

Second, the cross sectional design of this study prohibits any determination in causality of analyzed factors or process of disease due to certain circumstances. Moreover, although we measured many potential modifiers of pain, the descriptive design allows for possible influence by unknown and unmeasured confounders.

Third, we did not evaluate anxiety or depression, conditions which can worsen the disease course and influence pain perception and HRQOL[1]. Nor did we evaluate disease phenotype according to the Montreal classification, which would have allowed a better correlation with pain localization. Nevertheless, we believe that our data will encourage physicians to devise patient-specific therapy plans for a subgroup of patients suffering from pain independent of disease activity.

In conclusion, most IBD patients suffer from pain and have decreased HRQOL. Duration and localization vary widely and differ not only between CD and UC patients, but also between genders. Most interestingly, our data point out that pain levels and HRQOL do not necessarily correlate with disease activity. By using this newly designed questionnaire we now bring to light that the CDAI does not sufficiently consider pain as a major limitation of HRQL in IBD. On the other hand, when defining the absence of diarrhea and pain as true remission, a CDAI below 150 might not indicate remission in the perception of a CD patient. Our results focus new attention on currently insufficient pain therapy in IBD patients and stress the need for sufficient and continually reviewed individualized pain therapy. Whether this insufficient pain therapy is due to the inadequate effect of pain killers, additional IBS-like symptoms, underrepresented complaints of pain, or physicians' underestimation of pain intensity remains unclear and will require further studies.

ACKNOWLEDGMENTS

We thank the patients who participated in the trial and the supporting nursing staff at the Charité.

COMMENTS

Background

Despite recent advances in the treatment of inflammatory bowel diseases (IBD), acute and chronic pain continues to have a profound negative impact on patients' health-related quality of life (HRQOL). Moreover, the methods for evaluating pain and its impact on HRQOL in IBD patients are still insufficient.

Research frontiers

The development of a new questionnaire based on both the short inflammatory bowel disease questionnaire (SIBDQ) and the German pain questionnaire enabled us to investigate localization and levels of pain and their impact on

HRQOL in IBD patients. Many clinical studies have been performed to investigate the effect of anti-inflammatory therapies on HRQOL but there are no data available on the correlation of pain and HRQOL.

Innovations and breakthroughs

This study provides the first evidence that the majority of IBD patients suffer from pain despite anti-inflammatory therapy and that HRQOL is impaired by pain. Interestingly, ulcerative colitis pain and HRQOL are well correlated with disease activity, whereas Crohn's disease patients also suffer from pain during remission.

Applications

This study focuses new attention on pain as a burden to patients and urges physicians to devise sufficient and individualized pain therapy plans to improve patients' HRQOL.

Peer review

There is increasing focus upon QOL in patients with chronic IBD, and upon factors determining and modifying this. Pain is a frequent symptom in IBD, and may contribute adversely to QOL in these patients.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3178

World J Gastroenterol 2010 July 7; 16(25): 3178-3182
ISSN 1007-9327 (print)

BRIEF ARTICLE

Use of isolated Roux loop for pancreaticojejunostomy reconstruction after pancreaticoduodenectomy

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Received: March 19, 2010 Revised: April 12, 2010
Accepted: April 19, 2010
Published online: July 7, 2010

Abstract

AIM: To evaluate the efficacy of the isolated Roux loop technique in decreasing the frequency of pancreaticojejunal anastomosis failure.

METHODS: We retrospectively reviewed 88 consecutive patients who underwent pancreaticoduodenectomy (standard or pylorus-preserving). Single jejunal loop was used in 42 patients (SL group) while isolated Roux loop was used in 46 patients (RL group). Demographic characteristics (age, gender) and perioperative results (major/minor complications, mortality, hospital stay) were compared between the two groups.

RESULTS: Mortality was almost equal in both groups and overall mortality was 2.27%. Leak rate from the pancreaticojejunal anastomosis and hospital stay were lower in the RL group without significant difference. Morbidity was 39.1% in the RL group, insignificantly higher than the SL group. Operative time was almost 30 min longer in the RL group.

CONCLUSION: The isolated Roux loop, although an equally safe alternative, does not present advantages over the traditional use of a single jejunal loop. Randomized controlled studies are required to further clarify its efficacy.

Key words: Pancreaticojejunal anastomosis; Isolated Roux loop; Whipple pancreaticoduodenectomy; Pancreatic leak

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Ballas K, Symeonidis N, Rafailidis S, Pavlidis T, Marakis G, Mavroudis N, Sakantamis A. Use of isolated Roux loop for pancreaticojejunostomy reconstruction after pancreaticoduodenectomy. *World J Gastroenterol* 2010; 16(25): 3178-3182 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3178.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3178

INTRODUCTION

Pancreaticoduodenectomy (PD) is the procedure of choice for the treatment of peri-ampullary and pancreatic head malignancies and was first described by Allen Whipple *et al*[1] back in the 1930s. Early enthusiasm concerning the procedure was followed by skepticism because of the associated high morbidity and mortality rates[2]. However,

advances in operative techniques and perioperative patient care have resulted in lower hospital mortality and longer survival, making the procedure relatively safe in expert hands[3,4].

Despite recent favorable outcomes, leakage from the pancreaticojejunal anastomosis is still considered a significant source of morbidity and associated mortality. Various methods of surgical management of the pancreatic remnant have been proposed to address this serious problem. The rationale of creating an isolated Roux loop for the drainage of the pancreatic stump was initially introduced by Machado *et al*[5] in 1976. They proposed that this isolated Roux loop can prevent the activation of pancreatic fluid by the intestinal contents and bile, and therefore protect the pancreaticojejunal anastomosis from erosion.

The aim of this study was to assess the outcome of the pancreaticojejunal anastomosis formed with an isolated Roux loop compared to the standard single loop technique.

MATERIALS AND METHODS

Study design

We retrospectively studied all patients who underwent PD for malignancy in our department from 1994 to 2006. The medical records of 88 consecutive patients were reviewed. All PDs were performed by two experienced pancreatic surgeons. There have been two distinct periods in our study during which the management of the pancreatic remnant was different. In period I (1994 to 1999) the pancreatic stump was anastomosed sequentially to the single jejunal loop (followed by hepaticojejunal and gastrojejunal anastomoses) used for the reconstruction of all anastomoses (Group SL). During period Ⅱ (2000 to 2006) an isolated Roux loop (Group RL) was used for the pancreatic reconstruction. Informed consent for the surgical procedures was obtained from each patient.

Preoperative assessment

Preoperative diagnostic workup during the early period of study included abdominal computerized tomography scan with oral/intravenous contrast, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography and mesenteric angiography in selected patients. The advent of magnetic resonance imaging and magnetic resonance cholangio-pancreatography in the late 1990s provided an excellent adjunct to diagnosis and a safe alternative for biliary-pancreatic evaluation.

Surgical approach

Standard Whipple type operation was performed in 63 patients while the remaining 25 patients underwent a pylorus-preserving PD (PPPD) according to Traverso *et al*[6]. In the majority of cases an end-to-side, duct-to-mucosa pancreaticojejunal anastomosis with transanastomotic stent was preferred. Only in cases where the pancreatic remnant was considered to be very friable was invagination of pancreatic stump into the jejunal loop preferred.

Table 1 Demographic characteristics of the patients and type of operation for the two different groups

	Single loop group (SL)	Isolated Roux loop group (RL)	Total
Patients (*n*)	42	46	88
Age	60.9 ± 11.5	64.4 ± 9.5	62.7 ± 10.5
Gender			
Men/women	23/19	29/17	52/36
Type of operation			
Standard Whipple	25	38	63
Traverso-Longmire	17	8	25

Data collection

Patient data concerning postoperative complications, mortality and hospital stay were evaluated and compared between the two groups. Pancreatic anastomotic failure was initially as described according to the Heidelberg and Johns Hopkins groups as the drainage of more than 50 mL of fluid in 24 h, with an amylase content of more than 3 times the serum amylase activity for more than 10 d after operation[7,8]. In order to adopt a more universally uniform definition we used the ISGPF (International Study Group on Pancreatic Fistula) proposal which is based on the high amylase content of the drain fluid on or after the third postoperative day[9].

Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc., Chicago, IL). Demographic, operative data and postoperative outcome were collected retrospectively. Continuous variables were compared by using Mann-Whitney *U* test and categorical variables were compared by using the χ^2 or Fisher's exact test, depending on the frequency distribution. $P < 0.05$ was considered statistically significant.

RESULTS

Between 1994 and 2004, 88 patients underwent PD for malignancy. The underlying disease was pancreatic head carcinoma ($n = 59$), ampullary carcinoma ($n = 13$), cholangiocarcinoma ($n = 8$), duodenal carcinoma ($n = 6$) and two rare cases of ampullary carcinoid. The male to female ratio was 52:36. The patients' mean age was 62.7 ± 10.5 years (range 33-78). Period I (1994 to 1999-single loop group) included 42 patients, while period Ⅱ (2000 to 2004-isolated Roux loop group) included 46 patients. The demographic data of these patients are shown in Table 1.

Perioperative outcomes

Postoperative complications are demonstrated in Table 2. Mean operative time for the RL group was 366.1 min (range 270-520), which was significantly longer ($P = 0.046$) than the operative time of 338.8 min (range 240-470) recorded in the SL group. No major intraoperative compli-

Table 2 Major and minor complications in both groups *n* (%)

	Single loop group (SL) *n* = 42	Isolated Roux loop group (RL) *n* = 46	*P*
Major complications			
PJ anastomosis failure	3 (7.1)	2 (4.3)	NS
Hemorrhage	1 (2.3)	1 (2.2)	NS
Minor complications			
Wound infection	2 (4.8)	3 (6.5)	
Pulmonary infection	2 (4.8)	4 (8.7)	
Delayed gastric emptying	4 (9.5)	7 (15.2)	
Subhepatic fluid collection	-	1 (2.2)	
Cardiac failure	1 (2.3)	-	
Morbidity	13 (30.9)	18 (39.1)	NS
Mortality	1 (2.3)	1 (2.2)	NS
Operative time (min)	338.8 ± 52.7	366.1 ± 60.1	< 0.05
Hospital stay (d)	19.5 ± 10.1	14.6 ± 5.5	NS

PJ: Pancreaticojejunal; NS: Non-significant.

cation occurred in patients of either group. Two patients died in the early postoperative period (overall mortality; 2.27%). Mortality did not differ significantly between the two groups (P = 1.0). One fatality occurred in a patient of the SL group (mortality; 2.3%) who presented with postoperative pancreatic leak and subsequent massive gastrointestinal bleeding. Death in a patient of the RL group (mortality; 2.17%) resulted after the patient who had pancreaticojejunal anastomotic failure also developed postoperative intrabdominal bleeding accompanied by profound hypoglycemia. Although the patient was reoperated and hemostasis was achieved, he died a few days later as he developed sepsis and multiple organ failure.

Leak from the pancreaticojejunal anastomosis, as defined above, occurred in 3 patients of the SL group (7.1%) and in 2 patients of the RL group (4.3%). The overall leak rate was 5.7%. Comparison of the leak rate between the two groups showed no significant difference (P = 0.66). The incidence of the other grave complication of the procedure, hemorrhage, was not found significantly different between the two groups (P = 1.0) as it occurred in one patient each. Minor complications reported in both groups were wound infection, pulmonary infection and delayed gastric emptying, contributing to morbidity rates of 30.9% for the SL group and 39.1% for the RL group, without significant difference between the two groups (P = 0.422). One patient of the SL group developed cardiac failure, and in one patient of the RL group a subhepatic biloma was detected which was treated nonoperatively with CT-guided drainage.

Patients of the SL group remained in hospital postoperatively for a mean of 19.5 ± 10.1 d (range 9-49). The mean hospital stay of the patients of the RL group was shorter (14.6 ± 5.5 d, range 9-31), but without statistically significant difference between the two groups.

DISCUSSION

The present study failed to demonstrate any significant reduction of pancreatic anastomosis failure when the isolated Roux loop technique was performed for the construction of pancreaticojejunal anastomosis instead of the single loop technique.

The operative mortality rate of pancreaticoduodenectomy, which had remained at unacceptably high levels since the 1970s[6,10,11], dropped dramatically in the last two decades to less than 5% in many reports[3,12,13]. The improved mortality rates can be attributed to a variety of reasons including better perioperative care, accumulated experience on the part of the pancreatic surgeons, refinement of surgical instruments and materials and better anesthesiologic management[14].

Despite reductions in mortality after pancreaticoduodenectomy, the incidence of postoperative morbidity remains high, ranging between 30%-50%[12,13,15,16]. Common postoperative complications include pancreatic fistula, delayed gastric emptying and wound infection. Pancreatic anastomosis failure, which is a major source of morbidity, is considered as the "Achilles' heel" of the procedure. Pancreatic fistula rate can reach 20% even in specialized centers and does not seem to have declined in the same way as mortality rate has done over the last few decades[13,14,17]. Hemorrhage and sepsis are the most frequent sequels of pancreatic fistula, both of which contribute largely to the mortality (20%-40%) as well as to prolonged hospitalization and increased hospital cost[15,18,19].

We analyzed a series of 88 consecutive patients who underwent PD in our department. During the first period (1994-1999), the pancreatic stump was anastomosed sequentially to the single jejunal loop used for the reconstruction of all anastomoses. Mortality (2.3%), morbidity (30.9%) and failure rate of the pancreaticojejunal anastomosis (7.1%) did not differ significantly from those reported in the literature. Although initial reports concerning the use of an isolated jejunal loop for the construction of the pancreaticojejunal anastomosis were far from encouraging with high fistula rates[5,20], various studies published during this first period supported this alternative technique, presenting considerably improved results[21-24]. These studies demonstrated extremely low anastomotic leak rates ranging from 0% to 5.7% and zero fistula-related mortality. Table 3 demonstrates results of the isolated Roux loop technique from various studies[20-28].

The concept of isolation of the pancreatic anastomosis was based mainly on the rationale of diverting biliary from pancreatic secretions. On one hand, this results in avoidance of activation of pancreatic enzymes which could, in theory, erode the anastomotic line and weaken the anastomosis. On the other hand, the jejuno-jejunal anastomosis carries the risk of occlusion due to edema which could increase intraluminal pressure with probably detrimental consequences for the pancreatic anastomosis.

Influenced by these data and aiming to reduce the anastomotic leak rate, in the second period of the study (2000-2006) we adopted the isolated Roux loop technique. However, in our study no significant advantage of this method was found. Although many previous studies utilizing an isolated Roux loop reported zero anastomotic leaks[21-23,25,26], in our study the leak rate was higher (4.3%).

Table 3 Roux-en-Y pancreaticojejunostomy results from various studies

Series	Study type	Patients (total)	Fistulae (%)	Mortality related to fistula (%)	Overall mortality (%)	Hospital stay (d)
Machado *et al*[5]	CS	15	2 (13.3)	0	0	20.0
Funovics *et al*[20]	CS	48	9 (18.7)	0	3 (6.2)	NA
Kingsnorth *et al*[22]	CS	52	0	0	3 (5.8)	18.4
Albertson *et al*[23]	CS	25	0	0	0	12.2
Meyer *et al*[24]	CS	35	2 (5.7)	0	4 (11.4)	NA
Papadimitriou *et al*[21]	CS	109	0	0	1 (0.9)	7.6
Khan *et al*[24]	CS	41	0	0	1 (2.4)	19.6
Sutton *et al*[25]	CS	61	0	0	3 (5)	16.0
Jover *et al*[27]	CS	80	16 (20)	3 (60)	5 (6.6)	20.6
Kaman *et al*[28]	RC	60 (111)	6 (10)	2 (33.3)	5 (8.3)	17.75

NA: Not available; CS: Case series; RC: Retrospective comparative.

This rate was lower than the leak rate of the group in which a single loop of jejunum was used, but without statistical significance. In contrast to previous series of isolated Roux-en-Y pancreaticojejunal anastomosis, which presented no pancreatic fistula-associated mortality[5,20-26], both our fatalities (one from each group) died due to sequels of pancreatic anastomosis failure.

Overall postoperative morbidity in our study is in accordance with major series[29,30]. The lower anastomotic failure rate reported in the RL group possibly contributes to the shorter hospital stay, but also increases the duration of the operation (adding 30 min) and subsequently exposes patients with concomitant diseases to increased risk of complications not related to the operation. Delayed gastric emptying occurred at an increased frequency in patients of the isolated Roux loop group due to the small percentage of pylorus-preserving procedures taken place in this group.

Our study does not confirm that construction of the pancreaticojejunal anastomosis with an isolated Roux loop proves beneficial. Success may well depend on already known parameters, such as consistency of the pancreatic parenchyma and diameter of the pancreatic duct. Hard pancreatic tissue accompanied by wide, dilated duct, as seen in chronic pancreatitis, can result in a safer anastomosis than one constructed over soft tissue and thin duct[31,32].

In conclusion, creation of an isolated Roux-en-Y loop in 46 out of 88 patients in our study did not provide sufficient evidence of superiority over the single loop technique regarding the leak rate, morbidity, mortality and hospital stay.

COMMENTS

Background

Pancreaticojejunal anastomosis failure remains the leading cause of postoperative morbidity and mortality after pancreaticoduodenectomy. Various methods of surgical management of the pancreatic remnant have been proposed, addressing this serious problem. One of these methods utilizes an isolated Roux loop for the construction of the pancreaticojejunal anastomosis.

Research frontiers

This study investigated the outcome of the isolated Roux loop pancreaticojejunal anastomosis and compared it to the single loop technique.

Innovations and breakthroughs

Although the isolated Roux loop technique has been previously described and evaluated, its comparison with the standard single loop technique was not fully studied.

Applications

The isolated Roux loop technique has not been proven to reduce the incidence of pancreatic fistula formation and additionally prolonged the operation time. Single loop technique with sequentially constructed anastomoses remains the operation of choice for reconstruction after pancreaticoduodenectomy. Further randomized controlled studies could strengthen this conclusion.

Peer review

This is a nice series of pancreaticoduodenectomies. The mortality is acceptable and the authors note the limitations of their study.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3183

World J Gastroenterol 2010 July 7; 16(25): 3183-3186
ISSN 1007-9327 (print)

BRIEF ARTICLE

Technical problems produced by the Bravo pH test in nonerosive reflux disease patients

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Received: February 2, 2010 Revised: April 21, 2010
Accepted: April 28, 2010
Published online: July 7, 2010

Abstract

AIM: To evaluate the technical failures of the Bravo pH test in a population with nonerosive gastroesophageal reflux disease.

METHODS: Over the course of a year, we prospectively studied a population of 66 nonerosive reflux disease patients who received a Bravo pH test. The number and frequency of all technical failures were documented, quantified and analyzed.

RESULTS: A total of 66 patients, with a mean age of 41.7 years, were studied. Technical failures occurred in 15.15% of the sample. The most frequent failures were due to poor data reception (4.5%), early dislodgement (4.5%) and capsule removal (6.1%).

CONCLUSION: The Bravo capsule pH test involves a low but non-negligible rate of technical problems, a fact that must always be considered by physicians.

Key words: Bravo test; Capsule dislodgement; Nonerosive reflux disease; Poor data reception; Technical problems

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de Hoyos A, Esparza EA. Technical problems produced by the Bravo pH test in nonerosive reflux disease patients. *World J Gastroenterol* 2010; 16(25): 3183-3186 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3183.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3183

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a condition characterized by heartburn and regurgitation with or without esophageal lesions[1]. It is a common condition, with a prevalence of up to 52.8%, and occurs more frequently in women than in men[2]. Erosive esophagitis and nonerosive reflux disease (NERD) are the primary presentations, with NERD being the most common phenotype[3,4]. This group of patients must undergo pH measurements to confirm the diagnosis of acid reflux, as they lack visible esophageal reflux lesions[5].

To date, the two best methods employed for objectively demonstrating the presence of acid reflux are conventional ambulatory catheter pH-metry monitoring and the Bravo capsule (Medtronic, Minneapolis, MN, USA) catheter-free pH test[6-8]. Both methods are valid and reliable for the measurement of esophageal acid exposure[9,10].

The traditional catheter pH-metry remains the more commonly used of the two methods. However, it can cause undue burden on the patient due to the discomfort and embarrassment of the transnasal pH probe placement, which is known to be uncomfortable and is poorly tolerated[11]. Although the Bravo capsule is a device that is designed to overcome the disadvantages of traditional pH

monitoring, to improve acceptance of testing and to extend the period of monitoring, it is not exempt from technical problems and side effects[12,13]. The primary technical problems of this test are related to failures in transmission or early detachment of the Bravo capsule. Nevertheless, there are many case reports that describe a list of unusual and diverse problems. Both novice and expert gastroenterologists must be familiar with the various possible technical problems that can occur with this device so that they can solve them promptly and efficiently, avoiding additional complications.

In this paper, we quantify and describe our experience of the technical problems that can occur with the use and physical presence of the Bravo capsule in a sample of patients with NERD.

MATERIALS AND METHODS

Patients

We enrolled a total of 66 consecutive patients in a prospective study of 48-h ambulatory pH monitoring using the Bravo capsule, in order to record all technical failures. Before enrollment, each patient had undergone an upper endoscopy which showed an absence of lesions on the esophageal mucosa and were thus diagnosed as having NERD. Patients were asked to discontinue the use of proton pump inhibitors and histamine-2 receptor antagonists for one week prior to the study and to avoid the use of anti-acids 24 h prior to the examination.

None of the patients had significant comorbidities, such as coronary artery disease. The study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants.

Bravo procedure and pH recording

The Bravo capsule was placed 6 cm from the squamocolumnar junction, using standard techniques. All the capsules were placed orally. A second-look esophagoscopy documented suitable capsule attachment. Once the pH recording was initiated, patients were encouraged to engage in their usual daily activities and to consume their usual diet without restrictions. All subjects were instructed to document their food intake, sleep periods and the occurrence of GERD symptoms in a diary to complete the pH-metry evaluation. From the pH records of the first and second day, the average pH value was obtained.

To confirm any capsule dislodgment, a chest X-ray was obtained seven days after the the beginning of the study. Those patients who still showed the device in the esophagus were asked to undergo another plain radiography seven days later to record the detachment.

Assessment of technical problems and statistical analysis

Technical failures of the device were recorded in a "database" and analyzed with a statistical package. Quantitative data, including age and the fraction of time with pH < 4 on the first and second monitoring days were also added. The percentages, means and standard deviations were calculated for each variable. The analysis was performed with the statistical software package SPSS, version 9.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Technical failures, removals and feasibility of the Bravo capsule procedure *n* (%)

Technical problems	Patients (*n* = 66)
Technical failure	10 (15.15)
Transmission failure	3 (4.5)
Early dislodgement	3 (4.5)
Capsule removal	4 (6.1)
Absolute	
Intolerable chest pain	1 (1.5)
Relative	
Detachment failure	1 (1.5)
Transmission failure	1 (1.5)
Error in placement	1 (1.5)
Capsule replacements	2 (3.0)
Feasibility	
Fully finished tests (48 h)	59 (89.39)
Half-finished tests (24 h)	4 (6.0)
Tests without record (0 h)	3 (4.5)

RESULTS

Patient population

Between July 2007 and June 2008, a total of 66 patients were enrolled in the trial. These patients included 27 men (40.9%) and 39 (59.1%) women, aged 11-73 years (mean age = 41.7 years; standard deviation = 13.3 years).

Of the total population, 31 patients reported typical gastroesophageal reflux symptoms, 15 had atypical manifestations and 20 had a mixed symptomatology.

Technical failures and recording efficacy

All of the technical failures observed in our trial are described in Table 1. In the 66 patients, 10 technical failures (15.15%) were observed. Three probes had poor data reception (4.5%), and three more presented with early dislodgement (4.5%). In four cases (6.1%), the capsule had to be removed. We considered intolerable chest pain in one case as an absolute indication for removal. On the contrary, we considered cases of detachment failure (12 d after implantation, because a Heller myotomy was to be performed), transmission failure and placement error to be relative indications for removal. In the last two cases, new capsules (3%) were reinstalled so that those patients could complete the study.

Of the total sample, 89.39% completed the 48-h examination period, and 6% were able to complete only 24 h. However, 24 h was enough time to gather sufficient information to obtain a diagnosis; indeed, using 24 h of data, the Bravo test was accurate in 95% of patients. Only three (4.5%) patients lacked records.

DISCUSSION

This study describes our experience with the Bravo pH test in patients with NERD, a patient population with special features in terms of sensibility and treatment issues.

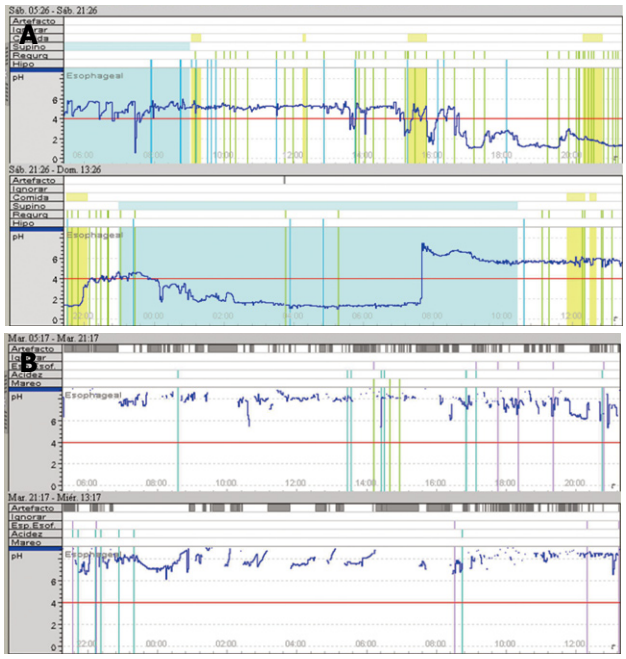

Figure 1 Tracings of problems. A: pH tracing with the typical prolonged drop in the pH line when the capsule drops into the stomach; B: pH tracing (in blue) showing gaps as a result of poor data reception.

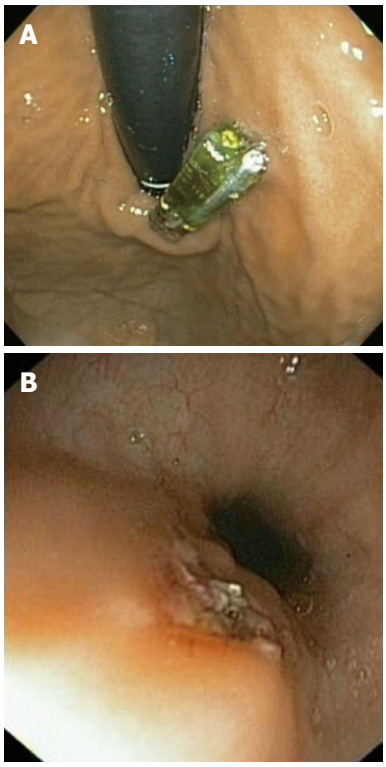

Figure 2 Improper delivery and removal. A: Capsule placement at an erroneous site; B: Mucosal appearance after a hot snare removal.

Technical problems occurred in a low but non-negligible proportion (15.15%) of the sample. Our most remarkable problems were early dislodgement, poor data reception and capsule removals, which occurred at similar rates.

In early capsule dislodgement, the Bravo probe detaches from its location and falls prematurely, i.e., before the 48 h monitoring period is complete. This finding can be observed in the pH tracing as a sudden prolonged drop in the pH value line when the capsule drops into the stomach, followed by a subsequent sharp rise in the pH line as the capsule enters the small intestine (Figure 1A). This early capsule dislodgement rate of 4.5% is similar to those in other reports, which registered rates between 0% and 3.22%[6,14]. Although early dislodgement is considered a failure, it is sometimes possible to complete at least 24 h of monitoring, a time period that still allows a diagnosis of NERD (Table 1).

In the same way, the poor data reception rate of 4.5% (3 of our 66 patients) presented a similar problem to that of early capsule dislodgment. Some authors have reported transmission failure rates of 8.2%[6]. This electromechanical flaw may be seen in the pH tracing as time periods during which data capture was interrupted and are shown as gaps on pH tracings (Figure 1B); these gaps may be interpreted as artifacts during the computerized data analysis. They are potentially attributable to malfunctions in the electronics or possibly the receiver being beyond the range of the signal emanating from the pH capsule.

The need for capsule removal is another frequently observed problem. The capsule removal frequency reached 6.1% (4 of our 66 patients), higher than previously documented frequencies, which ranged from 0% to 3.5%[6,15]. However, the higher incidence observed in our study may have been due to the fact that we considered absolute and relative indications, even though our only absolute indication was a patient with intolerable chest pain (1.5%). This symptom was the primary indication in most previous studies[12,16]. The other three patients had a relative indication. One of these patients presented with transmission failure, and another with a placement error (gastroesophageal junction) (Figure 2A). We decided to remove and replace the capsule with a new one in order to finish the test and to avoid the side effects produced by the presence of two probes in the esophagus, as the presence of the capsule produces esophageal contractions associated with pain[17,18]. Finally, a fourth capsule was retrieved twelve days after implantation, as a Heller myotomy was to be performed. Three of these capsules were retrieved by a cold snare technique, and the other by a hot snare technique (Figure 2B), as previously described[19].

Other studies have reported different and unusual technical problems. Ward *et al*[15] observed that the capsules did not attach properly on the first attempt in at least 7 of 60 (12%) patients. Although there are some reports of major complications, we did not observe any. The main complications previously described were trauma, severe bleeding, esophageal perforations and aspiration into the bronchus[20,21]. In addition, minor incidents have been reported, such as localized inflammation, capsules not deploying from the delivery system, the plunger being broken off during delivery, mucosal tearing and probes not being correctly calibrated[22].

In conclusion, the Bravo capsule results in a low but non-negligible rate of heterogeneous technical difficulties, and although most of them are not life-threatening,

gastroenterologists must be aware of these difficulties in order to interpret and address them appropriately.

COMMENTS

Background

The Bravo capsule pH test is a relatively new method for the assessment of reflux disease, and as a new method, the authors have tried to evaluate the possible technical problems that may occur during its application, course and interpretation.

Research frontiers

This article describes the most common technical failures. Gastroenterologists will find the causes of these technical failures useful in order to avoid them in the future.

Innovations and breakthrough

This trial focuses on patients with nonerosive reflux disease. Up to now, this population has not received much attention.

Applications

The present study will find application in making people aware of the possible problems and failures associated with the Bravo capsule pH test, giving people the opportunity to solve them.

Peer review

The paper is straightforward and clear.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3187

World J Gastroenterol 2010 July 7; 16(25): 3187-3195
ISSN 1007-9327 (print)

BRIEF ARTICLE

Thiopurine S-methyltransferase polymorphisms and thiopurine toxicity in treatment of inflammatory bowel disease

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Received: February 2, 2010 Revised: March 30, 2010
Accepted: April 6, 2010
Published online: July 7, 2010

Abstract

AIM: To evaluate the relationship between thiopurine S-methyltransferase (TPMT) polymorphisms and thiopurine-induced adverse drug reactions (ADRs) in inflammatory bowel disease (IBD).

METHODS: Eligible articles that compared the frequency of TPMT polymorphisms among thiopurine-tolerant and -intolerant adult IBD patients were included. Statistical analysis was performed with Review Manager 5.0. Sub-analysis/sensitivity analysis was also performed.

RESULTS: Nine studies that investigated a total of 1309 participants met our inclusion criteria. The incidence of TPMT gene mutation was increased 2.93-fold (95% CI: 1.68-5.09, P = 0.0001) and 5.93-fold (95% CI: 2.96-11.88, P < 0.00001), respectively, in IBD patients with thiopurine-induced overall ADRs and bone marrow toxicity (BMT), compared with controls. The OR for TPMT gene mutation in IBD patients with thiopurine-induced hepatotoxicity and pancreatitis was 1.51 (95% CI: 0.54-4.19, P = 0.43) and 1.02 (95% CI: 0.26-3.99, P = 0.98) *vs* controls, respectively.

CONCLUSION: This meta-analysis suggests that the TPMT polymorphisms are associated with thiopurine-induced overall ADRs and BMT, but not with hepatotoxicity and pancreatitis.

Key words: Methyltransferases; Inflammatory bowel diseases; Meta-analysis; Adverse drug reactions; Bone marrow toxicity

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Dong XW, Zheng Q, Zhu MM, Tong JL, Ran ZH. Thiopurine S-methyltransferase polymorphisms and thiopurine toxicity in treatment of inflammatory bowel disease. *World J Gastroenterol* 2010; 16(25): 3187-3195 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3187.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3187

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting disease of the gastrointestinal tract. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The thiopurine drugs azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used to treat patients with active, steroid-refractory, and steroid-dependent IBD, and have been proven to be effective for inducing and maintaining remission of CD[1,2]. Unfortu-

nately, the dose of thiopurine often has to be reduced or the therapy has to be discontinued in 9%-28% of patients because of adverse drug reactions (ADRs)[3]. Gastrointestinal disturbances, bone marrow toxicity (BMT), hepatotoxicity and pancreatitis are among the most frequent reasons to prevent their use in some patients.

Blood tests have been performed regularly to monitor the blood count and liver function to detect BMT and hepatotoxicity at an early enough stage to discontinue therapy and avoid life-threatening toxicity. However, the ADRs might develop suddenly and unpredictably during the interval between two tests. Thus, screening the patients before thiopurine treatment for genetic susceptibilities to predict the risk of toxicity has aroused considerable interest in several clinical centers.

Thiopurine S-methyltransferase (TPMT) is a very important enzyme for the metabolism of thiopurine compounds. Lack of its activity has been associated with the incidence of BMT induced by thiopurine therapy. Genetic polymorphisms that account for reduced (heterozygote) or absent (homozygote) TPMT activity have been confirmed. About 10% of the patients have intermediate activity due to heterozygosity of the TPMT, and 1 in 300 patients inherit TPMT deficiency as an homozygote[4]. TPMT*1 is the wild type, TPMT*3A is the most prevalent mutant allele (85%) in Caucasians[4] and TPMT*3C is the most common reported mutant alleles in African and South-East Asian populations[5]. Although several studies have shown that low TPMT activity is associated with BMT, the efficacy of the strategy of screening the TPMT gene mutation in all patients prior to initiating treatment with thiopurine drugs has not yet been definitively confirmed.

The aim of this meta-analysis was to evaluate whether there is a relationship between TPMT polymorphisms and incidence of ADRs in IBD patients.

MATERIALS AND METHODS

Search strategy

The databases PubMed (1966 to July 2009), Embase (1980 to July 2009), Cochrane Controlled Trials Register (Issue 1, 2009), Science Citation Index (1945 to July 2009) and Chinese Biomedical Database (1981 to July 2009) were used for systematic literature searches. We employed both MeSH and free-language terms for "TPMT", "thiopurine S-methyltransferase" AND "inflammatory bowel disease", "ulcerative colitis", OR "Crohn's disease" combined with each of the following: "thiopurine", "azathioprine", "imuran", "6-mercaptopurine" as search terms. A comprehensive search of reference lists of all review articles and original studies retrieved by this method was performed to identify additional studies. Furthermore, we also searched abstracts of major gastroenterological meetings, such as the Digestive Disease Week of the American Gastroenterological Association, the World Congress of Gastroenterology and British Society of Gastroenterology. Authors of some identified trials were asked whether they knew of additional studies, including unpublished ones.

Inclusion and exclusion criteria

The selection criteria were as follows: cross-sectional cohort, prospective cohort and case-control studies were included in the meta-analysis. Studies in abstract form or meeting reports, without publication of the full paper, were also included in the meta-analysis. Only IBD patients aged over 18 years were included.

Studies were included that compared TPMT polymorphism frequencies among thiopurine-tolerant and -intolerant adult IBD patients. Two of the most prevalent mutant types of the TPMT gene were studied, namely, TPMT*3A and TPMT*3C polymorphisms. Studies were included if they provided information on at least one outcome parameter as follows: proper OR of overall ADRs, BMT, hepatotoxicity or pancreatitis.

Furthermore, articles published in English and Chinese were included. Studies in other languages were excluded unless a translation was available.

Data extraction

Standardized data abstraction sheets were prepared. Data were extracted for author and year, location of trials, trial design, diseases, number of enrolled subjects, and dose of thiopurine, meanwhile, key outcome data, such as TPMT polymorphisms and thiopurine induced overall ADRs, BMT, hepatotoxicity and pancreatitis were abstracted from the selected studies. All papers were examined independently for eligibility by two reviewers (Dong XW and Zheng Q). Disagreements were resolved by consulting a third reviewer (Ran ZH). When the results of a particular study were reported in more than one publication, only the most recent and complete data were included in the meta-analysis. Finally, the manuscripts were studied for their comparability by Zhu MM and Tong JL.

Statistical analysis

Data were entered into the Cochrane Collaboration RevMan 5.0 (Copenhagen, 2008). OR with 95% CI was calculated for the TPMT*3A and TPMT*3C polymorphisms *vs* overall ADRs, BMT, hepatotoxicity and pancreatitis. Because of too small a number of patients, heterozygous and homozygous patients were combined as "polymorphism-positive". An OR of < 1 favored the control group, $P < 0.05$ and 95% CI that did not include the value 1 were considered to be statistically significant. The included studies displayed heterogeneity regarding study design, definition of ADRs, and follow-up periods. Therefore, it would be inappropriate to combine the data for further analysis with the fixed-effects model, whereas the random effects model was used for calculations. Finally, we used funnel plot asymmetry to detect any publication bias in the meta-analysis, and Egger's regression test to measure funnel plot asymmetry.

RESULTS

Description of the studies

We reviewed 181 citations and abstracts obtained from our computerized literature searches. Fifty-nine papers

Table 1 Characteristics of excluded studies

Author	Location	Study design	Participants	TPMT genotypes was determined	Dose of thiopurine	Reason for exclusion
Cao *et al*[6], 2009	China	Cross-sectional	43 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	AZA 1.35 mg/kg per day	Incomplete data
Takatsu *et al*[7], 2009	Japan	Cross-sectional	147 treated IBD patients	TPMT*2, TPMT*3B TPMT*3C, TPMT*8	AZA 25 mg/d or 75 mg/d or 100 mg/d	Incomplete data
Uchiyama *et al*[8], 2009	Japan	Cross-sectional	16 treated IBD patients with ADRs	TPMT*2, *3A, *3B TPMT*3C, *3D, *4 TPMT*5, *6, *7, *8	AZA < 50 mg/d or 6-MP < 30 mg/d	Incomparable control group No definition of ADRs Pediatric patients included
Ban *et al*[9], 2008	Japan	Case-control	70 treated IBD patients 41 healthy controls	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	Not mentioned	Dose of thiopurine not mentioned
Gearry *et al*[10], 2003	New Zealand	Case-control	56 treated IBD patients with ADRs 50 treated IBD patients without ADRs	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	Not mentioned	Dose of thiopurine not mentioned
Regueiro *et al*[11], 2002	USA	Cross-sectional	71 treated CD patients	TPMT*3A, TPMT*3B TPMT*3C	AZA 2.35 mg/kg per day or 1.28 mg/kg per day	38 patients had determination of TPMT by phenotype
Evans *et al*[12], 2001	USA	Cross-sectional	23 treated patients with ADRs	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	AZA 32 mg/m^2 per week or 175 mg/m^2 per week or 280 mg/m^2 per week	Non-IBD patients
Naughton *et al*[13], 1999	UK	Cross-sectional	15 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	Not mentioned	Dose of thiopurine not mentioned

Dose of thiopurine: mean or median dose of thiopurine. TPMT: Thiopurine S-methyltransferase; IBD: Inflammatory bowel disease; AZA: Azathioprine; ADRs: Adverse drug reactions; 6-MP: 6-mercaptopurine; CD: Crohn's disease.

were excluded on the basis of publication type and another 105 were excluded after examining the title and abstract. Among these 17 potentially appropriate studies, we found that all of them had studied the association between TPMT polymorphisms and thiopurine-induced ADRs. Abstracts and full texts of the remaining 17 papers were retrieved for further assessment. Of these potential eligible articles, we excluded another eight[6-13], which are listed in Table 1. Two studies were not able to extract the data from the published results, and we also failed to contact the authors of this manuscript, therefore, these two studies were excluded. Three studies were excluded for not mentioning the prescription dose of thiopurine. One study was excluded as it did not set a control group, such that a proper OR could not be calculated. One study was excluded since it included non-IBD patients. In one study, not all of the patients had determination of TPMT genotype. Finally, nine studies[3,5,14-20] met the inclusion criteria; all of which were published in the last 7 years. Three were prospective cohort studies, with the remaining six being cross-sectional. The flowchart of reviews showed the detailed process of selection (Figure 1). The characteristics of the nine trials included in the meta-analysis are summarized in Table 2. The outcomes of the meta-analysis are shown in Figure 2.

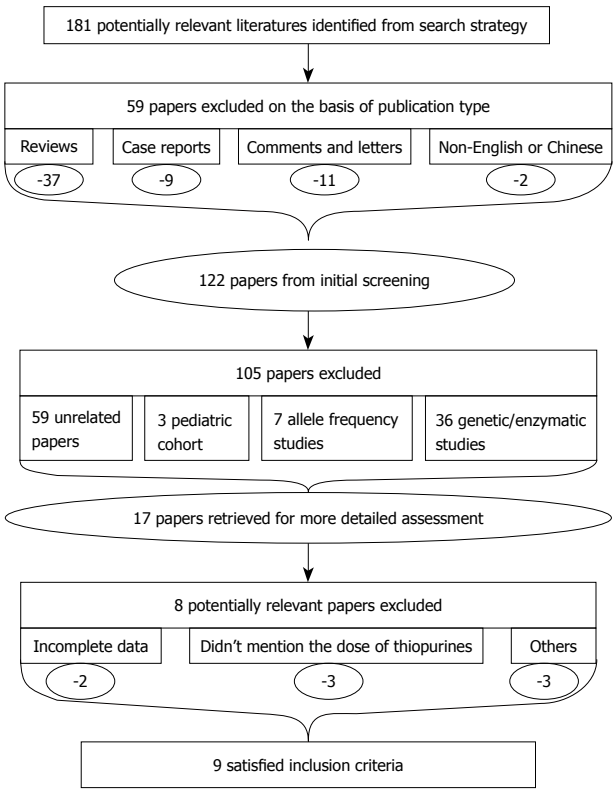


Figure 1 The flowchart of study selection for the meta-analysis.

OVERAll ADRs

Six[3,5,14,17,18,20] of the nine studies reported on the incidence of overall ADRs in 273 IBD patients exposed to thiopurine drugs *vs* 708 IBD patients without ADRs. ADRs included BMT, hepatotoxicity, pancreatitis, gastrointestinal disturbances and adverse reactions during treatment that required reduction of thiopurine dose or discontinuation of therapy. Three[3,5,20] of the studies demonstrated that the AZA/6-MP-induced ADRs were independent of the TPMT polymorphisms; the remaining three showed that TPMT polymorphisms strongly predicted ADRs. The TPMT polymorphisms were significantly associated with ADRs in the overall calculated OR (OR: 2.93, 95% CI: 1.68-5.09, P = 0.0001). When excluding hepatotoxicity and

Table 2 Characteristics of included studies

Author	Location	Study design	Participants	TPMT genotypes was determined	Dose of thiopurine	ADRs and definitions
Ansari *et al*[14], 2008	UK	Prospective cohort	215 treated IBD patients	TPMT*3A, TPMT*3B TPMT*3C	AZA 2 mg/kg per day	All ADRs BMT: WBC < 3.5×10^9 or Ne < 1.5×10^9 H: ALT > 2 × ULN P: AP and amylase > 4 × ULN and supportive radiological findings
Hawwa *et al*[15], 2008	UK	Cross-sectional	36 treated IBD patients	TPMT*3A, TPMT*3B TPMT*3C	AZA 1.49 mg/kg per day	BMT: WBC < 3.0×10^9 or Ne < 1.5×10^9 or PLT < 150×10^9
Winter *et al*[5], 2007	UK	Cross-sectional	130 treated IBD patients	TPMT*2, TPMT*3A TPMT*3C	AZA 1.6 mg/kg per day	All ADRs BMT: WBC < 3×10^9 H: No definition P: No definition
Stocco *et al*[16], 2007	Italy	Cross-sectional	70 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	AZA: 2 mg/kg per day	All ADRs BMT: WBC < 3×10^9 or PLT < 100×10^9 H: ALT, AST or ALP > 2 × ULN P: AP and amylase > 2 × ULN
Palmieri *et al*[17], 2007	Italy	Cross-sectional	422 treated IBD patients	TPMT*3A, TPMT*3B TPMT*3C	AZA 2-2.5 mg/kg per day or 6-MP 1-1.25 mg/kg per day	All ADRs BMT: WBC < 3×10^9 or PLT < 100×10^9 H: ALT > 2 × ULN P: Amylase, lipase > 2 × ULN and AP
Zelinkova *et al*[18], 2006	Netherlands	Cross-sectional	262 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	AZA 2-2.5 mg/kg per day[1]	BMT: WBC < 3×10^9 or PLT < 100×10^9 H: ALT > 2 × ULN
Hindorf *et al*[3], 2006	Sweden	Prospective cohort	60 treated IBD patients	TPMT*2, *3A, *3B TPMT*3C, *3D, *4 TPMT*5, *6, *7, *8 TPMT*10, *14, *15	AZA 2.5 mg/kg per day or 6-MP 1.25 mg/kg per day	All ADRs BMT: WBC < 3×10^9 or PLT < 100×10^9 or Ne < 1.5×10^9 P: No definition
Derijks *et al*[19], 2004	Netherlands	Prospective cohort	30 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, TPMT*3C	6-MP 0.71 mg/kg per day	BMT: WBC < 4×10^9 or PLT < 100×10^9
Schwab *et al*[20], 2002	Germany	Cross-sectional	93 treated IBD patients	TPMT*2, TPMT*3A TPMT*3B, *3C, *3D	AZA 1.5 mg/kg per day or 1.9 mg/kg per day	All ADRs BMT: WBC < 3×10^9 or PLT < 100×10^9 H: ALT or AST > 2 × ULN P: Amylase, lipase > 2 × ULN and AP

[1]The initial dose. White blood cells (WBC), neutrophils (Ne), and platelets (PLT) are expressed per liter; H: Hepatotoxicity; P: Pancreatitis; AP: Abdominal pain; BMT: Bone marrow toxicity; ALT: Alanine transaminase; ULN: Upper limit of normal; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

pancreatitis from the overall ADRs group, it still indicated that IBD patients exposed to thiopurine drugs, with ADRs, were more likely to have TPMT polymorphisms than the controls (OR: 4.37, 95% CI: 1.69-11.29, $P = 0.002$).

BMT

All of the nine studies[3,5,14-20] reported on the incidence of BMT in 1309 IBD patients treated with thiopurine. Twenty-three of 77 IBD patients with BMT and 87 of 1232 IBD patients without BMT had the TPMT gene mutation. Researchers from the United Kingdom[14] defined BMT as a white blood cells count of < 3.5×10^9/L or a neutrophil count of < 1.5×10^9/L. In the study of Winter *et al*[5], BMT was defined as a white blood cells count of < 3.0×10^9/L. Researchers from Sweden[3] have defined BMT as a white blood cells count of < 3.0×10^9/L, a neutrophil count of < 1.5×10^9/L, or a platelet count of < 100×10^9/L. Hawwa *et al*[15] have defined BMT as a white blood cells count of < 3.0×10^9/L, a neutrophil count of < 1.5×10^9/L, or a platelet count of < 150×10^9/L. Dutch researchers[19] have defined BMT as a white blood cells count of < 4.0×10^9/L or a platelet count of < 100×10^9/L. BMT was defined in the remaining four studies[16-18,20] as a white blood cells count of < 3.0×10^9/L or a platelet count of < 100×10^9/L. Of these selected studies, five reported that heterozygous TPMT genotype strongly predicted BMT, and the remaining four failed to demonstrate an association between TPMT gene mutation and risk of BMT. The pooled OR demonstrated that TPMT polymorphisms were significantly associated with BMT (OR: 5.93, 95% CI: 2.96-11.88, $P < 0.00001$).

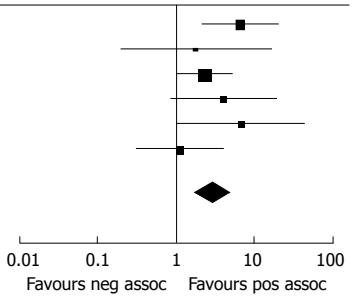
Hepatotoxicity

Six studies[5,14,16-18,20] have reported on the incidence of hepatotoxicity in IBD patients treated with thiopurines. Three[14,17-18] of these have defined hepatotoxicity as alanine transaminase (ALT) levels of at least twice the upper limit of normal (ULN). A German research group[20] has defined hepatotoxicity as either ALT levels or aspartate aminotransferase (AST) levels of at least twice the ULN. Stocco *et al*[16] have defined hepatotoxicity as ALT, AST or alkaline phosphatase (ALP) levels of at least twice the ULN. No definition of hepatotoxicity was provided in the study of Winter *et al*[5], therefore, this study was excluded in this sub-analysis. Only three of the 37 IBD patients with hepatotoxicity were TPMT heterozygotes/homozygotes, while 82 of 1017 IBD patients without hepatotoxicity were TPMT heterozygotes/homozygotes. All of the remaining five have shown that there was no association between AZA-related hepatotoxicity and TPMT poly-

A

Study or subgroup	All adverse drug reactions Events	Total	No adverse drug reactions Events	Total	Weight (%)	Odds ratio M-H, random (95% CI)
Ansari *et al*[14], 2008	15	83	4	124	21.0	6.62 (2.11-20.74)
Hindorf *et al*[3], 2006	5	44	1	15	6.0	1.79 (0.19-16.73)
Palmieri *et al*[17], 2007	9	73	20	349	36.2	2.31 (1.01-5.31)
Schwab *et al*[20], 2002	3	14	5	79	11.8	4.04 (0.84-19.31)
Stocco *et al*[16], 2007	3	15	2	55	8.2	6.63 (1.00-44.11)
Winter *et al*[5], 2007	4	44	7	86	16.9	1.13 (0.31-4.08)
Total (95% CI)		273		708	100.0	2.93 (1.68-5.09)
Total events	39		39			

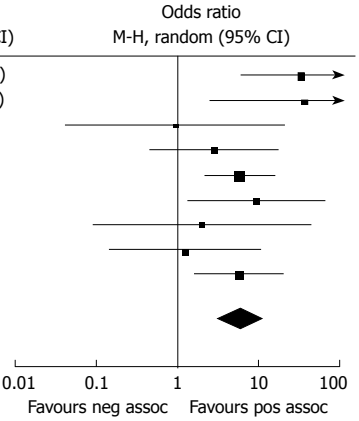
Heterogeneity: Tau^2 = 0.04; χ^2 = 5.44, *df* = 5 (*P* = 0.36); I^2 = 8%
Test for overall effect: *Z* = 3.80 (*P* = 0.0001)



B

Study or subgroup	Bone marrow toxicity Events	Total	No bone marrow toxicity Events	Total	Weight (%)	Odds ratio M-H, random (95% CI)
Ansari *et al*[14], 2008	5	7	14	200	12.3	33.21 (5.90-186.87)
Derijks *et al*[19], 2004	3	4	2	26	5.9	36.00 (2.46-527.06)
Hawwa *et al*[15], 2008	0	3	4	33	4.5	0.94 (0.04-21.31)
Hindorf *et al*[3], 2006	2	10	4	49	11.0	2.81 (0.44-18.00)
Palmieri *et al*[17], 2007	6	23	23	399	24.4	5.77 (2.08-16.02)
Schwab *et al*[20], 2002	2	5	6	88	10.0	9.11 (1.27-65.46)
Stocco *et al*[16], 2007	0	3	4	67	4.5	2.02 (0.09-45.36)
Winter *et al*[5], 2007	1	10	10	120	8.6	1.22 (0.14-10.65)
Zelinkova *et al*[18], 2006	4	12	20	250	18.7	5.75 (1.59-20.77)
Total (95% CI)		77		1232	100.0	5.93 (2.96-11.88)
Total events	23		87			

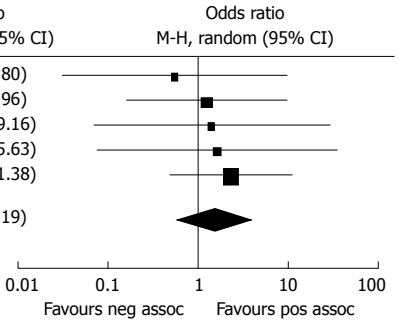
Heterogeneity: Tau^2 = 0.25; χ^2 = 10.32, *df* = 8 (*P* = 0.24); I^2 = 22%
Test for overall effect: *Z* = 5.02 (*P* < 0.00001)



C

Study or subgroup	Hepatotoxicity Events	Total	No hepatotoxicity Events	Total	Weight (%)	Odds ratio M-H, random (95% CI)
Ansari *et al*[14], 2008	0	8	19	199	12.5	0.54 (0.03-9.80)
Palmieri *et al*[17], 2007	1	12	28	410	24.1	1.24 (0.15-9.96)
Schwab *et al*[20], 2002	0	3	8	90	11.3	1.39 (0.07-29.16)
Stocco *et al*[16], 2007	0	3	5	67	11.0	1.62 (0.07-35.63)
Zelinkova *et al*[18], 2006	2	11	22	251	41.2	2.31 (0.47-11.38)
Total (95% CI)		37		1017	100.0	1.51 (0.54-4.19)
Total events	3		82			

Heterogeneity: Tau^2 = 0.00; χ^2 = 0.82, *df* = 4 (*P* = 0.94); I^2 = 0%
Test for overall effect: *Z* = 0.79 (*P* = 0.43)



D

Study or subgroup	Pancreatitis Events	Total	No pancreatitis Events	Total	Weight (%)	Odds ratio M-H, random (95% CI)
Ansari *et al*[14], 2008	0	8	19	199	22.5	0.54 (0.03-9.80)
Palmieri *et al*[17], 2007	0	16	29	406	23.3	0.39 (0.02-6.63)
Schwab *et al*[20], 2002	0	3	8	90	20.2	1.39 (0.07-29.16)
Stocco *et al*[16], 2007	1	7	4	63	34.1	2.46 (0.24-25.69)
Total (95% CI)		34		758	100.0	1.02 (0.26-3.99)
Total events	1		60			

Heterogeneity: Tau^2 = 0.00; χ^2 = 1.29, *df* = 3 (*P* = 0.73); I^2 = 0%
Test for overall effect: *Z* = 0.02 (*P* = 0.98)

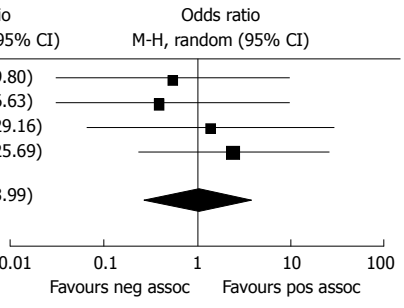


Figure 2 Association between thiopurine S-methyltransferase (TPMT) polymorphisms and all adverse drug reactions (ADRs) (A), bone marrow toxicity (B), hepatotoxicity (C) and pancreatitis (D). Total: Total number of patients within the ADRs or no ADRs group; Events: Number of patients with one or more TPMT alleles within the ADRs or no ADRs group. neg assoc: Favoured the no ADRs group; pos assoc: Favoured the ADRs group.

morphisms. The pooled OR indicated that no significant difference in TPMT polymorphisms was seen between IBD patients with thiopurine-induced hepatotoxicity and controls (OR: 1.51, 95% CI: 0.54-4.19, *P* = 0.43).

Pancreatitis

Six studies[3,5,14,17,18,20] have reported on the incidence of pancreatitis in IBD patients treated with thiopurine and detected TPMT polymorphisms. In the study of Ansari

et al[14], pancreatitis was defined as serum amylase of more than four times the ULN, associated with severe abdominal pain, and supportive radiological findings. Winter *et al*[5] and Hindorf *et al*[3] have not defined the criteria of pancreatitis, thus, we also excluded these two studies from the sub-analysis. The remaining studies[17,18,20] have defined pancreatitis as either serum amylase or serum lipase of more than two times the ULN, associated with severe abdominal pain. Only one of 34 IBD patients with pancreatitis and 60 of 758 without pancreatitis had the TPMT gene mutation. All of the four studies have demonstrated that there was no association between pancreatitis and TPMT polymorphisms. Pooled data have demonstrated that there was no significant difference in TPMT polymorphisms between IBD patients with thiopurine-induced pancreatitis and controls (OR: 1.02, 95% CI: 0.26-3.99, $P = 0.98$).

Subgroup analysis

We performed subgroup analysis in which we excluded the studies with different definitions of BMT to determine the effect on the test of heterogeneity and the overall pooled estimates. Because the results were nearly identical, only results based on the four studies[16-18,20] that have defined BMT as a white blood cells count of $< 3.0 \times 10^9$/L or a platelet count of $< 100 \times 10^9$/L were reported. The pooled OR of the four studies[16-18,20] was 5.05 (95% CI: 2.58-9.88, $P < 0.01$). This result still showed a significant difference in TPMT polymorphisms between IBD patients with and without BMT. Similarly, we also performed subgroup analysis in which we individually excluded the studies with different definitions of hepatotoxicity and pancreatitis, to determine the effect on the test of heterogeneity and the overall pooled estimates. Because the results were nearly identical, they were not reported.

Sensitivity analysis

Studies that prescribed AZA $\leqslant$ 2 mg/kg per day or 6-MP $\leqslant$ 1 mg/kg per day.

When the studies[5,14,16,19,20] that prescribed AZA > 2 mg/kg per day or 6-MP > 1 mg/kg per day were excluded, results remained consistent with original results. A significant difference in the incidence of TPMT polymorphisms was observed in the overall ADRs group (OR: 7.82, 95% CI: 2.18-28.12, $P < 0.01$) and BMT group (OR: 6.73, 95% CI: 3.52-12.85, $P < 0.01$) compared with the controls. However, no significant difference was found in the hepatotoxicity group (OR: 1.04, 95% CI: 0.18-5.90, $P = 0.96$) and pancreatitis group (OR: 1.36, 95% CI: 0.28-6.49, $P = 0.70$) compared with the controls.

Including the excluded studies

When the excluded studies[9,10,13] that provided information on at least one outcome parameter such as proper OR of overall ADRs, BMT, hepatotoxicity or pancreatitis were taken into account, there was still a significant difference in TPMT polymorphisms between IBD patients with or without ADRs. It also showed an increase in the risk of the incidence of TPMT polymorphisms in the overall ADRs group (OR: 2.76, 95% CI: 1.69-4.50, $P < 0.0001$) and BMT group (OR: 6.73, 95% CI: 3.52-12.58, $P < 0.00001$) compared with the controls. No significant difference in the incidence of TPMT polymorphisms was found in the hepatotoxicity group (OR: 1.27, 95% CI: 0.53-3.07, $P = 0.59$) and pancreatitis group (OR: 1.32, 95% CI: 0.46-3.76, $P = 0.60$) compared with the controls.

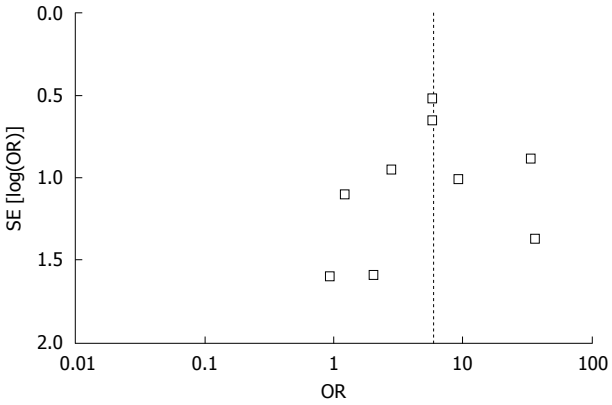


Figure 3 Funnel plot of included studies for this meta-analysis.

Studies reporting on $\geqslant$ 100 patients

Analysis of the studies[5,14,17,18] that reported on $\geqslant$ 100 IBD patients treated with thiopurine, and detected TPMT polymorphisms showed that the results were consistent with the overall results and the analysis of the original studies. A significant difference in the incidence of TPMT polymorphisms was observed in the overall ADRs group (OR: 2.64, 95% CI: 1.06-6.53, $P = 0.04$) and BMT group (OR: 6.53, 95% CI: 2.35-18.19, $P = 0.0003$) compared with the controls. No significant difference in the incidence of TPMT polymorphisms was found in the hepatotoxicity group (OR: 1.51, 95% CI: 0.47-4.82, $P = 0.49$) and pancreatitis group (OR: 0.46, 95% CI: 0.06-3.47, $P = 0.45$) compared with the controls.

Publication bias

Figure 3 shows a funnel plot of the studies used in this meta-analysis that have reported on the incidence of TPMT polymorphisms in BMT. We found that the funnel plot was slightly asymmetrical in distribution, but Egger's regression test suggested no significant asymmetry of the funnel plot ($P = 0.623$), which indicated no evidence of substantial publication bias.

DISCUSSION

This review evaluated 17 published studies that have studied the association between TMPT gene polymorphisms and the development of ADRs in thiopurine-treated IBD patients. Nine studies among adult IBD patients were included, while the random effects model was used for the meta-analysis. The results of the present meta-analysis suggested that IBD patients with TPMT polymorphisms are more likely to experience ADRs, in particular BMT, but not hepatotoxicity and pancreatitis. Notably, the result

remained significant after the sensitivity analysis.

The association of TPMT polymorphisms with thiopurine toxicity suggested by previous studies[14] is confirmed in this meta-analysis. The study of Ansari *et al*[14] was the largest prospective study to use full-dose AZA (2 mg/kg) without dose adjustment in IBD patients. They have found that heterozygous TPMT genotype strongly predicted ADRs (79% heterozygous *vs* 35% wild-type TPMT, $P < 0.001$), especially for gastric intolerance and BMT. Another study[21] has shown that heterozygous TPMT genotypes were significantly associated with IBD patients experiencing nausea and vomiting. Although several studies[3,5,20] have demonstrated that there was no association between thiopurine-related ADRs and TPMT polymorphisms, the retrospective nature of most of these studies may not have provided a true measure of ADRs. The OR for mutations of the TPMT gene in thiopurine-treated IBD patients suffering from overall ADRs was 2.93.

A higher frequency of TPMT mutation was seen in thiopurine-treated IBD patients with BMT than in those without BMT. This was corroborated in the sensitivity analysis. TPMT polymorphisms results in greater conversion of 6-MP to 6-thioguanine nucleotides (6-TGNs) and methylthioinosine monophosphate (meTIMP) *via* the hypoxanthine guanine phosphoribosyltransferase pathway. 6-TGNs and meTIMP are thought to be associated with BMT[3,22]. The study by Hindorf *et al*[3] has suggested that BMT might be dose-related; 67% of the 27 patients with ADRs tolerated long-term treatment with a lower dose (median 1.32 mg/kg per day AZA), and low-dose AZA/6-MP was as effective as the standard dose for remission induction and maintenance of remission in patients with UC and CD[23], but this was not confirmed in the sensitivity analysis of studies that prescribed AZA $\leqslant$ 2 mg/kg per day or 6-MP $\leqslant$ 1 mg/kg per day. That could be explained by the small sample sizes. Hawwa *et al*[15] have found that patients with a heterozygous TPMT genotype usually experienced early and severe myelosuppression when they were treated with AZA/6-MP at standard dosage, but all of the included studies showed that BMT occurred after an average exposure time of 1 mo, except the studies by Hawwa *et al*[15] and Derijks *et al*[19].

Previous studies[24] have found an association between heterozygotes for the TPMT*3A allele and hepatotoxicity in acute lymphocytic leukemia patients who received a standard dose of 6-MP/6-TG. However, it was not shown in this meta-analysis. The results of the present study showed no increase in the incidence of TPMT polymorphisms in thiopurine-treated IBD patients with hepatotoxicity compared with those without hepatotoxicity. This was confirmed in the sensitivity analysis. TPMT polymorphisms are seldom found in patients exhibiting thiopurine-related hepatotoxicity, and thiopurine-induced hepatotoxicity cannot be generally attributed to it[20]. It has been suggested that elevated concentrations of 6-methylmercaptopurine (6-MMP) are associated with thiopurine-induced hepatotoxicity, and elevated concentrations of 6-MMP could be due to high TPMT activity but not TPMT polymorphisms[25].

No significant difference was found in the incidence of TPMT polymorphisms between the thiopurine-treated IBD patients with and without pancreatitis. The results of the sensitivity analysis also showed no significant difference. Thiopurine-induced pancreatitis is dose-independent and seems to be independent of TPMT polymorphisms. AZA is a drug that can induce pancreatitis, especially when it is used in the treatment of CD, which might be due to an immune-mediated idiosyncratic drug reaction because of a genetic predisposition[26].

Recently, Higgs *et al*[27] have published a meta-analysis of myelosuppression in patients with intermediate TPMT activity compared with wild-type. They have found that individuals with intermediate TPMT activity or one TPMT variant allele had an increased risk of developing thiopurine-induced BMT, compared with individuals with normal activity. The search was not limited to a specific disease or condition. They included a total of 67 studies, but most of them were retrospective cohorts. The genotype and/or phenotype tests were described in 57 studies. As we know, the overall concordance rate between the genetic and phenotypic tests for TPMT was 71.6%[28]. In addition, the cutoff points of TPMT activity have varied in different groups. The phenotype test might also be influenced by blood transfusion during the previous 3 mo. The most important thing is that prospective studies that have evaluated the cost-effectiveness of the phenotype test used in IBD treatment have been rather scarce.

This meta-analysis has several weaknesses, most of which relate to heterogeneous definitions of BMT, hepatotoxicity and pancreatitis. Therefore, we had to base our analysis on studies that were vulnerable to bias. Second, all of the included studies were performed in populations of European descent. Further studies are needed in other countries because of ethnic differences in TPMT polymorphisms. Third, the low number of ADRs made statistical precision difficult. Finally, it should be noted that not all of the ADRs could be explained only by TPMT genotyping, but also by other reasons, such as concurrent viral infections[29] or co-medication (e.g. mesalamine or sulfasalazine)[30], which can interfere with thiopurine metabolism.

In conclusion, this meta-analysis provides strong evidence that TPMT polymorphisms are associated with overall ADRs and BMT in patients taking 6-MP or AZA, and thus may be useful in the management of these patients. The present study also clearly demonstrates that thiopurine-related hepatotoxicity and pancreatitis cannot be explained by TPMT polymorphisms. Although there is no consensus that TPMT genotype should be measured before embarking on therapy with thiopurine in IBD patients[31], based on our meta-analysis, we believe that TPMT genotyping is warranted before embarking on therapy with thiopurine in IBD patients. This is also suggested by the US Food and Drug Administration recommendations[32] and by one socioeconomic study of IBD patients[33].

COMMENTS

Background

The efficacy of the thiopurines has been well established for the treatment of

inflammatory bowel disease (IBD). However, they have severe adverse drug reactions (ADRs) such as gastrointestinal disturbances, bone marrow toxicity (BMT), hepatotoxicity and pancreatitis that prevent their use in some patients. The impact of genetic variation of the thiopurine S-methyltransferase (TPMT) gene on its toxicity has been evaluated in several studies, with varying outcomes.

Research frontiers

Recent pharmacogenetic advances have led to the development of novel strategies to optimize and individualize therapy with azathioprine (AZA) and 6-mercaptopurine (6-MP). The strategy of screening the TPMT gene mutation might maximize efficacy while minimizing toxicity.

Innovations and breakthroughs

The results of the previous studies on TPMT polymorphisms and thiopurine toxicity have been inconsistent. Recently, one meta-analysis has found that individuals with both intermediate and absent TPMT activity had an increased risk of developing thiopurine-induced BMT. However, there is still no prospective study that has evaluated the cost-effectiveness and utility of the TPMT phenotyping test before taking thiopurine medication, while TPMT genotyping is recommended by one socioeconomic study in IBD patients. This meta-analysis has shown that IBD patients with TPMT polymorphisms are at risk of increased thiopurine toxicity when taking thiopurine medications.

Applications

The meta-analysis provides strong evidence that the TPMT polymorphisms are associated with overall ADRs and BMT in patients taking 6-MP or AZA. Based on the meta-analysis, it is believed that TPMT genotyping is warranted before embarking upon therapy with thiopurine in IBD patients.

Terminology

TPMT is a cytosolic enzyme that methylates thiopurine compounds. This gene encodes the enzyme that metabolizes thiopurine drugs *via* S-adenosyl-L-methionine as the S-methyl donor and S-adenosyl-L-homocysteine as a byproduct. Genetic polymorphisms that affect this enzymatic activity are correlated with variations in sensitivity and toxicity to such drugs within individuals.

Peer review

The authors describe a meta-analysis of associations between TPMT genetic polymorphisms and ADRs, including BMT, hepatotoxicity, and pancreatitis. The authors' attempt at meta-analysis is interesting, considering current contradictory data about the efficacy of TPMT genotyping in predicting AZA/6-MP toxicity. Moreover, their claims are reasonable.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3196

World J Gastroenterol 2010 July 7; 16(25): 3196-3201
ISSN 1007-9327 (print)

BRIEF ARTICLE

Role of diffusion-weighted magnetic resonance imaging in the diagnosis of extrahepatic cholangiocarcinoma

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Received: January 4, 2010 Revised: March 17, 2010
Accepted: March 24, 2010
Published online: July 7, 2010

Abstract

AIM: To determine the clinical value of diffusion-weighted imaging (DWI) for the diagnosis of extrahepatic cholangiocarcinoma (EHCC) by comparing the diagnostic sensitivity of DWI and magnetic resonance cholangiopancreatography (MRCP).

METHODS: Magnetic resonance imaging examination was performed in 56 patients with suspected EHCC. T1-weighted imaging, T2-weighted imaging, MRCP and DWI sequence, DWI using single-shot spin-echo echo-planar imaging sequence with different b values (100, 300, 500, 800 and 1000 s/mm^2), were performed. All cases were further confirmed by surgery or histopathological diagnosis. Two radiologists jointly performed the analysis of the DWI and MRCP images. Apparent diffusion coefficient (ADC) value and signal-noise ratio were calculated for EHCC. Sensitivity, specificity, accuracy, positive predictive value and negative predictive value were tested using DWI with a b value of 500 s/mm^2 and MRCP images, respectively.

RESULTS: Histopathological diagnosis confirmed that among the 56 cases, 35 were EHCC (20 hilar and 15 distal extrahepatic), 16 were cholangitis, and 5 were calculus of bile duct. Thirty-three out of the 35 EHCC cases were detected by DWI. EHCC exhibited differential levels of high signal intensity in DWI and low signal intensity in the ADC map. The mean value for ADC was (1.31 ± 0.29) × 10^{-3} mm^2/s. The detection rate of EHCC was significantly higher by DWI (94.3%) than by MRCP (74.3%) (P < 0.05). There was a significant difference in sensitivity (94.3% *vs* 74.3%), specificity (100% *vs* 71.4%), accuracy (96.4% *vs* 73.2%), positive predictive value (100% *vs* 81.3%), and negative predictive value (91.3% *vs* 62.5%) between DWI and MRCP in diagnosing EHCC.

CONCLUSION: DWI has a high sensitivity for the detection of EHCC as it shows the EHCC lesion more unambiguously than MRCP does. DWI can also provide additional clinically important information in EHCC patients when added to routine bile duct MR imaging protocols.

Key words: Diffusion magnetic resonance imaging; Cholangiocarcinoma; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography

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Cui XY, Chen HW. Role of diffusion-weighted magnetic resonance imaging in the diagnosis of extrahepatic cholangiocarcinoma. *World J Gastroenterol* 2010; 16(25): 3196-3201 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3196.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3196

INTRODUCTION

Extrahepatic cholangiocarcinoma (EHCC) refers to cancers occurring at the left or right hepatic duct to the

ampulla. In clinical practice, the lack of typical early symptoms often results in a delayed or incorrect diagnosis. In recent years, advances in imaging technology have significantly improved the detection rate and accuracy of definitive diagnosis for EHCC.

With the continuous development and improvement of magnetic resonance imaging (MRI) hardware and software, the use of parallel acquisition techniques to reduce scan time and improve spatial resolution, and the wide application of the shortened echo chain technique in spin-echo echo-planar imaging (SE-EPI), diffusion-weighted imaging (DWI) has been increasingly used in a wide range of clinical applications[1,2]. Although the application of DWI to the diagnosis of numerous conditions has attracted increased attention and is the focus of many researches, to our knowledge, there exists no literature on the clinical application of DWI to the diagnosis of EHCC.

MATERIALS AND METHODS

Patients

A total of 56 patients (34 male and 22 female) were examined between March 2008 and December 2009. The patients had a suspected diagnosis of EHCC, and the main clinical symptom was jaundice. Patient ages ranged from 38 to 75, with an average age of 61.0. Thirty-three patients had an elevated level of carbohydrate antigen 19-9 (CA19-9).

MRI scan sequence and parameters

All imaging was performed on a 1.5T MRI scanner (Signa Excite HD, General Electric Healthcare) and a body phased array coil. Patients were asked to fast 4-6 h before imaging, were coached in breathing and breath-hold and were imaged in the supine position. All patients underwent MR axial fast spoiled gradient echo (FSPGR) T1-weighted (T1W), fast recovery fast spin echo (FRFSE) fat-suppressed T2-weighted (T2W), coronal fast imaging employing steady-state acquisition (FIESTA), 3D MRCP and DWI axial scans. 3D MRCP using fast recovery fast spin echo sequence (FRFSE-XL), heavily T2W with respiratory gating, triggered at exhale, and thin multi-plane collection. The scan parameters were: TR and TE time adjusted according to the respiratory rate, and set at approximately 2000-4500 ms and 550-750 ms, respectively; bandwidth 62.5 kHz, echo chain length (ETL) 8; field of view (FOV) 360-400 mm, matrix 320 × 256; slice thickness 3 mm, without interval (-1.5 mm), 80 slices, NEX 1, acquisition time was 2 min and 20 s to 3 min. The original 2D multi-plane formation image was reconstructed into 3D MRCP images using the maximum intensity projection (MIP) and volume-rendering (VR) functions in the workstation, thereby allowing the reconstructed 3D MRCP images to be rotated in any direction. Axial DWI used a single-shot SE-EPI sequence, space parallel acquisition and array spatial sensitivity encoding technique (ASSET), and the scan was performed under breath-hold. Diffusion gradients were applied along three orthogonal directions: frequency encoding (X), phase encoding (Y) and slice-select (Z). The diffusion sensitivity coefficients were b = 100, 300, 500, 800, and 1000 s/mm^2. The DWI scan parameters were: TR 1300 ms, effective TE 41.0-69.2 ms; bandwidth 166.67 kHz, flip angle 90°, slice thickness 5 mm, slice gap 1 mm, single acquisition, FOV 340-400 mm, matrix 128 × 128, NEX 4, acceleration factor 2.

Image analysis

All MR images were jointly viewed and analyzed by two senior radiologists with more than 5 years of combined experience in abdominal MRI diagnosis. All image post-processing was performed using the Advantage Workstation 4.3 (AW4.3, General Electric Healthcare) and the FuncTool software package.

The apparent diffusion coefficient (ADC) values and signal-noise ratio (SNR) of the lesions imaged in the DWI sequence were calculated for different b values. The formula[3] for calculating the value of ADC is given by: $ADC = [\ln (S_{low}/S_{high})]/(b_{high}-b_{low})$. Where S_{low} and S_{high} represent low signal intensity and high signal intensity, respectively, and b_{high}, and b_{low} represent the high b value and low b value, respectively.

After calculating the ADC map using AW4.3, the ADC values can be automatically measured by mapping regions of interest (ROI). The ROI is mapped by selecting lesion areas with high signal intensity in DWI and low signal intensity in ADC. Preferably, the size of ROI is between 8-20 mm^2. Two radiologists performed these tests three times each to derive the mean value. The SNR represents the ratio of lesion signal intensity to the standard deviation of background noise along the phase-encoding direction.

For DWI-based detection and confirmation of diagnosis of EHCC, the b value used was 500 s/mm^2. The evaluation criteria for EHCC were the status of the malignant lesions, the intensity of the malignant lesion signal, the degree of reduction in the malignant lesion signal with the increase of b value, and the ADC value of the malignant lesion. In detail, the following descriptions were assigned[4]: malignant lesions demonstrate mild to moderate high signal intensity in DWI with a b value of 0 s/mm^2; compared with the surrounding tissue, malignant lesions continue to demonstrate high signal intensity in DWI with a b value of 500 s/mm^2; ADC map demonstrates low signal intensity and has a relatively low ADC value. Otherwise, the lesion was considered benign.

In MRCP, the malignant stricture is characterized by an irregular narrow edge, non-symmetry of the narrow and an abrupt cut-off[5,6].

Statistical analysis

All data were processed using the SPSS 13.0 statistical package. Measurement data are represented by mean ± SD. To compare the ADC value and SNR under different b values, the Friedman test was performed over

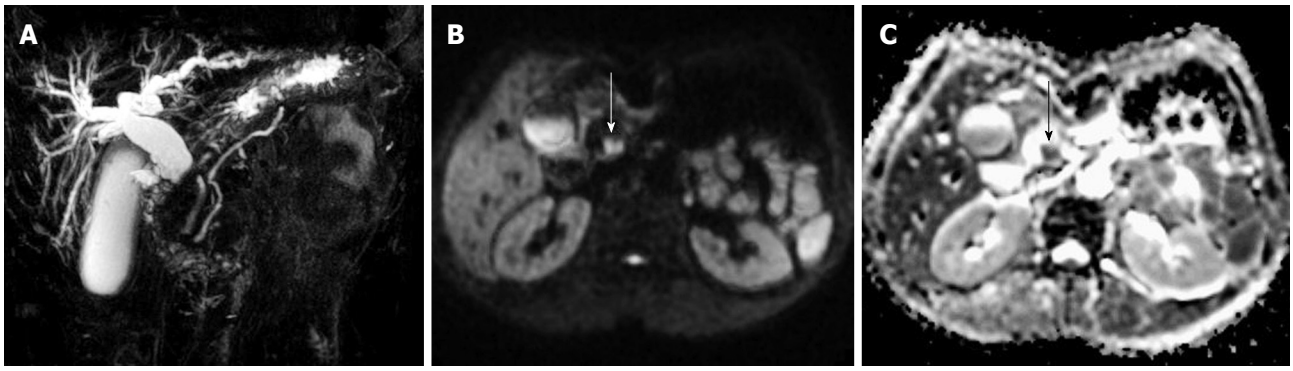

Figure 1 A 55-year-old man with extrahepatic cholangiocarcinoma. A: Magnetic resonance cholangiopancreatography (MRCP) demonstrates the dilatation of intrahepatic ductal and gallbladder with abrupt cutoff of the common bile duct; B: The diffusion-weighted image (b = 500 s/mm^2) shows the cholangiocarcinoma lesion to be a high-signal intensity lesion (arrow); C: Apparent diffusion coefficient (ADC) map (b = 500 s/mm^2) image shows a low-signal intensity area (arrow) and the ADC value of this cholangiocarcinoma was 1.30 × 10^{-3} mm^2/s.

Table 1 ADC value and SNR for EHCC with different b value

B value (s/mm^2)	ADC (× 10^{-3} mm^2/s)	SNR
100	2.73 ± 0.55	135.81 ± 33.27
300	1.84 ± 0.42	99.17 ± 22.63
500	1.31 ± 0.29	69.31 ± 16.36
800	1.22 ± 0.29	47.90 ± 12.35
1000	0.89 ± 0.23	29.48 ± 9.72
M	136.23	142.79
P	< 0.01	< 0.01

All *P* values are less than 0.05 for each two b values exhibited above. ADC: Apparent diffusion coefficient; SNR: Signal-noise ratio; EHCC: Extrahepatic cholangiocarcinoma.

multiple relevant samples, and the detection sensitivity of DWI and MRCP for EHCC was determined using the χ^2 test. A *P* value of < 0.05 was used as the statistical significance level of the observed differences.

RESULTS

Clinical and pathological results

All 56 cases collected in the study were confirmed by histopathological diagnosis. Of the 56 cases, 35 were EHCC (20 located near the hepatic hilum, 15 located at the far end of the extrahepatic bile duct), 16 were cholangitis, and 5 were bile duct stones.

EHCC presentation in DWI imaging and ADC map

Out of the 35 histopathologically-confirmed EHCC cases, 33 cases were detected by DWI. Compared with the surrounding tissues, these cases of EHCC exhibited differential levels of high signal intensity in the DWI image and exhibited low signal intensity in the ADC map (Figure 1). The average size of the EHCC was 28.5 mm (range, 5.0-67.2 mm). For EHCC, the mean ADC value (n = 33) was (1.31 ± 0.29) × 10^{-3} mm^2/s (95% CI, 1.20-1.47). For bile duct stones, the mean ADC value was (0.48 ± 0.22) × 10^{-3} mm^2/s (95% CI, 0.21-0.75). The two cases of EHCC undetected by DWI were due to the interference of artifacts around the bile duct and the small size of the malignant transformation of the bile duct adenoma (5 mm), respectively.

Table 2 Evaluation of diagnostic accuracy of DWI and MRCP for EHCC

	Confirmed pathological diagnosis		Total
	EHCC	Non-EHCC	
DWI			
EHCC	33	0	33
Non-EHCC	2	21	23
MRCP			
EHCC	26	6	32
Non-EHCC	9	15	24
Total	35	21	56

DWI: Diffusion-weighted imaging; MRCP: Magnetic resonance cholangio pancreatography.

ADC value and SNR for EHCC lesion under different b values

For the DWI sequence, the SNR gradually decreased in the lesion of EHCC as the b value was increased (Table 1). The difference was statistically significant (P < 0.01). With the increase in b value, the ADC value for the lesion gradually decreased (Table 1), and a *P* value of less than 0.05 was considered statistically significant.

Detection and definitive diagnostic accuracy of DWI and MRCP for EHCC

MRCP correctly diagnosed 4 out of 5 cases of bile duct stones, 11 out of 16 cases of cholangitis (5 cases were incorrectly diagnosed as EHCC), and 26 out of 35 cases of EHCC (2 cases were not detected and 7 were incorrectly diagnosed). The overall rate of correct diagnosis was 73.2% (41/56) (Table 2). DWI correctly diagnosed all 5 cases of calculi, all 16 cases of inflammation and 33 out of 35 cases of EHCC (1 case was not detected and 1 was incorrectly diagnosed). The overall rate of correct diagnosis was 96.4% (54/56) (Table 2). The detection sensitivity and specificity of MRCP was 74.3% and 71.4%, respectively.

For DWI, the sensitivity and specificity was 94.3% and 100%, respectively. The differences in detection sensitivity, specificity, and accuracy between the two examination methods for EHCC were of statistical significance ($P < 0.05$). The positive predictive value and negative predictive value of DWI (100%, 91.3%) were higher than those of MRCP (81.3%, 62.5%). The differences between the two sets of values were of statistical significance ($P < 0.05$).

DISCUSSION

DWI reflects the micro-movements of water molecules. It produces images by comparing the inter-tissue diffusion coefficient. It can be used to investigate tissue organization, structure and functional status of a biological object at the cellular and molecular level. Unlike conventional MRI examination, DWI enables imaging of the human body to be performed at a microscopic level, thereby allowing for accurate, rapid and reliable characterization of the spatial organization and composition of tissues, and the functional status of water exchange between tissue components affected by pathological or physiological status.

The extent of water molecule diffusion ability in a tissue is negatively correlated to that tissue's cell density and integrity of the cell membranes. The degree of water molecule movement was found to positively correlate with signal reductions in DWI. The movement of water molecules is more restricted as the cell membrane becomes more intact or if the cell density is higher (i.e. tumor tissue). Therefore, the observation of a relative reduction of signal in tissue imaged with DWI can assist in the detection and confirmation of tumor. The solid portion of the tumor has a higher cell density than normal tissues, and thus should continuously exhibit relatively higher signal intensity on DWI. However, given the fact that DWI is inherently T2-weighted, an extra-long T2 relaxation time can also show as continuously higher signal intensity in DWI, which may then be misdiagnosed as restricted diffusion. This is known as the T2 shine-through effect[7,8]. To avoid this effect, ADC values can be calculated using two images obtained under different b values. The ADC map can then be used to overcome the T2 shine-through effect by observing the relative reduction in signal in images obtained under different b values. Confirmation of tumor through differences in tissue water molecule diffusion is thus made possible. Higher cell densities restrict diffusion and exhibit low ADC values, otherwise, high ADC values are present. The b value is the sensitivity coefficient for diffusion; it can be used to determine the sensitivity for detection of diffusion of water molecules in the tissue under examination. The larger the b value used in DWI, the greater the dispersion of water molecules, and the more obvious the signal intensity reduction. This work focused on observing different ADC values for EHCC under different b values, followed by a comparison of the impact of b value on the ADC value measurement. The ADC value for EHCC was $(1.84 \pm 0.42) \times 10^{-3}$ mm^2/s under a b value of 300 smm^2; the ADC value was $(0.89 \pm 0.23) \times 10^{-3}$mm^2/s under a b value of 1000 s/mm^2. Using a smaller b value can produce a relatively higher ADC value, and with the increase in b value, the ADC value for lesions decreased. This correlation was of statistical significance. In biological tissues, the signal reduction in DWI is determined not only by the diffusion effect of water molecules, but also by reperfusion from the capillary microcirculation. In the work by Yamada *et al*[9], when a low b value was used, the derived ADC value mainly reflected the blood perfusion status of the tissue. Hence, large b values should be used to reflect the true diffusion ability of water molecules in the lesion. The larger the b value used, the better the image will reflect the true diffusion ability of water molecules in the lesion. In theory, when deriving the ADC value, a larger b value should cause the lesion to exhibit relatively higher signal intensity in DWI, producing greater lesion contrast in the ADC map, and increasing the accuracy of the derived ADC value. However, it is worth noting that increasing the b value will likely result in an increase in the diffusion gradient pulse time. The increased TE value can reduce the SNR of DWI and impact on the image quality. Our results show that the SNR of the lesion in DWI decreases following an increase in b value. Hence, the b value should be set between 500 and 800 s/mm^2 to ensure good image quality and an accurate ADC value, which is relatively close to the true diffusion value.

Previously, image quality for abdominal imaging has been relatively poor due to the impact of such factors as abdominal breathing, cardiac motion and chemical shifts. With the development of fast imaging techniques, i.e. single-shot echo planar imaging[10-14], the technology for performing abdominal DWI has improved rapidly[15,16]. DWI has been widely applied to the detection and confirmation of cancer in liver, pancreas, kidney, colon and prostate[17-21]. It has been reported that the characterization sensitivity of MRCP combined with an enhanced 3D MRI scan for EHCC is 87%, and the characterization specificity is 51%[22]. Our results demonstrate that DWI has a greater detection sensitivity and specificity than MRCP in terms of detection of EHCC. DWI reflects microstructural changes in tumor cells, where increases in the DWI signal are mainly caused by diffusion contrast, while T2W and T1W imaging cause signal increases by relaxation time differences. EHCC leads to increased cell density, diminished extracellular space, and restricted movement of water molecules due to an increase of macromolecular materials, all of which increase the EHCC signal. DWI possesses good background suppression effects; blood vessels, the bile duct and intra-abdominal fat all exhibit obvious low signal intensities. This increases tumor contrast to surrounding tissues, which improves lesion detection and facilitates observation of the size and scope of EHCC. DWI also provides a good image-based reference for clinical treatment. By contrast, MRCP only performs analysis over the site and shape of the bile duct stricture, and does not provide intuitive data by which to judge the characteristics and scope of the disease. When

the cholangiocarcinoma is of an infiltrating growth along the bile wall, MRCP shows the lesion as funnel-shaped or a gradually narrowing cone shape, which is difficult to differentiate from chronic inflammatory bile duct stricture. It is also hard to distinguish non-typical cholangiolithiasis and cholangiocarcinoma growing in the bile cavity, as both exhibit low signal intensity filling defects in the high signal intensity bile of MRCP. In theory, different compositions and spatial distributions of lesion tissue and cells lead to different diffusion coefficients, which can contribute to the identification of bile duct lesions. This work shows that with a b value of 500 s/mm^2, the ADC value for EHCC was (1.31 ± 0.29) × 10^{-3}mm^2/s, while the ADC value for cholangiolithiasis was (0.48 ± 0.22) × 10^{-3}mm^2/s. Hence, the measurement of lesion ADC values is useful for distinguishing non-typical cholangiolithiasis and cholangiocarcinoma growing in the bile cavity. However, due to the fact that the size of the ROI often exceeds the size of the thickened bile wall, it is difficult to derive an accurate ADC value for cholangitis. Distinguishing between cholangitis and EHCC only depends on changes in DWI signal but lacks quantitative indicators.

There remain challenges in the use of DWI for examination of the bile duct. The EPI-DWI technique currently in use employs small matrix scanning methods, which produce a low spatial resolution and SNR. The high frequency switching of the high-intensity gradient field can easily produce eddy currents and is highly sensitive to magnetic field inhomogeneities, which will result in unavoidable reverse geometry of space artifacts and partial image signal loss. These artifacts and signal loss become more obvious at air-tissue interfaces. As the b value increases, these artifacts worsen. In this study, one instance of undetected EHCC was attributed to the interference of artifacts around the common bile duct. In addition, the use of fat-suppression techniques to avoid artifacts due to chemical shifts can further degrade image quality. The other exception in the study was due to the partial malignant transformation of bile duct adenoma, where the lesion was too small for the DWI signal change to be obvious. Improvements to MR technology may further enhance image quality in DWI. Comparing with MRCP, DWI is a more intuitive approach to detect and confirm the diagnosis for EHCC. The cholangiocarcinoma DWI results are still preliminary and more research should be conducted to determine its value in clinical applications.

COMMENTS

Background

Extrahepatic cholangiocarcinomas account for 65% of primary neoplasms of the biliary tract. In recent years, improvement in imaging technology has significantly improved the detection rate and accuracy of definitive diagnosis for extrahepatic cholangiocarcinoma (EHCC). Diffusion-weighted imaging (DWI) has recently emerged as a method for detecting cancers in the abdomen. This study aimed to evaluate the usefulness of DWI in the differential diagnosis of extrahepatic cholangiocarcinomas.

Innovations and breakthroughs

DWI has been increasingly used in a wide range of clinical applications. Although the application of DWI in the diagnosis of various conditions has attracted increased attention and has become the focus of many researches, literature on clinical application of DWI for the diagnosis of EHCC has not been observed.

Applications

The authors suggest that all patients presenting with an EHCC or who are suspected to have EHCC, should have an abdominal DWI scan as a regular diagnostic test. DWI can also provide additional clinically important information in EHCC patients when added to routine bile duct MR imaging protocols.

Terminology

DWI is used to observe the random motion of water molecules in the body. The degree of restriction to water diffusion in biologic tissue is inversely correlated to the tissue cellularity and integrity of cell membranes. The motion of water molecules is more restricted in tissues with a high cellular density, associated with numerous intact cell membranes (e.g. tumor tissues). The degree of water motion has been found to be proportional to the degree of signal attenuation in DWI. Visual assessment of the relative tissue signal attenuation in DWI has been applied to tumor detection and tumor characterization. By observing the relative attenuation of signal intensity on images obtained at different b values, tissue characterization based on differences in water diffusion becomes possible. More cellular solid tumor areas will continue to show relatively high signal intensity in DWI. Therefore, the observation of the relative reduction of tissue signal in DWI can help with the detection and confirmation of tumors.

Peer review

The authors compare DWI with magnetic resonance cholangiopancreatography in 56 patients with suspected extrahepatic cholangiocarcinoma. Basically this is an extremely interesting study for assessing the new DWI in more tumor entities. It is nicely performed and provides a histopathological correlation.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3202

World J Gastroenterol 2010 July 7; 16(25): 3202-3205
ISSN 1007-9327 (print)

BRIEF ARTICLE

Perfusion CT findings in liver of patients with tumor during chemotherapy

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Received: April 4, 2010 Revised: April 26, 2010
Accepted: May 3, 2010
Published online: July 7, 2010

Abstract

AIM: To investigate the microcirculation changes in liver of patients with tumor during chemotherapy by perfusion computed tomography (CT).

METHODS: Sixty patients with tumor and 20 controls were enrolled in this study. Perfusion CT parameters of patients and controls were compared, including hepatic perfusion index (HPI), mean transit time (MTT), and permeability-surface area product (PS). Correlation between perfusion CT parameters, treatment cycle and alanine aminotransferase (ALT) level was studied.

RESULTS: No difference was found in HPI (25.68% ± 7.38% *vs* 26.82% ± 5.13%), MTT (19.67 ± 5.68 s *vs* 21.70 ± 5.43 s) and PS (17.00 ± 4.56 mL/100 mL per min *vs* 19.92 ± 6.35 mL/100 mL per min) between patients and controls. The HPI and MTT were significantly higher in patients undergoing 2 cycles of chemotherapy than in controls and those undergoing 1 cycle of chemotherapy (29.76% ± 5.87% *vs* 25.68% ± 7.38% and 25.35% ± 4.05%, and 25.61 ± 5.01 s *vs* 19.67 ± 5.68 s and 19.74 ± 4.54 s, respectively, $P < 0.05$). The HPI was higher in patients with hepatic steatosis than in controls and those without hepatic steatosis (30.85% ± 6.17% *vs* 25.68% ± 7.38% and 25.70% ± 4.24%, $P < 0.05$). Treatment cycle was well correlated with HPI and MTT ($r = 0.40$, $r = 0.50$, $P < 0.01$). ALT level was not correlated with perfusion CT parameters.

CONCLUSION: HPI and MTT are significantly increased in patients with tumor during chemotherapy and well correlated with treatment cycle. Chemotherapy affects hepatic microcirculation in patients with tumor. Changes in hepatic microcirculation can be quantitatively assessed by perfusion CT.

Key words: Liver; Microcirculation; Chemotherapy; Tomography, X-ray computed; Perfusion imaging

Peer reviewer: Paul E Sijens, PhD, Associate Professor, Radiology, UMCG, Hanzeplein 1, 9713GZ Groningen, The Netherlands

Zhang Q, Yuan ZG, Wang DQ, Yan ZH, Tang J, Liu ZQ. Perfusion CT findings in liver of patients with tumor during chemotherapy. *World J Gastroenterol* 2010; 16(25): 3202-3205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3202.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3202

INTRODUCTION

Liver damage secondary to chemotherapy in patients with

tumor is common, and hepatic toxicity differs from mild inflammatory changes of nonspecific hepatitis to fibrosis and marked cirrhosis[1,2]. Although early liver damage causes no symptoms and is reversible in most patients, it occasionally progresses to more severe liver impairment, which may be irreversible. It is, therefore, necessary to demonstrate the presence and severity of drug-related parenchymal changes with effective imaging.

Early liver damage cannot be demonstrated by traditional computed tomography (CT). Perfusion CT is a noninvasive method showing hemodynamic changes in living tissue and has been used in evaluation of liver diseases. However, microcirculation changes in liver on perfusion CT image during chemotherapy have not been described. In this study, we investigated the hemodynamic changes in liver during chemotherapy, and estimated the correlation between the perfusion CT parameters and alanine aminotransferase (ALT) level.

MATERIALS AND METHODS

Patients and study design

Eighty consecutive individuals including 60 patients with tumor and 20 controls were enrolled in this study. Although history, physical examination, laboratory test, and Doppler sonography of liver showed that the 20 controls including 8 women at a mean age of 47.3 ± 8.6 years (range 35-64 years) had no evidence of liver disease, they underwent abdominal perfusion CT for unrelated causes. Inclusion criteria included patients with tumor confirmed by appropriate clinical and laboratory examinations, those still on chemotherapy when perfusion CT was performed, those with hepatic carcinoma or metastasis who had an adequate space in liver parenchyma for later calculation, those who had no history of alcohol abuse, viral hepatitis, liver cirrhosis or other hepatic/biliary diseases, and those who had adequate heart, renal and liver function for perfusion CT. The 60 patients including 24 women at a mean age of 53.2 ± 7.1 years (range 25-71 years) met the criteria. All patients received 1-2 cycles of chemotherapy (6-8 courses in one treatment cycle). Perfusion CT parameters were compared between controls and patients. The diagnostic criteria for hepatic steatosis were the density of liver parenchyma lower than that of spleen or the CT value of liver parenchyma less than 40 Hu on CT image without contrast agent.

The study was approved by the institutional ethics committee. Written informed consent was obtained from each patient or his or her family members after the nature of procedures was fully explained.

Perfusion CT imaging and data processing

Perfusion CT was performed for patients at the end of each treatment cycle. After an overnight fast, patients and controls lying supine on a table underwent perfusion CT with a 64-row multi-detector CT scanner (Light Speed VCT, GE Healthcare, Chalfont St. Giles, UK). Before perfusion CT, patients underwent an abdominal scan without contrast medium during a breath hold at the end of expiration. We selected a slice with the right hepatic lobe, spleen and portal trunk clearly visualized. Then, multiple-slice dynamic sequences lasting 80 s were scanned 8 s after injection of a contrast material (iohexol, 350 mg I/mL, Changfujiejing Pharmaceutical Co. Ltd., Shandong, China). The CT perfusion protocol comprised 80 scans, for 320 images. Because blood vessels of patients with tumor during chemotherapy became too fragile to endure a high injection pressure, a bolus infusion of 40 mL contrast material was given at a speed of 2.5-3.0 mL/s *via* a 20-gauge intravenous catheter in the antecubital vein with a power injector (SCT-210, Medrad Inc., Indianola, PA, USA). The patients kept normal respiration during the perfusion study. The CT parameters used in this study were 120 kV, 250 mA, 5-mm slice thickness, 1-s cycle time and standard reconstruction algorithm.

After image acquisition, the data were transferred to an image processing workstation (AW4.1, GE Healthcare, Chalfont St. Giles, UK) and analyzed with the integrated software of CT Perfusion 3. Four regions of interest (ROI) were set on the abdominal aorta, portal vein trunk, spleen and right liver lobe. The ROI of right liver lobe was drawn on the whole visible right lobe carefully, avoiding blood vessels, margin of liver parenchyma and possible lesions. Hepatic perfusion index (HPI), mean transit time (MTT), and permeability-surface area product (PS) were calculated.

Statistical analysis

All data were expressed as mean ± SD. Independent-sample *t* test was used to determine differences between patients and controls. Data about controls and patients were compared by one-way ANOVA. Spearman correlation coefficient was used to assess the correlation between perfusion parameters, treatment cycle and ALT level. All tests were two-tailed. $P < 0.05$ was considered statistically significant. Data processing and analysis involved use of SPSS v11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Differences in perfusion parameters

No significant difference was found in HPI (25.68% ± 7.38% *vs* 26.82% ± 5.13%, $t = 0.77$, $P = 0.44$), MTT (19.67 ± 5.68 s *vs* 21.70 ± 5.43 s, $t = 1.43$, $P = 0.16$) and PS (17.00 ± 4.56 mL/100 mL per min *vs* 19.92 ± 6.35 mL/100 mL per min, $t = 1.90$, $P = 0.06$) between patients and controls.

Patients were divided into two subgroups: one receiving 1 cycle of chemotherapy ($n = 40$) and the other receiving 2 cycles of chemotherapy ($n = 20$). The HPI and MTT were higher in patients receiving 2 cycles of chemotherapy than in those receiving 1 cycle of chemotherapy and controls (29.76% ± 5.87% *vs* 25.35% ± 4.05% and 25.68% ± 7.38%, and 25.61 ± 5.01 s *vs* 19.74 ± 4.54 s and 19.67 ± 5.68 s, $P < 0.05$). No significant difference was observed in HPI and MTT between patients receiving 1 cycle of chemotherapy and controls, and in PS between patients and controls ($F = 1.78$, $P = 0.18$) (Table 1).

Table 1 Comparison of perfusion parameters between controls and patients by treatment cycle (mean ± SD)

Parameters	Controls (n = 20)	1 cycle (n = 20)	2 cycles (n = 40)	F value	P value
HPI (%)	25.68 ± 7.38	25.35 ± 4.05	29.76 ± 5.87	4.62	0.01
MTT (s)	19.67 ± 5.68	19.74 ± 4.54	25.61 ± 5.01	10.59	< 0.01
PS (mL/100 mL per min)	17.00 ± 4.56	19.95 ± 6.76	19.86 ± 5.60	1.78	0.18

Of the 60 patients, 13 (21.7%) showed hepatic steatosis. The incidence of hepatic steatosis was higher in patients receiving 2 cycles of chemotherapy than in those receiving 1 cycle of chemotherapy (P = 0.01). The HPI was higher in patients with hepatic steatosis than in those without hepatic steatosis and controls (30.85% ± 6.17% *vs* 25.70% ± 4.24% and 25.68% ± 7.38%, P < 0.05). No significant difference was found in HPI between patients without hepatic steatosis and controls, and in MTT or in PS between patients and controls (Table 2).

Correlation between CT perfusion parameters, treatment cycle and ALT level

Treatment cycle was well correlated with HPI (r = 0.40, P < 0.01) and MTT (r = 0.50, P < 0.01) but not with PS (r = 0.02, P = 0.89). ALT level was not correlated with treatment cycle (r = -0.05), HPI (r = -0.06), MTT (r = 0.19), and PS (r = 0.18).

DISCUSSION

Hepatic toxicity is often encountered in patients with tumor following chemotherapy. Chemotherapy agents can impair many vital functions of liver cells and cause their death[3]. Severe cell death is followed by nodular regeneration and obstruction of sinusoids, including transformation of fenestrated sinusoids into continuous capillaries and deposition of collagen in extravascular tissue spaces located between sinusoidal endothelium and hepatocytes[4,5]. These morphologic alterations modify blood transit time and distribution volume of small and large molecules[6,7], increase vascular resistance and reduce portal perfusion[8,9]. The reduction in portal perfusion is then buffered by liver arterialization, thereby increasing the arterial fraction of liver perfusion[8,10,11]. These alterations following chemotherapy are non-specific and can mimic any form of acute or chronic liver disease.

Perfusion CT can be used to evaluate the hemodynamic changes in liver disease. However, since the results of previous studies are varied[12-14], no established conclusion is available on the change in liver perfusion, especially in early chronic liver disease. Some studies showed that HPI and MTT values are significantly increased in patients or rats with chronic liver disease[12,13]. However, another study showed that the MTT is lower while the PS is higher in patients than in controls[14]. In the present study, the hepatic HPI and MTT values were higher in patients during chemotherapy than in controls, and well correlated with treatment cycle. No significant difference was observed in PS between patients and controls.

Table 2 Comparison of perfusion parameters between controls and patients with or without hepatic steatosis (mean ± SD)

Parameters	Controls (n = 20)	Patients without steatosis (n = 47)	Patients with steatosis (n = 13)	F value	P value
HPI (%)	25.68 ± 7.38	25.70 ± 4.24	30.85 ± 6.17	4.79	0.01
MTT (s)	19.67 ± 5.68	21.33 ± 5.31	23.03 ± 5.83	1.52	0.23
PS (mL/100 mL per min)	17.00 ± 4.56	20.23 ± 6.08	18.79 ± 7.43	2.09	0.13

Steatosis is a common manifestation of drug hepatotoxicity and a form of liver damage most readily recognized on CT image. Several studies have shown that the incidence of hepatic steatosis is increased in patients undergoing chemotherapy[15-18]. In this study, hepatic steatosis was observed in 13 (21.7%) of the 60 patients, which was higher in patients receiving 2 cycles of chemotherapy than in those receiving 1 cycle of chemotherapy. The HPI was higher in patients with hepatic steatosis than in those without hepatic steatosis and controls. No difference was found in MTT or in PS between patients and controls.

At present, ALT level is the main index of drug-induced hepatic damage[19]. However, the ALT level was found to be a less sensitive index of hepatic damage and could not thoroughly reflect hepatic toxicity in this study. In clinical practice, many liver function tests remain normal despite obvious liver changes seen on CT images. In the present study, only 4 of 13 patients with hepatic steatosis had abnormal ALT, which was not correlated with HPI, MTT or PS. However, it has been shown that perfusion CT parameters are correlated with the severity of hepatic disease[13], indicating that further study is needed to classify the severity of liver damage with perfusion CT parameters.

Our study has some limitations. First, the injection rate of contrast agent was low. Because the detection of perfusion parameters is affected by many factors such as the type and injection rate of contrast agent, drawing of ROI and calculation method[20,21], the low injection rate may be a major obstacle to comparison with other results. Second, our sample size was small. Third, we only investigated the changes in perfusion CT in liver of patients during chemotherapy, and did not observe the entire changing characteristics of hepatic microcirculation during and after chemotherapy.

In conclusion, hepatic microcirculation is changed in patients with tumor during chemotherapy and can be quantitatively assessed by perfusion CT. Perfusion CT may be used as a noninvasive tool in detection of hepatic toxicity.

ACKNOWLEDGMENTS

The authors thank Ji-Quan Du, Hospital of Shandong Armed Police Forces for his efforts in the research, and

Gui-Lin Liu, Affiliated Hospital of Shandong Academy of Medical Science for her invaluable help in data processing and manuscript preparation.

COMMENTS

Background

Liver damage is common in patients with tumor following chemotherapy. Although early liver damage causes no symptoms and is reversible in most patients, it occasionally progresses to more severe liver impairment, which may be irreversible, it is thus necessary to demonstrate the presence and severity of drug-related parenchymal changes.

Research frontiers

Hepatic microcirculation changes in patients with tumor during chemotherapy were evaluated by perfusion computed tomography (CT). The hotspot of this research is whether hepatic microvascular changes can be quantified with perfusion CT and what kind of modifications can be detected.

Innovations and breakthroughs

At present, alanine aminotransferase (ALT) level is the main index in diagnosis of drug-induced hepatic damage. In this study, however, ALT level was found to be a less sensitive index and could not thoroughly reflect hepatic toxicity. The aim of this study was to investigate the microcirculation changes in liver of patients with tumor during chemotherapy, showing that hepatic perfusion index and mean transit time are significantly increased in patients undergoing 2 cycles chemotherapy.

Applications

The findings of this study may underscore the possibility of using perfusion CT parameters as indicators of hepatic microcirculation alteration in drug-induced liver damage. Perfusion CT may be used as a noninvasive tool in detection of hepatic toxicity.

Peer review

The paper is a concise documentation of a good study.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3206

World J Gastroenterol 2010 July 7; 16(25): 3206-3210
ISSN 1007-9327 (print)

CASE REPORT

Annular pancreas associated with duodenal carcinoma

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Received: January 25, 2010 Revised: March 19, 2010
Accepted: March 26, 2010
Published online: July 7, 2010

Abstract

Annular pancreas (AP) is a rare congenital anomaly. Coexisting malignancy has been reported only in a few cases. We report what is, to the best of our knowledge, the first case in the English literature of duodenal adenocarcinoma in a patient with AP. In a 55-year old woman with duodenal outlet stenosis magnetic resonance cholangiopancreatography showed an aberrant pancreatic duct encircling the duodenum. Duodenojejunostomy was performed. Eight weeks later she presented with painless jaundice. Duodenopancreatectomy revealed a duodenal adenocarcinoma, surrounded by an incomplete AP. Thus, co-existent malignancy with AP can be present without obstructive jaundice and without being visible through preoperative diagnostics.

Key words: Duodenal carcinoma; Annular pancreas; Duodenopancreatectomy; Whipples operation

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Brönnimann E, Potthast S, Vlajnic T, Oertli D, Heizmann O. Annular pancreas associated with duodenal carcinoma. *World J Gastroenterol* 2010; 16(25): 3206-3210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i25/3206.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i25.3206

INTRODUCTION

Annular pancreas is a rare congenital anomaly, which consists of a ring of pancreatic tissue, confluent with the head of the pancreas, and partially or completely encircling the second segment of the duodenum. It was first described by Tiedemann in 1818[1] and first named "pancreas anulare " by Ecker in 1862[2].

Studies based on cases where the diagnosis was made on autopsy or during surgery estimate the incidence of annular pancreas of 3 in 20 000[3,4]. When the diagnosis is based on modern imaging methods, such as multislice computed tomography (MSCT), magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP), the incidence seems to be higher; up to 1 in 250[5,6]. About 700 cases of annular pancreas have been reported in the literature[7]. During normal organogenesis, the pancreas is formed by merging the ventral with the dorsal pancreatic bud during the first four to nine weeks of gestation. Two main theories, sustained by Lecco[8] and Badwin[9] respectively, describe the pathogenesis of annular pancreas. Lecco[8] explained the development of the anomaly as an adherence of the ventral pancreatic bud to the duodenum, which leads to an improper rotation of the ventral bud, resulting in the encirclement of the duodenum. Badwin[9] contends that the persistence and enlargement of the left ventral bud causes the pancreatic obstructing ring. Although the pancreatic band usually adheres to the muscularis of the du-

odenum, it may also be loosely applied to the serosa of the duodenum[10]. The annular pancreas has been associated with other congenital anomalies, such as trisomy 21, tracheoesophageal fistula, cardiac anomalies, and intestinal malrotation[7]. Association of annular pancreas with malignancy is rare. It has been described only 14 times in the English literature (five pancreatic carcinomas, six ampullary carcinomas, two cholangiocarcinomas, and one Insulinoma)[11-22]. A review of 151 cases of annular pancreas in Japan by Ogawa *et al*[11] revealed an association with malignant tumors of the duodenopancreatobiliary system in 15 patients (five cholangiocarcinomas, four gallbladder carcinomas, four duodenal, and two pancreatic carcinomas). Primary carcinoma of the duodenum is rare, representing about 0.3% of all malignant neoplasms of the gastrointestinal tract and 25%-45% of malignant neoplasms of the small intestine[23].

We report what is, to the best of our knowledge, the first case in the English literature of duodenal adenocarcinoma in a patient with annular pancreas.

CASE REPORT

A 55-year-old Caucasian woman was admitted to our hospital with a six-week history of persistent postprandial nausea and vomiting, associated with a weight loss of 7 kg compared to a total weight of 70 kg. There was no associated pain, hematemesis, fever, diarrhoea, or jaundice. Domperidone therapy was ineffectual. Four years prior to the diagnosis, the patient had intermittent occasional mild epigastric pain. She also reported occasional vomiting during the previous 15 years, which was not medically investigated.

On clinical examination, the patient appeared weak and dehydrated. Her abdomen was soft, without tenderness or palpable masses. Laboratory results revealed a hypokalemia of 1.9 mmol/L, which was substituted, and a metabolic alkalosis with an initial pH of 7.65. Serum amylase, bilirubin, and C-reactive protein (CRP) levels were not elevated.

Gastroscopy showed a reflux esophagitis, a massive distension of the stomach and the first part of the duodenum, respectively, and a stenosis of the second part of the duodenum, which could not be passed with the thin gastroscope.

Abdominal MSCT depicted a fluid filled stomach and second segment of the duodenum (Figure 1). The pancreatic head was enlarged, surrounding the second segment of the duodenum; no dilation of the main pancreatic duct and common bile duct was noticed. For further evaluation, an MRCP was done. This showed an aberrant pancreatic duct encircling the duodenum, linked to the main pancreatic duct (Figure 2). No dilation of the common hepatic bile duct or the main pancreatic duct was evident. Due to the annular pancreas, there was an obstruction at the level of the second segment of the duodenum. During surgery, we found a massively distended and elongated first segment with a conic ste-

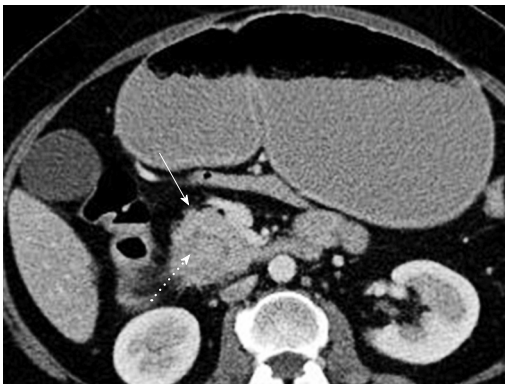

Figure 1 Multislice computed tomography. A fluid filled stomach and enlargement of the pancreatic head (arrow) are detected, which encircles the second segment of the duodenum (dotted arrow).

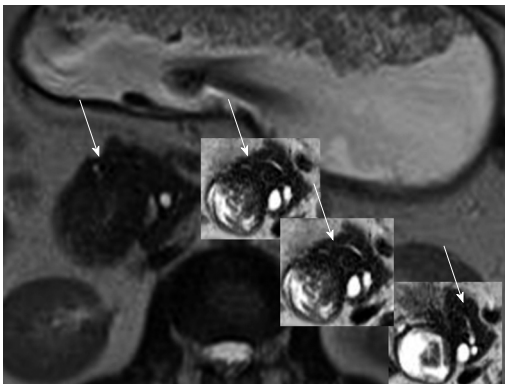

Figure 2 T2 weighted magnetic resonance cholangiopancreatography images depict the aberrant pancreatic duct, which encircles the duodenum and connects with the main pancreatic duct (arrows).

nosis of the second segment of the duodenum due to the annular pancreas (Figure 3). A duodenojejunostomy was performed. There was no visible prestenotic tumor in the duodenum and the duodenal mucosa appeared to be normal. No tumor mass was palpated. The patient had an uneventful recovery and was discharged home with no complaints nine days later. After eight weeks, the patient was readmitted presenting with painless jaundice. She had no other complaints. No further weight loss was reported. Laboratory investigations showed elevated total bilirubin (112 μmol/L; reference range: 5-18) and alkaline phosphatases (178 U/L; reference range: < 104). An MRCP demonstrated dilatation of the common bile duct (Figure 4). On explorative laparotomy, a hard mass was palpated in the pancreatic head region. The frozen section of one of several suspicious superior pancreatic lymph nodes revealed adenocarcinoma cells. A duodenopancreatectomy was performed. The tumor was clearly located in the duodenum, with infiltration of the surrounding structures (Figure 5). No tumor was observed in the head of the pancreas. Pathological examination showed a poorly differentiated, infiltrating adenocarcinoma of the duodenum, surrounded by the incomplete annular pancreas (Figure 5). The tumor stage was pT4,

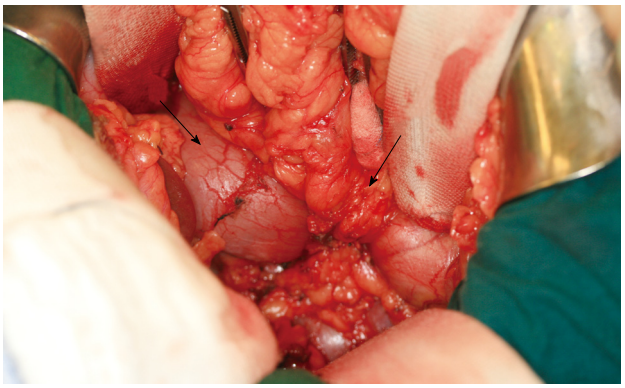

Figure 3 During surgery, a massively distended and elongated first segment (left arrow) and a conic stenosis of the second segment of the duodenum were observed due to the annular pancreas (right arrow).

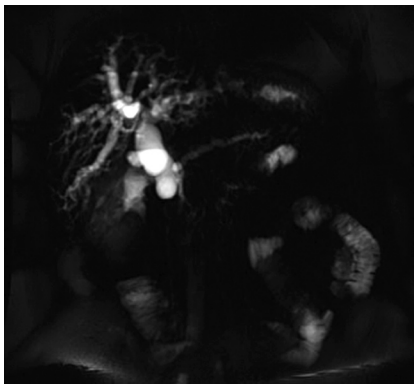

Figure 4 Eight weeks later, T1 w post contrast axial MR shows dilatation of the common bile duct.

pN1 (8/12), G3, L0, V0, R0. The tumor seemed to have arisen from the duodenal epithelium.

DISCUSSION

Symptoms

Clinical manifestation of annular pancreas can occur at any age, from neonatal period to adulthood. When the duodenal constriction is minimal or absent, annular pancreas can remain asymptomatic lifelong[24]. In infants, the common clinical symptoms are vomiting and feeding intolerance[7]. Clinical manifestation in adults can present with cramping epigastric pain, postprandial fullness, vomiting, and weight loss[10,25,26]. Association with pancreatitis, or a gastric/duodenal ulcer, can also occur[7,25]. The most frequent symptom in adult annular pancreas seems to be abdominal pain[7,10]. Most adults become symptomatic between the ages of 20 and 50[25]. The differential diagnosis includes other upper gastrointestinal pathologies, such as peptic ulcer disease, duodenal web, pancreatitis, or pancreatic carcinoma[19,27]. Obstructive jaundice is described as a rare direct result of annular pancreas, but appears in coexisting periampullary malignancies[19]. Of the above-mentioned 14 cases of carcinoma associated with annular pancreas, 10 presented obstructive jaundice. The most

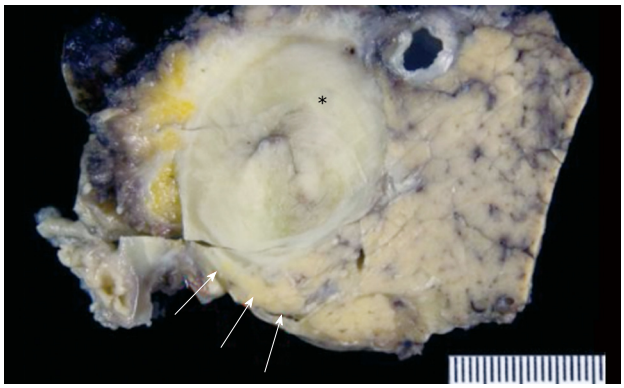

Figure 5 Cross sectional view showing the tumor within the duodenum (asterisk). The duodenum is surrounded by the incomplete annular pancreas (arrows).

common symptoms of primary carcinoma of the duodenum are abdominal pain and weight loss[28]. Upon her first hospitalisation, our patient's main symptoms were postprandial nausea and vomiting, associated with a significant weight loss. No abdominal pain was reported. Neither pancreatitis nor obstructive jaundice was apparent. Four years prior to diagnosis, the patient had been complaining intermittent occasional mild epigastric pain. In addition, over the previous 15 years, she had reported occasional vomiting. Fifteen years had passed from first symptoms until diagnosis of annular pancreas. Upon her second hospitalisation, her only symptom was jaundice.

Diagnosis/radiology

Annular pancreas is diagnosed by ERCP or by CT[29]. Since MRCP has become widely used, annular pancreas can be diagnosed non-invasively[30,31]. A recent study reviewed 55 cases with annular pancreas in adults[7]. Diagnosis was made with ERCP in 47%, MSCT in 18%, with MRCP in 16%, and in 13% of the patients, diagnosis was made at the time of surgery. We were able to make the diagnosis of annular pancreas non-invasively by abdominal MSCT (Sensation 16, Siemens Medical Solutions, Erlangen, Germany) and MRCP using a 1.5T scanner (1.5T Symphony, Siemens Medical Solutions, Erlangen, Germany). Due to the duodenal obstruction by the pancreatic ring, ERCP was not a feasible diagnostic alternative. Upon readmission, dilatation of the common bile duct was demonstrated by MRCP. Malignancy was found during explorative laparotomy. The final diagnosis was made by the pathologist. During her first hospitalisation, malignancy was not suspected either by preoperative diagnostics nor laparotomy. Upper gastrointestinal tract endoscopy is the most frequently performed test to diagnose duodenal adenocarcinoma[28]. In the present case, the tumor was hidden behind the annular pancreas and not within reach of the endoscope.

Therapy and prognosis

The treatment of annular pancreas is always surgical. The goal is to relieve the duodenal or gastric outlet obstruc-

tion. Dissection of the pancreatic ring should be avoided due to a high incidence of complications, including duodenal leak, pancreatic fistula, and postoperative pancreatitis[10]. Different surgical approaches have been described. Bypass surgery, such as duodenojejunostomy in pediatric surgery, and duodenoduodenostomy or gastrojejunostomy in adults is preferred. The safest and most successful way of bypassing the annular constriction seems to be duodenoduodenostomy or duodenojejunostomy[10]. The presence of a periampullary malignancy must be considered in adult patients with annular pancreas presenting with obstructive jaundice[19]. In cases of annular pancreas associated with proven or suspected periampullary malignancy, duodenopancreatectomy might be the treatment of choice[19]. This should also be performed when annular pancreas is associated with pancreatolithiasis and localized chronic pancreatitis[32]. Long-term survival of patients with duodenal adenocarcinoma can be achieved with a surgical procedure that produces negative resection margins, such as pancreaticoduodenectomy[28]. Adjuvant therapy seems not to improve the survival rate[33]. Initially, our patient did not show jaundice and imaging did not present a tumoral mass.

We decided to perform a duodenojejunostomy to bypass the stenosis. The postoperative course was uneventful. Five days after intervention, the patient was able to eat solid food.

After eight weeks, painless jaundice appeared and MR imaging revealed dilatation of the common bile duct but no tumoral mass. Intraoperatively, a hard mass was palpated in the pancreatic head region and adenocarcinoma cells were found in a pancreatic lymph node at frozen section. We performed a duodenopancreatectomy and the patient received no adjuvant therapy.

In conclusion, annular pancreas should be kept in mind when symptoms of upper gastrointestinal obstruction occur, but the presence of annular pancreas should not distract from a possible coexisting malignancy. As in the present case, such a malignancy can also be present without obstructive jaundice and without being visible through preoperative diagnostics. In such a case, pancreaticodudoenectomy might be the only way to exclude the coexistence of duodenal carcinoma with annular pancreas.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3211

World J Gastroenterol 2010 July 7; 16(25): 3211-3214
ISSN 1007-9327 (print)

CASE REPORT

Surgical treatment of hepatocellular carcinoma with severe intratumoral arterioportal shunt

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Received: March 10, 2010 Revised: April 14, 2010
Accepted: April 21, 2010
Published online: July 7, 2010

Key words: Arterioportal shunt; Hepatocellular carcinoma; Esophagogastric varices

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Abstract

We report a case of hepatocellular carcinoma (HCC) that caused a severe arterioportal shunt (APS). A 49-year-old man was admitted to hospital due to esophagogastric variceal hemorrhage and HCC, and underwent endoscopic variceal ligation (EVL) and endoscopic injection sclerotherapy (EIS). He was then referred to our hospital. Abdominal computed tomography revealed a low-density lesion in the posterior segment of the liver and an intratumoral APS, which caused portal hypertension. Although the patient underwent EVL, EIS, Hassab's operation, and transcatheter arterial embolization for APS, he vomited blood due to rupture of esophagogastric varices. Right hepatectomy was performed for the treatment of HCC and APS, although the indocyanine green retention value at 15 min after intravenous injection was poor (30%). The patient's postoperative course was uneventful. Eventually, APS disappeared and the esophagogastric varices improved.

INTRODUCTION

Hepatocellular carcinoma (HCC) can easily invade the portal vein and form a direct communication between the hepatic artery and portal vein, which results in the formation of an arterioportal shunt (APS). Severe APS leads to life-threatening conditions, such as esophagogastric varices, refractory ascites, and hepatic encephalopathy, as a result of portal hypertension[1-3]. Furthermore, indocyanine green retention at 15 min after intravenous injection (ICG-R15) for APS is worse than the true value[4]. We report a patient who underwent successful hepatectomy for the treatment of HCC and an intratumoral APS that was not controlled with various treatments.

CASE REPORT

A 49-year-old man was admitted to hospital because of vomiting blood, which was diagnosed as esophagogastric

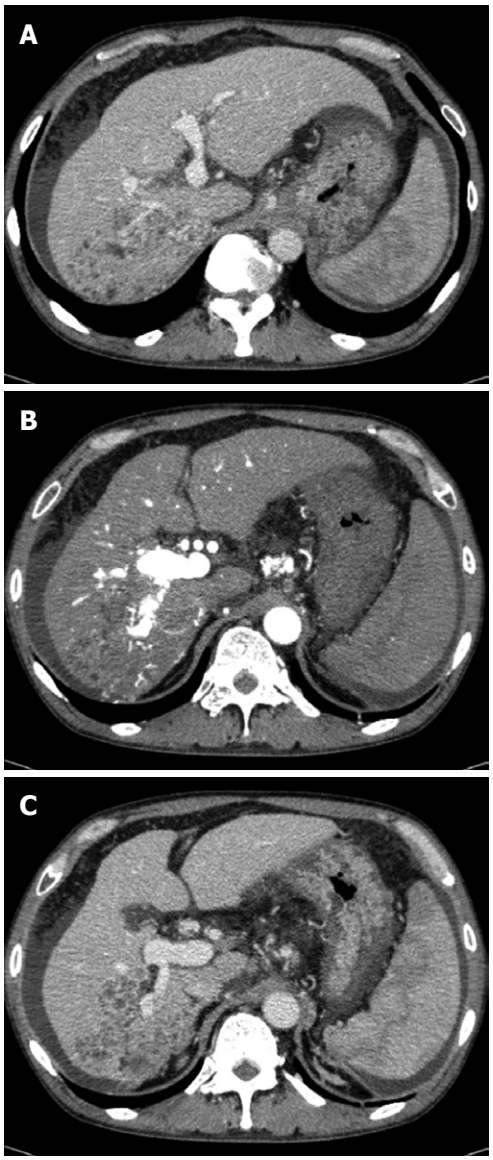

Figure 1 Computed tomography examination. A: Low-density lesion in the posterior segment of the liver, which pressed the vena cava and right hepatic vein and opposed the right anterior superior portal vein; B: Hyper-enhanced portal vein during the arterial phase; C: Tumor thrombus in the posterior branch of the portal vein. A tumor opposed the right anterior inferior portal vein.

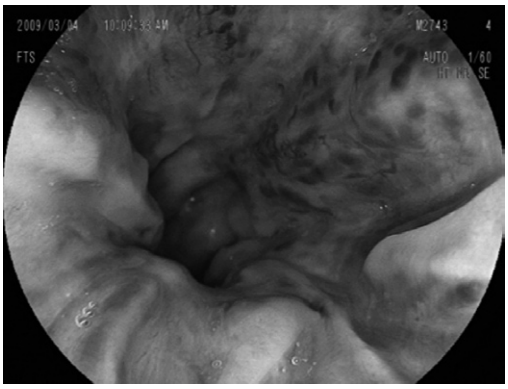

Figure 2 Upper gastrointestinal endoscopy revealing severe esophageal varices.

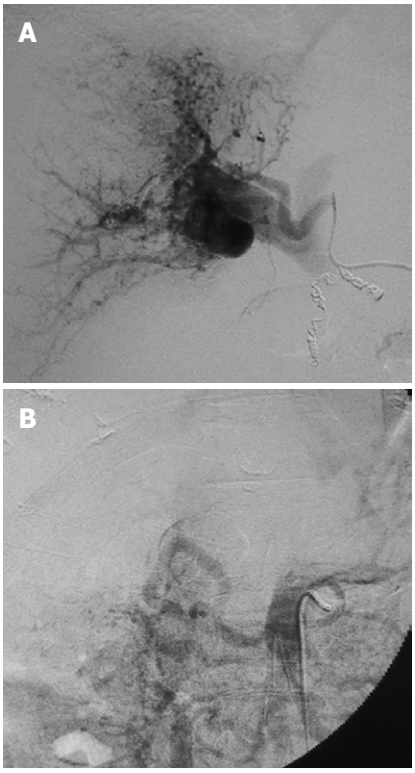

Figure 3 Arteriography. A: Right hepatic arteriography exhibiting the right portal vein, caused by severe intratumoral arterioportal shunt; B: Transarterial portography revealing non-enhancement of the portal vein because of portal hypertension.

varices and HCC. He underwent emergency endoscopic variceal ligation (EVL), followed by endoscopic injection sclerotherapy (EIS) three times at the hospital. The patient was then referred to our hospital for treatment of HCC.

The laboratory data obtained at the time of hospitalization in our hospital are shown in the Table 1. The Child-Pugh classification status was class B (8 points) and the degree of liver damage on the scoring system designed by the Liver Cancer Study Group of Japan[5] was class B. Abdominal dynamic computed tomography (CT) revealed a low-density lesion at any phases that existed in the posterior segment of the liver. The lesion was 11 cm in diameter and apposed the vena cava, the right hepatic vein, and the right anterior superior and inferior portal vein (Figure 1A and C). CT also demonstrated a hyper-enhanced portal vein during the arterial phase (CT attenuation of the portal vein was 284 HU and that of the proper hepatic artery was 294 HU) (Figure 1B); a filling defect caused by tumor thrombus in the posterior branch of the portal vein (Figure 1C); esophagogastric varices despite previous EIS; ascites and splenomegaly. Upper gastrointestinal endoscopy revealed severe esophagogastric varices (Figure 2). Right hepatic arteriography revealed severe intratumoral APS accompanied by reflux into the main portal vein (Figure 3A). Transarterial portography revealed non-enhancement of the portal vein due to portal hypertension (Figure 3B). The patient was diagnosed with HCC with severe intratumoral APS, which caused portal hypertension that lead to esophagogastric varices and hypersplenism.

Table 1 Laboratory data obtained at time of hospitalization and after TAE

	At time of hospitalization	After TAE
AST level (IU/L)	86	70
ALT level (IU/L)	66	20
Total bilirubin level (mg/dL)	0.98	0.71
Albumin level (g/dL)	3.4	3.8
White blood cell count (/μL)	1800	3900
Hemoglobin level (g/dL)	9.9	9.2
Hematocrit (%)	30.7	29.8
Platelet count (/μL)	7.5×10^4	18.2×10^4
Prothrombin activity (%)	71	80
HBsAg	Negative	-
HCV Ab	Positive	-
AFP level (ng/mL)	5570	-
PIVKA-Ⅱ level (mAU/mL)	5360	-
ICG-R15 (%)	36	30

TAE: Transcatheter arterial embolization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen; HCV Ab: Hepatitis C virus antibody; AFP: α-fetoprotein; PIVKA-Ⅱ: Protein induced by vitamin K absence or antagonist Ⅱ; ICG-R15: Indocyanine green retention at 15 min after intravenous injection.

We first performed Hassab's operation (splenectomy and devascularization of the distal esophagus and proximal stomach) due to uncontrollable esophagogastric varices and hypersplenism. Despite the Hassab's operation, the esophagogastric variceal hemorrhage prevailed. Consequently, we performed transcatheter arterial embolization (TAE) with fiber platinum coils and n-butyl 2-cyanoacrylate for the APS. Although we performed TAE twice, the esophagogastric variceal hemorrhage persisted because of recanalization of the APS.

The laboratory data obtained after TAE are shown in the Table 1.

The Child-Pugh classification status was class B (7 points) and the degree of liver damage was class B. Because the liver function data other than ICG-R15 were good, we surmised that severe APS led to an ICG-R15 value that was worse than that of the actual liver function. Subsequent therapy for HCC and APS was required, and we performed right hepatectomy for resection of HCC and APS 4 mo after hospitalization. Although the tumor was located mainly in the right posterior section of the liver, the tumor apposed the right anterior superior and posterior portal vein and the right hepatic vein. Therefore, we selected right hepatectomy. The portal vein pressure improved after liver resection. The pre- and post-liver resection state was 55 and 37 cmH_2O, respectively. Pathological examination revealed a moderately differentiated HCC in the posterior section of the liver, a tumor thrombus in the right posterior branch of the portal vein, and chronic hepatitis in the non-tumorous area. The postoperative course was uncomplicated, and APS and esophagogastric varices improved. The patient is alive at 12 mo after right hepatectomy, without esophagogastric variceal hemorrhage, and has been undergoing systemic chemotherapy for multiple recurrent HCC and lymph node metastases.

DISCUSSION

HCC is frequently associated with APS. Kido *et al*[6] and Ngan *et al*[7] have determined that APS occurs in 60% of patients with HCC, and Okuda *et al*[8] have reported that marked APS of the main, right or left portal vein occurs in 30% of HCC patients. Severe APS leads to or aggravates portal hypertension, which causes life-threatening conditions, such as rupture of esophagogastric varices, refractory ascites, and hepatic encephalopathy[1-3]. Luo *et al*[9] have reported that severe APS exhibits strong enhancement of the main portal trunk and/or the first-order branches at the hepatic arterial phase of CT. HCC with tumorous APS is frequently unenhanced at the arterial phase in dynamic CT[9,10] because APS reduces arterial flow to the tumor. In our case, the patient presented with rupture of esophagogastric varices. CT revealed a low-density lesion in the posterior segment of the liver, and tumor staining at the arterial phase was not visible because of the tumorous APS.

Endoscopic treatment is the standard treatment for esophageal varices. EVL is safer and more convenient to perform compared to EIS, but does not completely disrupt the interconnecting perforating and feeder vessels[11]. For this reason, EIS is used additionally to improve the clinical results of EVL. Hassab's operation[12] is performed when esophagogastric varices are not controlled with endoscopic treatment and/or if hypersplenism is present. In the present case, we performed Hassab's operation because esophagogastric varices were not controlled with endoscopic treatment and hypersplenism was present; however, the esophagogastric variceal hemorrhage prevailed. We suspect that the esophagogastric variceal hemorrhage occurred as a result of the development of submucosal collateral vessels. APS needs to be treated to improve portal hypertension caused by severe APS. Although TAE with several embolic materials such as gelatin sponge, coil, and ethanol is an effective treatment for APS, Huang *et al*[13] have reported that the recanalization rate of APS was 18% and 86% in the ethanol and gelatin sponge groups, respectively. In our patient, unfortunately, APS was resistant to two TAE procedures, and the esophagogastric variceal hemorrhage prevailed. Only right hepatectomy is effective for the treatment of HCC and intratumoral APS. Although the ICG-R15 value was poor (30%), we performed right hepatectomy. The ICG clearance test is one of the most commonly used liver function tests[14], and Imamura *et al*[15] have reported that the cutoff value of ICG-R15 that allows safe right hepatectomy is 10%, and patients with 30%-39% ICG-R15 are treated with limited hepatic resection alone. However, this is premised on the assumption that there is no portosystemic shunt and that the intrahepatic blood flow is even, and thus, the ICG-R15 value is worse than the true value in patients with APS[4]. In such cases, technetium-99m diethylenetriaminepentaacetic acid galactosyl human serum albumin (^{99m}Tc-GSA) scintigraphy demonstrates great potential and is used more widely because the results are not affected by the presence of a shunt. Many institutions

have described methods for predicting hepatic functional reserve with ^{99m}Tc-GSA scintigraphy; however, a consensus on the best method has not yet been reached[16]. Thus, we did not perform ^{99m}Tc-GSA scintigraphy. We judged that the poor value of ICG-R15 resulted from APS because the value of ICG-R15 was improved by TAE for APS, despite an incompletely occluded APS (ICG-R15 was 36% before TAE and 30% after). Thus, we judged that the patient could tolerate right hepatectomy because the standard liver function test results were almost in the normal range. The patient had no major postoperative complications and his quality of life was improved due to correction of APS.

In conclusion, we reported a patient with HCC and intratumoral APS. We encountered difficulty in controlling esophagogastric varices and APS and evaluating hepatic functional reserve; however, he was treated successfully with hepatectomy. In patients with severe APS, we must estimate hepatic functional reserve, and hope that a consensus on the best method for evaluating hepatic functional reserve will be obtained.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i25.3215

World J Gastroenterol 2010 July 7; 16(25): 3215-3218
ISSN 1007-9327 (print)

CASE REPORT

Hepatocellular carcinoma occurring in a Crohn's disease patient

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Received: March 4, 2010 Revised: March 30, 2010
Accepted: April 6, 2010
Published online: July 7, 2010

Abstract

We report a case of hepatocellular carcinoma (HCC) occurring in a patient with Crohn's disease (CD) without chronic hepatitis or liver cirrhosis, and review the clinicopathological features of HCC in CD patients. A 37-year-old Japanese man with an 8-year history of CD and a medication history of azathioprine underwent resection of a liver tumor. The histopathology of the liver tumor was pseudoglandular type HCC. In the non-neoplastic liver, focal hepatocyte glycogenosis (FHG) was observed, however, there was no evidence of liver cirrhosis or primary sclerosing cholangitis. Only nine cases of HCC in CD patients have been reported previously in the English-language literature. Eight of 10 cases (including the present case) had received azathioprine treatment, and four of these cases also showed FHG, which is considered a preneoplastic liver lesion, within the non-neoplastic liver. Although the precise mechanism of the development of HCC in CD patients is controversial, these results suggest that azathioprine therapy and FHG in the non-neoplastic liver contribute to the development of HCC. These findings also indicate that it is important to survey CD patients treated with prolonged azathioprine therapy for potential liver tumors.

Key words: Crohn's disease; Hepatocellular carcinoma; Azathioprine; Focal hepatocyte glycogenosis; Hepatocarcinogenesis

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INTRODUCTION

Fatty liver and primary sclerosing cholangitis are known to be associated with Crohn's disease (CD), and patients with CD also experience an increased risk of malignant lymphoma and cancers of the small intestine or colon[1,2]. Nonetheless, hepatocellular carcinoma (HCC) in CD pa-

tients is extremely rare, with only nine cases reported in the English-language literature[3-11]. Here, we report one additional case of HCC in a CD patient without established chronic liver disease, and review the clinicopathological features of HCC in CD patients. In addition, we discuss the tumorigenesis of HCC in CD patients and the relationship between HCC and azathioprine treatment.

CASE REPORT

A 37-year-old Japanese man with an 8-year history of CD was admitted to our hospital for examination of a liver tumor. He had been diagnosed with CD at age 29 years, when he required surgery for a bowel fistula. He had been treated with elemental diet, prednisolone, azathioprine, and 5-aminosalicylic acid. Two years prior to this admission, magnetic resonance imaging (MRI) showed a liver tumor in S7, which measured 4 cm × 3 cm. The liver tumor enlarged gradually in follow-up computed tomography (CT) and MRI. Preoperative abdominal contrast-enhanced CT disclosed the S7 tumor that measured 8 cm × 5 cm, which showed early arterial enhancement (Figure 1).

Upon admission, biopsy of the S7 tumor was performed, and histopathological study showed HCC. Then, the patient underwent hepatic resection of the posterior segment.

Preoperative colorectal endoscopic examination revealed mucosal redness and pseudopolyposis throughout the entire colorectum. Histopathological findings of the colorectal mucosa corresponded to CD, with the presence of discontinuous lymphoplasmacytic infiltrate in the lamina propria and a few non-caseating granulomas, unrelated to crypt rupture (Figure 2A).

Preoperative laboratory data revealed mild anemia (hemoglobin 11.1 g/dL; range 12.4-17.0). Liver enzymes were within normal limits (aspartate aminotransferase 14 IU/L; range 7-38, and alanine aminotransferase 15 IU/L; range 4-43). C-reactive protein was slightly elevated (2.57 mg/dL; range < 0.3). Although serum alpha-fetoprotein level was normal (7.7 ng/mL; range < 20), protein induced by vitamin K absence Ⅱ (PIVKA Ⅱ) level was markedly elevated (757 mAU/mL; range < 40). Serology was negative for hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B core antibody, and hepatitis C antibody. In addition, he had no history of alcohol consumption.

Microscopically, the resected specimen of the S7 tumor was almost well-circumscribed by a fibrous capsule, but focal extracapsular invasion was observed. The tumor displayed pseudoglandular to focal trabecular growth of tumor cells with rich eosinophilic cytoplasm and enlarged, round to oval nuclei with a nucleolus (Figure 2B). These histopathological findings were typical of pseudoglandular type HCC.

Non-neoplastic resected liver tissue showed no evidence of liver cirrhosis, chronic hepatitis, or primary sclerosing cholangitis. However, some foci of benign-appearing clear hepatocytes were observed (Figure 2C).

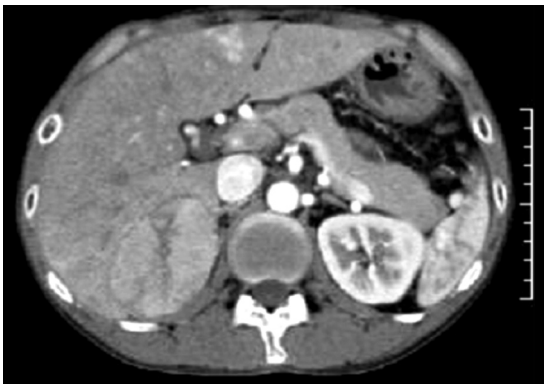

Figure 1 Contrast-enhanced abdominal computed tomography. A well-circumscribed tumor showing early arterial enhancement is present in S7.

These clear hepatocytes were confirmed to have glycogen accumulation (focal hepatocyte glycogenosis; FHG), because they stained positive for periodic acid-Schiff and were digested by diastase. In addition, no histopathological evidence suggestive of non-alcoholic steatohepatitis, such as macrovesicular steatosis, pericellular fibrosis, and neutrophils infiltration, was observed in the non-neoplastic liver tissue.

The postoperative course was uneventful, and no tumor recurrence has been observed during 2 years follow-up.

DISCUSSION

HCC generally occurs in patients with established chronic liver disease, such as liver cirrhosis and viral hepatitis. HCC in CD patients is extremely rare; to the best of our knowledge, only nine cases have been reported previously in the English-language literature[3-11]. The Table 1 summarizes the clinicopathological features of HCC in CD patients. The mean duration from the onset of CD to the development of HCC is 15.1 years (range: 0-36). Although no patients, including the present case, demonstrated apparent risk factors for developing HCC, such as liver cirrhosis or viral hepatitis, two patients had primary sclerosing cholangitis (Table 1)[6,8]. In the five cases in which histological typing of HCC was available, there were three cases of pleomorphic type HCC and two of trabecular type HCC (Table 1). The present case is believed to be the first case of pseudoglandular type HCC occurring in CD.

Most of the CD patients showed a history of medication with one or more drugs (Table 1). Eight of 10 cases had been treated with azathioprine and six had a medication history with 5-aminosalicylic acid. Azathioprine is an inhibitor of purine synthesis, and is prescribed widely in patients with organ transplantation or inflammatory bowel disease[12]. Patients who undergo long-term azathioprine-based immunosuppressive treatment have been reported to show an increased risk of non-Hodgkin lymphoma and cutaneous squamous cell carcinoma[13,14].

The occurrence of HCC in patients who received azathioprine therapy in the absence of liver cirrhosis and viral hepatitis remains exceptional; however, one case of

Table 1 Clinicopathological summary of HCC occurring in CD patients

Case No.	Sex	Age at: onset of CD/discovery of HCC (yr)	Medication for CD	Serum AFP/PIVKA Ⅱ (ng/mL, mAU/mL)	Histology of HCC	Histology of non-neoplastic liver	Outcome	Ref.
1	F	29/43	AZA, PSL	NA/NA	NA	No cirrhosis	DOD with intraabdominal and intrathoracic metastases	[3]
2	F	9/22	5-ASA, AZA	55000/NA	Trabecular	No cirrhosis FHG (+)	Recurrence at 6 mo, liver transplantation performed	[4]
3	M	13/33	5-ASA, AZA	NA/NA	NA	No cirrhosis	Lung metastases	[5]
4	F	63/63	5-ASA	NA/1100	NA	PSC	No evidence of recurrence	[6]
5	F	14/28	AZA, IFx	26.9/NA	Trabecular, pleomorphic	No cirrhosis FHG (+)	NA	[7]
6	M	17/33	AZA	Normal range/NA	NA	No cirrhosis PSC	Mediastinal and abdominal metastases; died 1 year after	[8]
7	M	19/37	5-ASA, AZA, PSL	15/NA	Trabecular to sinusoidal, pleomorphic	No cirrhosis (only CT imaging)	DOD 3 mo after surgery	[9]
8	M	16/52	5-ASA	13.9/16300	Trabecular	Chronic liver damage	No recurrence was found on CT imaging, but PIVKA Ⅱ was 1980	[10]
9	M	13/25	AZA, IFx, PSL	78/NA	Pleomorphic	No cirrhosis FHG (+)	No metastasis or recurrence 1 yr after surgery	[11]
Present report	M	29/37	5-ASA, AZA, PSL	7.7/757	Pseudoglandular	No cirrhosis FHG (+)	No recurrence 2 yr after surgery	

5-ASA: 5-aminosalicylic acid; AZA: Azathioprine; DOD: Died of disease; FHG: Focal hepatocyte glycogenosis; HCC: Hepatocellular carcinoma; CD: Crohn's disease; IFx: Infliximab; NA: Not available; PSC: Primary sclerosing cholangitis; PSL: Prednisolone; CT: Computed tomography.

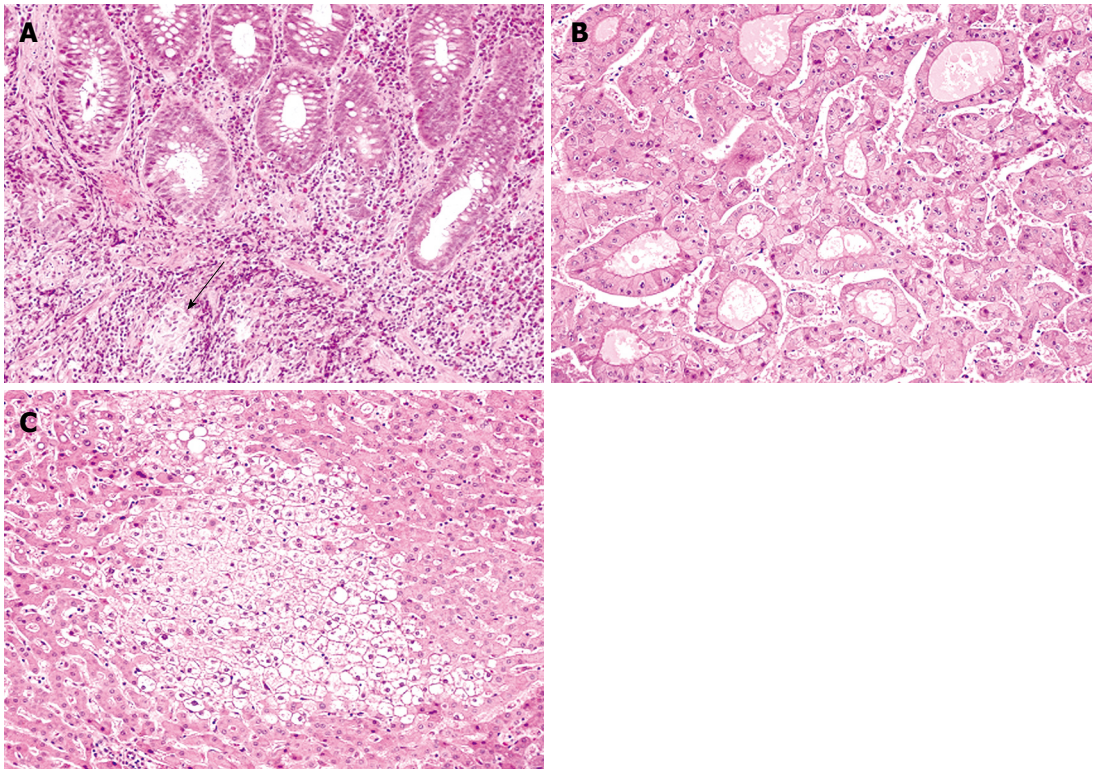

Figure 2 The histopathology of colorectal mucosa (A), the liver tumor (B) and non-neoplastic liver (C) (hematoxylin and eosion stain, × 100). A: Lymphoplasmacytic infiltrate and a tiny granuloma without association with crypt rupture (arrow) are observed; B: The neoplastic hepatocytes show pseudoglandular growth; C: Focal hepatocyte glycogenosis is observed.

HCC in a renal transplant patient without apparent risk factors other than prolonged azathioprine therapy has been reported (no hepatitis C studies were available at that time)[15], and one case of HCC after azathioprine therapy has also been reported in a patient with ulcerative colitis but no hepatitis B and C[16]. In addition, 80% of CD patients who developed HCC had a medication history of azathioprine (Table 1).

The precise mechanism involved in the development of HCC in CD patients is controversial. In one experi-

mental study, azathioprine was shown to increase hepatocyte turnover[17]. It was hypothesized that the degree of immunosuppression by prolonged azathioprine therapy was associated with the incidence of neoplasia[15]. These results suggest that azathioprine therapy is associated with the development of HCC in CD patients.

FHG was observed in the present case, as well as in three other CD patients previously reported to have developed HCC[4,7,11]. Although the cause and significance of FHG are not well established, glycogenated foci can be considered preneoplastic lesions, because FHG and lesions observed during drug-induced hepatocarcinogenesis have histological and biochemical similarities, and the presence of FHG within non-malignant hepatocellular nodules of cirrhotic liver can predict their malignant transformation[18]. Cattan *et al*[4] have reported previously on a CD patient with HCC who had both a medication history of azathioprine as well as disseminated FHG in the non-neoplastic liver. They suspected the pathogenic link between long-term azathioprine therapy and FHG. These results suggest that FHG could be related to hepatocarcinogenesis in CD patients.

In conclusion, we reported a case of pseudoglandular type HCC in a CD patient without established chronic liver disease. The patient had a medication history of azathioprine and FHG in the non-neoplastic liver. Although the precise mechanism of the development of HCC in CD patients is controversial, azathioprine therapy and FHG are likely to be involved. Therefore, accumulation of cases is necessary to clarify the precise mechanism of the development of HCC in CD patients, and we stress the importance of surveying CD patients with prolonged azathioprine therapy for liver tumors.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2010 July 7; 16(25): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 July 7; 16(25): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

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Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
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New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Case-Based Approach to the Management of Inflammatory Bowel Disease

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World J Gastroenterol 2010 July 7; 16(25): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
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Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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Chinese journal article (list all authors and include the PMID where applicable)

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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ISSN 1007-9327
CN 14-1219/R

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Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 26
July 14, 2010
World J Gastroenterol
2010 July 14; 16(26): 3219-3346

Online Submissions
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 26 July 14, 2010

EDITORIAL

3219 Black esophagus: Acute esophageal necrosis syndrome
Gurvits GE

3226 *Helicobacter pylori* and gastric cancer in the Middle East: A new enigma?
Hussein NR

GUIDELINES FOR BASIC SCIENCE

3235 Two-layer cold storage method for pancreas and islet cell transplantation
Fujino Y

REVIEW

3239 Interstitial cells of Cajal, the Maestro in health and disease
Mostafa RM, Moustafa YM, Hamdy H

ORIGINAL ARTICLE

3249 *Nardostachys jatamansi* extract protects against cytokine-induced β-cell damage and streptozotocin-induced diabetes
Song MY, Bae UJ, Lee BH, Kwon KB, Seo EA, Park SJ, Kim MS, Song HJ, Kwon KS, Park JW, Ryu DG, Park BH

3258 CD74 is a survival receptor on colon epithelial cells
Maharshak N, Cohen S, Lantner F, Hart G, Leng L, Bucala R, Shachar I

3267 Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions
Dong XJ, Zhang H, Pan RL, Xiang LX, Shao JZ

3279 Randomized controlled trial of minimally invasive surgery using acellular dermal matrix for complex anorectal fistula
A ba-bai-ke-re MMTJ, Wen H, Huang HG, Chu H, Lu M, Chang ZS, Ai EHT, Fan K

BRIEF ARTICLE

3287 Safety of same-day endoscopic ultrasound and endoscopic retrograde cholangiopancreatography under conscious sedation
Che K, Muckova N, Olafsson S, Srikureja W

3292 Functional magnetic resonance imaging in an animal model of pancreatic cancer
Lewandowski RJ, Eifler AC, Bentrem DJ, Chung JC, Wang D, Woloschak GE, Yang GY, Ryu R, Salem R, Larson AC, Omary RA

3299 Wireless capsule endoscopy and proximal small bowel lesions in Crohn's disease
Petruzziello C, Onali S, Calabrese E, Zorzi F, Ascolani M, Condino G, Lolli E, Naccarato P, Pallone F, Biancone L

3305 Experience with the surgical treatment of hepatic hydatidosis: Case series with follow-up
Losada Morales H, Burgos San Juan L, Silva Abarca J, Muñoz Castro C

3310 Comparison of different nutritional assessments in detecting malnutrition among gastric cancer patients
Ryu SW, Kim IH

3318 Oncological outcome of unresectable lung metastases without extrapulmonary metastases in colorectal cancer
Li WH, Peng JJ, Xiang JQ, Chen W, Cai SJ, Zhang W

3325 Relationship between let-7a and gastric mucosa cancerization and its significance
Zhu YM, Zhong ZX, Liu ZM

3330 Paclitaxel ameliorates fibrosis in hepatic stellate cells *via* inhibition of TGF-β/Smad activity
Zhou J, Zhong DW, Wang QW, Miao XY, Xu XD

CASE REPORT

3335 Vanishing bile duct syndrome in human immunodeficiency virus: Nevirapine hepatotoxicity revisited
Kochar R, Nevah MI, Lukens FJ, Fallon MB, Machicao VI

3339 Isolated ileal perforation due to cytomegalovirus reactivation during management of terbinafine hypersensitivity
Sano S, Ueno H, Yamagami K, Yakushiji Y, Isaka Y, Kawasaki I, Takemura M, Inoue T, Hosoi M

3343 Abdominal separation in an adult male patient with acute abdominal pain
Liu BL, Chen Y, Liu SQ, Zhang XB, Cui DX, Dai XW

World Journal of Gastroenterology
Volume 16 Number 26 July 14, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
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PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
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Beijing 100025, China
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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
July 14, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

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http://www.wjgnet.com

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3219

World J Gastroenterol 2010 July 14; 16(26): 3219-3225
ISSN 1007-9327 (print)

EDITORIAL

Black esophagus: Acute esophageal necrosis syndrome

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Received: March 15, 2010 Revised: April 2, 2010
Accepted: April 9, 2010
Published online: July 14, 2010

Abstract

Acute esophageal necrosis (AEN), commonly referred to as "black esophagus", is a rare clinical entity arising from a combination of ischemic insult seen in hemodynamic compromise and low-flow states, corrosive injury from gastric contents in the setting of esophago-gastroparesis and gastric outlet obstruction, and decreased function of mucosal barrier systems and reparative mechanisms present in malnourished and debilitated physical states. AEN may arise in the setting of multiorgan dysfunction, hypoperfusion, vasculopathy, sepsis, diabetic ketoacidosis, alcohol intoxication, gastric volvulus, traumatic transection of the thoracic aorta, thromboembolic phenomena, and malignancy. Clinical presentation is remarkable for upper gastrointestinal bleeding. Notable symptoms may include epigastric/abdominal pain, vomiting, dysphagia, fever, nausea, and syncope. Associated laboratory findings may reflect anemia and leukocytosis. The hallmark of this syndrome is the development of diffuse circumferential black mucosal discoloration in the distal esophagus that may extend proximally to involve variable length of the organ. Classic "black esophagus" abruptly stops at the gastroesophageal junction. Biopsy is recommended but not required for the diagnosis. Histologically, necrotic debris, absence of viable squamous epithelium, and necrosis of esophageal mucosa, with possible involvement of submucosa and muscularis propria, are present. Classification of the disease spectrum is best described by a staging system. Treatment is directed at correcting coexisting clinical conditions, restoring hemodynamic stability, nil-per-os restriction, supportive red blood cell transfusion, and intravenous acid suppression with proton pump inhibitors. Complications include perforation with mediastinal infection/abscess, esophageal stricture and stenosis, superinfection, and death. A high mortality of 32% seen in the setting of AEN syndrome is usually related to the underlying medical co-morbidities and diseases.

Key words: Acute esophageal necrosis; Black esophagus; Acute necrotizing esophagitis; Ischemia; Endoscopy; Gastrointestinal hemorrhage

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Gurvits GE. Black esophagus: Acute esophageal necrosis syndrome. *World J Gastroenterol* 2010; 16(26): 3219-3225 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3219.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3219

INTRODUCTION

Acute esophageal necrosis (AEN), commonly referred to as "black esophagus" or "acute necrotizing esophagitis", is a rare clinical disorder classically characterized by a striking endoscopic image of diffuse, circumferential, black-appearing, distal esophageal mucosa on esophagogastroduodenoscopy (EGD) (Figure 1) that stops abruptly at the gastroesophageal junction (GEJ)[1,2]. Proximal esophageal extension is common. Biopsy is recommended although not required to make the diagnosis. First described in 1990 by Goldenberg *et al*[3], this medical phenomenon was again brought into the spotlight by our large review series

in 2007, in which we analyzed all cases reported in the English literature to date and formulated the concept of a distinct clinical syndrome[2]. Its etiology is likely multifactorial. AEN is thought to arise from a combination of an ischemic insult to the esophagus, impaired mucosal barrier systems, and a backflow injury from chemical contents of gastric secretions[2,4-6]. Male sex, older age, chronic medical conditions, including diabetes mellitus, hematologic and solid organ malignancy, malnutrition, renal insufficiency, cardiovascular compromise, trauma, and thromboembolic phenomena place a patient at a higher risk for developing AEN[4,7-13]. Clinical presentation is almost universally related to upper gastrointestinal bleeding. Complications may include stenosis or stricture formation in the distal esophagus, perforation, mediastinitis, and death. Overall mortality is largely related to the underlying medical condition and approaches 32%[2].

In this latest review, we discuss the epidemiology of AEN syndrome, its pathophysiological and clinical features, etiology, presentation, distinction from other mimickers of "black esophagus", natural course of the disease, staging, complications, suggested treatment options, and prognosis.

EPIDEMIOLOGY

Literature analysis shows that the estimated prevalence of AEN is low. Two large autopsy series from the United States and France have reported zero cases in a series of 1000 adult autopsies[14] and 0.2% in 3000 autopsies, respectively[15]. Two large retrospective series that have reviewed the findings in > 100 000 endoscopies have estimated the incidence at approximately 0.01% (12 patients)[5,12], and another retrospective analysis of 10 295 endoscopies has shown an incidence of 0.28% (29 patients)[13]. A 1-year prospective study of EGD findings in 3900 patients has identified eight cases of AEN (0.2%)[6]. However, these numbers are likely underestimated and the true prevalence of AEN remains unknown, largely due to the potential of subclinical presentation of the disease and the early healing of the mucosa that can be seen with transient ischemic or chemical injury. An intriguing paper by Japanese authors has reported AEN as the fourth leading cause of coffee ground emesis, hematemesis, or melena in a series of 239 hospital admissions for upper gastrointestinal bleeding, which accounted for approximately 6% of the cases[16].

AEN clearly shows sex and age predilection. Men are four times more commonly affected than women, and although the disease has been documented in every age group, the peak incidence occurs in the sixth decade of life with an average age of 67 years[2]. General debilitation and poor clinical condition with multiple medical co-morbidities are associated with increased likelihood of development of AEN. Personal history of diabetes mellitus (24%), malignancy (20%), hypertension (20%), alcohol abuse (10%), and coronary artery disease (9%) places a patient at risk of developing AEN[2].

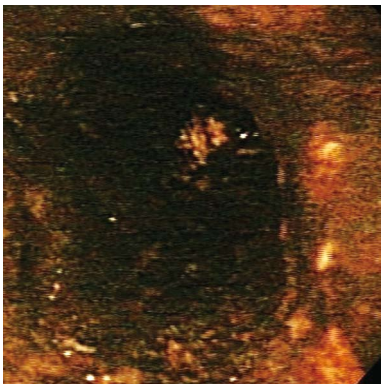

Figure 1 Black esophagus: acute esophageal necrosis.

ANATOMY AND PATHOPHYSIOLOGY

The esophagus is an expandable muscular organ lined by a stratified squamous epithelium. Characteristically, esophageal wall is composed of four distinct layers: mucosa, submucosa, muscularis propria, and adventitia. Unlike the rest of gastrointestinal tract, the esophagus lacks serosa and is separated from the adjacent organs by few millimeters of connective tissue, an important factor in potential development of extrinsic necrotic injury to the organ and spread of the infectious processes.

The esophagus receives an intricate segmental vascular supply that separates this organ into three parts: upper, middle, and distal esophagus. The arterial network of the upper esophagus is derived from the descending branches of the inferior thyroid arteries. Additional variable supply may come from branches of the subclavian, thyroidea ima, common carotid, or superior thyroid arteries. Mid-esophageal blood supply is derived from the bronchial arteries, right third or fourth intercostal arteries, and numerous small esophageal arteries off the descending aorta. Distal esophagus receives its blood flow from branches off the left gastric artery or left inferior phrenic artery, but variations are common and small branches off the celiac, splenic, short gastric, or left hepatic arteries may provide additional or alternative blood supplies[17,18]. Relative to the densely vascularized proximal and middle parts of the organ, the distal esophagus is slightly more "watershed" and therefore the first signs of the ischemic injury to the esophagus typically appear there. Overall, the complex anastomotic vascular network that is present in the esophageal submucosa generally makes esophageal infarction a rare clinical disease[18].

An important physiological property of the esophagus is the ability to use intricate protective barrier systems to shield itself from the injurious chemical reflux of the stomach contents. A delicate microarray of the epithelial tight junctions, zonula adherens, intercellular glycoconjugated material, intrinsic cellular buffering systems and anion exchangers, and extensive desmosomal network, coupled with bicarbonate, lubricant, mucus, and epidermal growth factor production by submucosal glands account for comprehensive defense mechanisms and restorative

function of the organ[18]. Compromise or inadequacy of these barriers may lead to cell damage, and ultimately, death. Local blood supply to the lamina propria and submucosa, which is essential in maintaining effective buffering systems, is influenced by a variety of factors, including autoregulation in response to the intraluminal acidic pH of gastric contents[19,20]. Initial augmentation in blood delivery needed for maintenance of protective and reparative processes may be significantly downregulated by an overwhelming back diffusion of hydrogen ions[19,21]; an important factor in pathogenesis of AEN, particularly in the setting of an already compromised blood flow. At the cellular and molecular level, the mechanism of the esophageal injury is similar to the development of ischemic changes in the small and large bowel. Reactive oxygen metabolites formed as a result of reperfusion injury and leukocytic migration may directly and indirectly damage important mechanisms of cellular viability and function, which results in cellular lysis and death[18,22].

Finally, anti-reflux barriers and luminal clearance are important factors in preventing significant esophageal injury. Integrity of the lower esophageal sphincter mechanism, gravity and esophageal peristalsis are vital. Compromise or ineffectiveness of any one of these may deeply potentiate necrotic injury[23].

ETIOLOGY

Tissue insult seen in AEN is likely multifactorial and usually results from a combination of tissue hypoperfusion, impaired local defense barriers, and massive influx of gastric contents that acutely overwhelm the already vulnerable esophageal mucosa[2]. Perfusion injury in the development of AEN is best demonstrated by an avascular distal esophagus reported by Goldenberg *et al*[24]. Significant vasculopathy that is typically associated with diabetes mellitus, atherosclerosis, cardiovascular and renal diseases is an important risk factor that may predispose some patients to rapid tissue injury, as well as general susceptibility to hemodynamic instability and compromise. Low-flow state related to sepsis, cardiac arrhythmias, congestive heart failure, severe third spacing, systemic inflammatory response syndrome due to severe pancreatitis, lactic- and ketoacidosis, acute blood loss, hypothermia, trauma and shock may lead to ischemic compromise of the esophagus[2,12,16,25-31]. Thromboembolic phenomena and underlying coagulopathy seen in patients with solid tumor or hematological malignancies, anticardiolipin antibody syndrome, and atherosclerosis have also been implicated in the development of AEN[8,9,11,16,29]. Striking predilection to the distal esophagus is likely explained by its lesser degree of vascularization relative to the proximal and middle portions of the organ[12].

Vascular compromise to the distal esophageal tissue can also explain some of the duodenal pathology commonly seen in association with AEN, namely, duodenal bulb ulcers, erosions, inflammation, and edema[3,6,24]. The common blood supply from the branches off the celiac axis makes distal esophageal and duodenal pathologies less of a coincidence, but rather possibly related entities. Characteristic relative sparing of the gastric mucosa can be explained by the acidic impact on the ischemic esophageal and duodenal surface[7], as well as typically rapid repair of the injured gastric mucosa (within hours) compared to the esophagus (may take days)[18]. The duodenal bulb ulcers and edema may result in gastric outlet obstruction that, in turn, potentiates the development of mucosal necrosis of the distal esophagus[5,32]. A transient form of non-obstructive gastropathy can be seen in acute alcohol intoxication and diabetic ketoacidosis, two medical conditions that are linked to the development of AEN[25,26,33,34]. Back flow injury from the acid, pepsin, and other gastric contents is augmented by the increased transient lower esophageal sphincter relaxation, decreased resting lower esophageal pressure, prolonged recumbence, decreased esophageal peristalsis, and increased gastroesophageal reflux that may be related to the multiple medical conditions seen in the patient with AEN (debilitated condition, significant hiatal hernia, alcohol use, diabetes mellitus, postoperative state, *etc.*).

Alteration in physiological processes is an important co-factor in the development of AEN. Critical illness, poor nutritional status, and general deconditioning may contribute to the diminished mucosal buffering, compromised local protective barriers, and impaired defensive mechanisms that may potentiate ischemic and chemical injury to the esophagus[6]. Such risk factors include malignancy, cirrhosis, chronic pulmonary disease, neutropenia, renal insufficiency or failure, diabetes mellitus, hypoalbuminemia, immunosupression after solid organ transplantation, acquired immune deficiency syndrome (AIDS), postoperative status, and sepsis[2,10,12,13,27,35-39].

Diffuse AEN may be associated with local infection and tissue biopsy is essential for proper management, which includes antiviral or antimicrobial agents. Exact distribution is variable and probably depends on mode of transmission of the infectious agent, from external spread reported from a superior mediastinitis that affects cervical and thoracic esophagus[40], to primary esophageal intraluminal infection seen in viral and fungal disease in immunocompromised patients[35,36,41]. Reported pathogens include *Klebsiella pneumoniae*[40] and cytomegalovirus[36,41], herpes simplex virus[37], *Penicillium chrysogenum*[35], *Candida*[7], and other fungal species.

Other diseases and medical conditions associated with the development of AEN include erythema multiforme or Stevens-Johnson syndrome, gastric volvulus, hematoma from traumatic transection of thoracic aorta, hypersensitivity to antibiotics, acute fatty liver of pregnancy, drug-induced hepatitis, photodynamic therapy, polyarteritis nodosa, pulmonary lobectomy with paraesophageal lymph node dissection, severe infectious mediastinitis, emphysematous gastritis, hypoxia, and pancreatitis[2,7,12,39,40,42-49]. A single case of isolated proximal black esophagus has been reported in a patient who underwent cardiac catheterization, an event that was possibly related to the procedure[50,51].

PRESENTATION

A typical patient with AEN is an elderly male with mul-

tiple medical comorbidities, who manifests with signs of upper gastrointestinal bleeding. Clinical presentation of AEN ranges from hematemesis, coffee ground emesis, and melena (overall, accounting for nearly 90% of the cases) to asymptomatic black esophagus that was noted during a percutaneous gastrostomy tube placement[2,52]. Review of systems may be remarkable for epigastric/abdominal pain, vomiting, dysphagia, nausea, low-grade fever, lightheadedness, and syncope. Other associated clinical conditions or endoscopic findings may include multiorgan failure[53], cardiac, pulmonary, and renal disease, diabetes mellitus and ketoacidosis, vasculopathy, coagulopathy, peptic ulcer disease, general debilitation and malnourishment, cirrhosis, acute alcoholic hepatitis, acute fatty liver of pregnancy, acute pancreatitis, sepsis, ischemic processes (stroke, ischemic bowel, ischemic gangrene), and trauma. Physical findings in a patient with AEN are usually confounded by the underlying medical conditions but may be notable for cachectia, fever, hypoxia, hemodynamic instability or compromise, including arrhythmia and hypotension, pallor, abdominal tenderness, and guaiac-positive stools. Laboratory analysis may show leukocytosis and anemia and computed tomography (CT) may reveal thickened distal esophagus, hiatal hernia, distended fluid-filled stomach with possible gastric outlet obstruction, and rarely, external compression of the esophagus by a large mediastinal process.

DIFFERENTIAL DIAGNOSIS

Classic AEN presents as a circumferential, black-appearing, diffusely necrotic esophageal mucosa, preferentially affecting the distal esophagus, of variable length, that stops abruptly at the GEJ. Proximal extension of the mucosal injury is common and the entire esophagus can appear black. Differential diagnosis includes malignant melanoma, acanthosis nigricans, coal dust deposition, pseudomelanosis, and melanocytosis of the esophagus[54-63]. Detailed history and physical examination provide essential clues to the diagnosis. Classic endoscopic appearance may be supported by brush cytology or biopsy specimen that will confirm the diagnosis. This is especially important in rare cases of isolated involvement of proximal and middle esophagus. Additionally, a history of corrosive ingestion[64] should be sought for and diligently excluded.

CLINICAL DIAGNOSIS

The characteristic endoscopic appearance described above makes the diagnosis of AEN. Almost universal predilection to the distal esophagus (97%) is a remarkable feature of this disorder[2]. Additional endoscopic findings may include duodenal peptic ulcer disease, erosions, edema, and sings of gastric outlet obstruction[23]. Early evaluation may also be notable for signs of bleeding, including active oozing from the distal esophagus and blood clots and "coffee grounds" material in the stomach. Esophageal biopsy or brushings of the affected esophageal tissue are supportive but not required to make the proper diagnosis. Histological findings include absence of viable epithelium, abundant necrotic debris, and necrotic changes in the mucosa, possibly extending into the submucosa and even muscularis propria. Full-thickness necrosis has been described in the surgical specimens. Associated findings may include heavy leukocytic infiltrate, visible vascular thrombi, deranged muscle fibers, and severe inflammatory changes[2]. Biopsy specimens should be sent for bacterial, fungal, and viral cultures to exclude infectious etiologies associated with AEN or a superimposed infection[7,26]. Special care should be taken to evaluate for the presence of multinucleated giant cells or viral inclusion bodies, particularly in immunocompromised patients. At this time, the role of angiography for diagnosis of AEN is not clearly defined, likely related to its potential risks, lack of effective therapeutic intervention in the absence of a recognizable lesion, and tendency for a spontaneous recovery with correction of the underlying medical illness.

Upper gastrointestinal bleeding in a patient with multiple medical conditions, vasculopathy, hemodynamic compromise, general debilitation, malignancy, ischemic disease, and hypercoagulable state should raise the possibility of AEN among other diagnoses. A strong association between diabetic ketoacidosis and AEN has been suggested by some authors, with four cases of black esophagus seen in 29 hospital admissions (14%)[16].

DISEASE COURSE AND STAGING

Generally, uncomplicated AEN follows an indolent clinical course and has a predictable endoscopic and histopathological trajectory. The recently proposed staging system attempts to classify disease progression and detail the distinct phases of AEN[2]. Stage 0 designates a prenecrotic viable esophagus. Stage 1 refers to an acutely diseased organ with an endoscopic picture dominated by a striking diffuse, circumferential, black-appearing esophageal mucosa with occasional yellow exudates and signs of friability, loss of light reflex, rigidity and under-distension of the lumen. These endoscopic findings nearly universally start at the GEJ, involve the distal esophagus, and variably extend proximally, potentially covering the entire organ. Histological appearance is notable for lack of viable squamous epithelium, presence of necrotic debris, and pronounced necrosis of the mucosa with possible extension into the submucosa and muscularis propria. Associated findings may include heavy leukocytic infiltrate with severe inflammatory changes, visible vascular thrombi, deranged muscle fibers, and mucosal infection with viral, fungal, and bacterial pathogens. Stage 2 describes the healing phase of the AEN dominated by residual black areas in the esophagus and thick white exudates composed of necrotic debris that cover friable pink mucosa. This "chess-board" appearance can sometimes be the presenting endoscopic picture of AEN on delayed endoscopy. The exact timing of this change is unknown and it likely parallels the underlying general condition of the patient. However, it has been observed as late as 1 mo after the diagnosis. Stage 2 is also notable for some improvement

in histological appearance of the esophageal tissue, with scattered areas of necrosis among underlying cellular regeneration, granulation tissue, and inflammatory changes. As early as 1-2 wk after diagnosis, the esophageal mucosa acquires its normal endoscopic appearance in stage 3 of the AEN with only microscopically present granulation tissue, a sequelae of recent injury.

COMPLICATIONS

The most serious complication of AEN is perforation, which can occur in severe cases that result in full-thickness necrosis of the esophageal tissue. Its reported incidence is just below 7%[2], and should be suspected in rapidly decompensating patients. Traumatic or spontaneous rupture of the thoracic aorta resulting in extrinsic esophageal compression by hematoma, photodynamic therapy for esophageal cancer, herniated gastric volvulus, and severe thromboembolic disease place a patient at a higher risk for this complication. Perforation can be seen in stage 1 of the disease, and it may lead to rapid clinical deterioration, mediastinitis, mediastinal abscess formation, empyema, and generalized sepsis. Prompt recognition, intravenous antibiotics, and surgical intervention are life-saving. Mortality may be especially high given a generally poor underlying medical condition and a limited reserve in many patients with AEN.

Another possible sequela of AEN is the formation of stenotic areas or strictures, which can be seen in > 10% of patients. These findings typically occur in stages 2 and 3 of the disease and likely result from protective scar formation during the acute, reparative, and healing phase of AEN. Acid suppression followed by possible endoscopic dilatation, if needed, typically produces resolution of reported symptoms.

Microbial superinfection of necrotic media is an important consideration in treating patients with AEN[26]. Patients should be diligently monitored for early signs of clinical decompensation and sepsis with prompt institution of intravenous antimicrobial therapy.

MANAGEMENT

Development of AEN carries a generally poor prognosis and the goal of therapy should be directed at treating the coexisting medical diseases. Timely systemic resuscitation and patient stabilization play pivotal roles in the management of this disease. Intravenous hydration, correcting anemia with packed red blood cell transfusion, and keeping the patient nil-per-os should be instituted. Parenteral support may be needed in malnourished patients to improve their nutritional status and expedite healing. Passage of the nasogastric tube should not be undertaken, to avoid perforation.

Medical management of AEN includes aggressive intravenous proton pump inhibitor or histamine receptor blocker therapy until improvement in clinical status, at which time, a change to an oral formulation is appropriate. Probably, oral therapy should be continued for a few months after the resolution of symptoms to aid prevention of stricture formation, in the absence of underlying reflux disease. Recent reports of esophageal manometry and pH monitoring in patients with AEN have shown a lack of significant acid reflux and normal motility at 5-7 mo after recovery[23,65]. Oral suspension of sucralfate may be used as a single therapy or as a supplement to the intravenous acid-reducing agents, excluding the cases with concomitant renal failure.

Antimicrobial therapy is important in the setting of positive esophageal cultures, stains for fungal agents, or visualization of multinucleated giant cells or inclusion bodies on histological evaluation of the biopsy specimen. Empirical antibiotics should be initiated in cases of suspected esophageal perforation, rapid clinical decompensation, unexplained fevers, and immune compromise in AIDS, cirrhosis, transplant recipient, and dialysis patients. Prophylactic use of antibiotics in sterile necrosis is probably not necessary and few reports have potentially linked the antibiotic use itself to the development of AEN[49].

Surgical intervention in patients with AEN is reserved for perforated esophagus with resultant mediastinitis and abscess formation. Esophagectomy, decortication, lavage, and delayed reconstruction may be performed in addition to standard surgical approaches in the setting of gastric volvulus or transected thoracic aorta. Primary closure of the perforated esophageal tissue should not be attempted. Subtotal esophagectomy and esophagogastrostomy have been reported in AEN-induced esophageal stricture that was refractory to repeated dilatations[3,26].

PROGNOSIS AND ROLE OF REPEAT ENDOSCOPY

Prognosis of AEN largely depends on coexisting medical conditions and ordinarily parallels the general state of health of a patient. As a rule, overall prognosis is poor with nearly one third of patients succumbing to the underlying critical illness. However, mortality specific to the AEN is much lower, at around 6%[2]. Important risk factors include esophageal perforation, diabetic ketoacidosis, and compromised immune system. Overall, reversible phenomena of the disease and its tendency for spontaneous healing are favorable factors in predicting complete recovery in otherwise healthy individuals. Relapsing AEN has been reported only once[66] but may be an important diagnosis to consider in the appropriate clinical setting. It may also explain some of the observed color changes of black esophagus reported in the literature[4]. Repeat endoscopic evaluation should be done to assess mucosal healing and document resolution of AEN, as well as to determine the duration of antacid therapy. Endoscopic sessions with balloon dilatation may be necessary in patients suffering from dysphasia in the setting of esophageal stenosis or stricture formation, which are well-known complications of AEN.

CONCLUSION

AEN is a rare syndrome that arises in the characteristic

clinical setting of combined ischemia from hemodynamic compromise, gastric outlet obstruction with backflow chemical injury, and inadequate protective barriers and physiological reserve due to critical illness and general deconditioning. It presents with signs of upper gastrointestinal bleeding and has a characteristic appearance on upper endoscopy of circumferential, diffuse, black-appearing, friable esophageal mucosa, almost universally affecting the distal part of the organ and abruptly ending at the GEJ. Histological tissue necrosis confirms the diagnosis. Treatment relies on aggressive resuscitation, correction of underlying medical conditions, institution of therapy with proton pump inhibitors and sucralfate, and monitoring for signs of infection or perforation. Mortality is high and is largely related to the principal medical conditions and the gravity of the overall state of health.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3226

World J Gastroenterol 2010 July 14; 16(26): 3226-3234
ISSN 1007-9327 (print)

EDITORIAL

Helicobacter pylori and gastric cancer in the Middle East: A new enigma?

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Supported by The University of Nottingham
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Received: February 8, 2010 Revised: April 19, 2010
Accepted: April 26, 2010
Published online: July 14, 2010

Abstract

The Middle East is the home of ethnic groups from three main backgrounds: Semitic (Arabs and Jews), Indo-European (Persians and Kurdish) and Turkic (Turkish and Turkmens). Its geographic location, which has been under continuous influences from Asia, Europe and Africa, has made it an ideal site for epidemiological studies on *Helicobacter pylori* (*H. pylori*) infection and genotyping. The gastric cancer rate differs in this region from very high in Iran ($26.1/10^5$) to low in Israel ($12.5/10^5$) and very low in Egypt ($3.4/10^5$). Epidemiological studies showed that the prevalence of *H. pylori* is almost similar in those countries with a high level of infection in childhood. Importantly, the frequency of *vacA* s1 and m1 regions and *cagA*+ genotypes were higher in non Semitic populations who inhabit the North than Semitic populations, the inhabitants of Southern parts of the Middle East. *H. pylori* infection prevalence, distribution pattern of virulence factors, diet and smoking could not have explained the difference in cancer rate. This reflects the multifactorial aetiology of gastric cancer and suggests that *H. pylori* infection does not always directly correlate with the risk for gastrointestinal disease, such as gastric cancer. Further detailed investigations and international comparative studies of each risk factor need to be performed to investigate whether this represents a true enigma.

Key words: *Helicobacter pylori*; Middle East; Gastric cancer; *dupA*; *cagA*; *vacA*; *iceA*

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Hussein NR. *Helicobacter pylori* and gastric cancer in the Middle East: A new enigma? *World J Gastroenterol* 2010; 16(26): 3226-3234 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3226.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3226

INTRODUCTION

Helicobacter pylori (*H. pylori*) causes gastritis and peptic ulceration and it is an important risk factor for gastric adenocarcinoma, the second highest cause of cancer deaths worldwide. The disease process is thought to have a multifactorial aetiology, and bacterial strain type, pattern of gastritis, and environmental conditions, are all thought to contribute[1]. *H. pylori* strains differ, and possession of specific virulence factors greatly increases the risk of disease. The best recognised of these are the cag pathogenicity island and active forms of the vacuolating cytotoxin (VacA). Duodenal ulcer promoting gene A (*dupA*) is a recently described gene shown to be associated with duodenal ulceration and protective against gastric cancer[2]. It was observed that the early acquisition of *H. pylori* infection in childhood resulted in pangastritis in adulthood. This pattern of gastritis is usually associated with mucosal atrophy which is a precancerous condition[3]. Therefore, acquiring the infection at an early age is a recognised risk factor for the development of

gastric cancer[4]. Additionally, antral predominant gastritis is usually associated with duodenal ulcer. Furthermore, different environmental factors such as high salt intake and inadequate consumption of fruit and vegetables containing vitamin C has been regarded as risk a factor for development of gastric cancer[5].

The Middle East is home to ethnic groups from three main backgrounds: Semitic (Arabs and Jews), Indo-European (Persians and Kurdish) and Turkic (Turkish and Turkmens)[6-8]. Its geographic location, which has been under continuous influences from Asia, Europe and Africa, has made it an ideal site for epidemiological studies on *H. pylori* infection and genotyping. The prevalence of *H. pylori* infection has been reported from many Middle Eastern countries, including Iraq, Iran, Turkey, Libya, Egypt, Israel, Bahrain, Oman, Saudi Arabia, and the United Arab Emirates. It has been shown that the prevalence rates of infection in these countries are almost similar to each other and to the reported prevalence from Europe and United States. However, gastric cancer and other *H. pylori* related diseases rates vary from very high in Iran to rare in Iraq and Egypt (Table 1)[9]. It is not known whether the difference is due to host, environment, or bacterial factors or a combination of these factors. In this review I tried to find a reason for the difference in *H. pylori* related diseases amongst Middle Eastern countries by discussing the prevalence of *H. pylori* and its virulence determinants, the pattern of gastritis, and environmental factors that might influence the disease process in the Middle East.

PREVALENCE OF *H. PYLORI*

The prevalence of *H. pylori* infection varies between countries; generally, the prevalence is about 30% in developed and up to 80% in developing countries[5]. Diagnosis of *H. pylori* can be achieved by taking biopsies by endoscopy. However, this procedure is invasive and might not give accurate results if colonisation is patchy[10]. Furthermore, it does not suit population based studies. For population screening, serodiagnosis remains one of the methods of choice for detecting the prevalence of infection[11]. Systemic humoral immunoglobulin G (IgG) immune responses to the organism are developed by humans infected with *H. pylori*[12-14]. Serological tests are useful tools for the diagnosis of *H. pylon* infection because all *H. pylori*-infected patients produce an antibody response which can be detected in the serum[14]. The technique of choice is currently enzyme-linked immunosorbent assay because it is a simple, quick, and low-cost technique that permits immunoglobulin class-specific determinations[14].

In a study conducted in Iraq, the prevalence varied in various ages (age: percentage, 6 mo: 0%; 6-24 mo: 27%; 2-18 years: 58%). In the same study, it was shown that 78% of adults were infected with *H. pylori* which was significantly higher than children. The prevalence of *H. pylori* increased markedly with age with the maximum colonization (81.5%) occurring in adults (40-60 years)[15]. The same scenario was found in Saudi Arabia, Iran, Libya and Israel. In Saudi, the prevalence of *H. pylori* infection markedly increased with age. The prevalence of *H. pylori* infection rose from 32.4% in those aged 0-10 years to more than 66.4% in those aged 20-30 years and 75% in those over 50 years[16]. In Iran, the prevalence of *H. pylori* infection increased with age [age (years): percentage, 6-10: 46%; 10-20: 50%; 20-30: 67%; 30-40: 80%; 40-50: 85%; 50-60: 84%; 60-70: 81%; over 70: 83%][17]. In Libya, overall prevalence was 67% with a steady increase with age [age (years): percentage, 0-10: 50%; 10-20: 84%; 20-30: 66%; 30-40: 80%; 40-50: 88%; 50-60: 83%; 60-70: 83%; over 70: 94%][18]. In a study conducted in Israeli rural communal settlements with an age range from 6 to 90 years[19], the prevalence *H. pylori* infection was shown to be 72%. It was also found that the prevalence of *H. pylori* increased with age [age (years): percentage, 6-10: 10%; 10-20: 39%; 30-40: 60%; 40-60: 70%; over 60: 85%]. In the same study, a significant association was shown between *H. pylori* infection and the country of origin of Israeli migrants. The highest prevalence (85%) was found in migrants from the Mediterranean and Asia. While 80% of East European migrants were *H. pylori* positive, the prevalence in West Europeans was 57%. The prevalence in people born in Israel was 66%. The association between *H. pylori* infection and country of origin was not changed after age adjustment[19].

In Turkey[20], the overall prevalence of *H. pylori* infection was 81%. There is no marked difference in *H. pylori* prevalence in different ages [age (years): percentage, 0-10: 70%; 10-20: 83%; 20-30: 77%; 30-40: 87%; 40-50: 88%; 50-60: 90%].

In some countries in the Middle East, the prevalence of *H. pylori* infection has been studied using polymerase chain reaction and histopathology. The prevalence of *H. pylori* infection in Jordan and Bahrain was 77.5% and 79%, respectively[21,22]. In Kuwait and Egypt, *H. pylori*, as detected by H and E and *H. pylori* special stains, was present in 84% and 86% of the biopsy samples, respectively[23,24]. There was no significant difference in the prevalence of infection between male and female subjects in this region. In a study conducted in Western Saudi, the prevalence of VacA and CagA were significantly elevated in males *vs* females[25]. In another study in Jordan, there was a clear trend that females were infected with less virulent *H. pylori* strains, though the correlation was not significant[22].

INCIDENCE OF GASTRIC CANCER IN THE MIDDLE EAST

Despite declining incidence rates in Western countries, gastric cancer remains the second most common cancer type and second cause of cancer-related death worldwide. *H. pylori* infection is strongly associated with gastric cancer risk. Gastric cancer rate varies from country to country and from region to region. For example, it is very high in Japan ($62.7/10^5$) and estimated to be 12 times higher than India[9]. Gastric cancer occurs nearly 7 times more frequently in Iran than in Iraq (Table 1). These data might not be very accurate because of incompetent di-

Table 1 Prevalence of atrophy, gastric cancer and the distribution of *vacA* allelic types and *cagA* status among *H. pylori* strains isolated in the Middle East

Country	s1 (%)	s2 (%)	m1 (%)	m2 (%)	i1 (%)	i2 (%)	*cagA* (%)	Atrophy (%)	Male gastric cancer
Saudi Arabia	58.3[61]	41.7[61]	12.6[61]	87.4[61]	No data	No data	52.0[43]	3-19[77]	$5.7/10^5$
Kuwait	46.4[23]	53.6[23]	No data	No data	No data	No data	41.0[23]	28[75]	$4.8/10^5$
Jordan	45.3[22]	54.7[22]	48.9[22]	51.1[22]	No data	No data	26.4[22]	65[76]	$6.6/10^5$
Iran	69.2[39]	30.8[39]	30.8[39]	69.2[39]	36.5[39]	63.5[39]	76.0[39]	22-39[74]	$26.1/10^5$
Iraq	88.6[39]	11.4[39]	25.7[39]	74.3[39]	28.5[39]	71.5[39]	71.0[39]	3[69]	$4.5/10^5$
Turkey	94.8[40]	5.2[40]	22.2[40]	77.8[40]	No data	No data	78.0[40]	43-75[72,73]	$12.2/10^5$
Egypt	42.9[41]	57.1[41]	14.3[41]	85.7[41]	No data	No data	35.7[41]	54[24]	$3.4/10^5$

agnostic methods, limitation of medical services, and the lack of unique reporting systems. However, I searched through all published literature and could not find any major discordance. Despite the geographical proximity, the gastric cancer rate varies from very low in Iraq and Egypt to intermediate in Israel and Turkey to high in Iran (Table 1)[9,26-28]. Interestingly, *H. pylori* infection prevalence in these countries is relatively high and almost the same. This discordance between gastric cancer incidence and *H. pylori* seroprevalence might imply that *H. pylori* infection is not the only factor related to gastric cancer risk[29,30]. A 6-fold variation was found in the association between gastric cancer risk and *H. pylori* infection[31]. *H. pylori* virulence factors, immune response, diet, environment, and other factors should be considered.

BACTERIAL VIRULENCE FACTORS

Cytotoxin associated gene A

The cytotoxin associated gene A (CagA) protein, which is encoded by the *cagA* gene, is a highly immunogenic protein. *H. pylori* strains possessing *cagA* are associated with a significantly increased risk for the development of atrophic gastritis, peptic ulcer diseases and gastric cancer[32-36]. The *cagA* gene is situated at one end of a 40 kb DNA insertion called the *cag* PAI and may have been acquired from a non-*Helicobacter* origin[33,37]. The *cag* PAI contains approximately 30 genes which are multicistronic. The difference in the ability of *H. pylori* strains to trigger chemokines from gastric mucosa depends upon the expression of genes within the *cag* PAI[33,37,38].

Strains from Iraq, Turkey and Iran possessing *cagA* were found in 71%, 78% and 76% of the samples analysed, respectively. *cagA* presence was significantly associated with peptic ulcer disease incidence in Iraq and Turkey but not in Iran[39,40]. In Iraq, the majority of the population are Arabs. However in a study by Hussein *et al*[39], samples were collected from Kurdish majority (Kurdistan) region.

In Jordan, the *cagA* genotype was detected in 26.4%[22]. While Kuwaitis and other Arabian Gulf Arabs had essentially the same prevalence rate of about 41%, Egyptians had a modest positivity of 35.7%[23,41]. In a study conducted in Israel, *cagA* and *cagE* genes were present in only 25.5% and 24.5%, respectively[42]. The prevalence of *cagA* positivity in Saudi was 52%[43]. *cagA* was associated with gastric cancer and/or peptic ulceration in Iran, Iraq, Saudi, Turkey and Israel[39,42-45]. However, Hussein *et al*[39] could not find significant relationships between *cagA* status and disease risk in the Iranian population.

Argent *et al*[46], suggested that the presence or absence of *cagA* is not enough to understand the relationship between *cagA* and clinical outcomes. It was found that there was a size variation of the CagA protein and this variation was shown to be related to the presence of the repeat tyrosine phosphorylation motifs (TPM) sequences containing the EPIYA within the 3' variable ends[47]. It was found that *H. pylori* strains in Western and East Asian countries carry the EPIYA-A, EPIYA-B. While Western *H. pylori* strains carry Western *cagA*-specific EPIYA-C segments which vary in number ranging from 1-3[48], East Asian strains carry the CagA-specific EPIYA-D motif. We previously studied the TPM of the *cagA* in Iraq and Iran. The presence of *cagA* alleles with more than 3 phosphorylation motifs was significantly higher amongst Iranian strains than those from Iraq (there was no Iraqi *cagA*-positive strain with more than 3 TPM, 12% in Iran). We thought that the presence of *cagA* with more phosphorylation motifs in Iran may help explain the higher cancer incidence rate in Iran[39]. However, a recent study from Turkey, where the gastric cancer rate is much lower than Iran, found that 34% of *cagA*-positive Turkish strains carried more than three motifs. For the first time, in this study, they reported a *cagA* positive strain with 5 C motifs[49]. The absence of *cagA* with more than 3 motifs in Iraq can be due to a type 2 error.

There is clear discrimination in *cagA* distribution between Semitic (Arab and Jew) and non Semitic (Kurd, Turk and Persian) populations[39,40,50]. Semitic populations tend to carry less virulent *H. pylori*[22,23,41]. The *cagA* positivity in Saudi Arabia is higher than other Semitic countries. This might be due the fact that Saudi society has been influenced by Hajj.

VacA

The VacA is another *H. pylori* virulence factor[35]. Unlike *cagA*, almost all *H. pylori* strains posses the vacuolating cytotoxin gene (*vacA*). Vacuolating cytotoxin activity is related to the mosaic structure of *vacA*. In general, type s1/m1 and s1/m2 strains produce high and moderate levels of toxin activity, respectively, whereas s2/m2 strains produce no vacuolating activity[51]. A 12-amino-acid hydrophilic amino-terminal segment, present in type s2 but absent from type s1 *VacA* proteins, slows the capacity of *VacA* to form membrane channels and abolishes vacuolation.

Some type s1/m2 *VacA* toxins show cytotoxic activity toward selected cell types, including RK-13, but relatively little activity for HeLa or AGS cells[51-53]. Heterogeneity among *vacA* alleles may be an important factor in understanding variations in clinical manifestations among *H. pylori*-infected subjects. Several studies have demonstrated that gastric infection with *H. pylori* strains containing type s1 *vacA* alleles is associated with a higher risk for development of peptic ulcer disease than infection with strains containing type s2 *vacA* alleles[51]. This relation is not seen in East Asia as the vast majority of East Asian strains are *vacA* type s1[51,54-56]. Thus in these countries, s1 cannot be used as a marker for the presence of peptic ulcer disease because the prevalence of the s1 genotype is uniformly high.

Rhead *et al*[45] have described a novel determinant of VacA toxicity, called the intermediate or i-region. They showed that two allelic variants of this region existed, i1, and i2. Furthermore, only s1/m2 strains varied in i-type; s1/m1 and s2/m2 strains were exclusively i1 and i2, respectively. This novel region determines vacuolating activity among these s1/m2 strains. More importantly, a significant correlation has been established between the i1 region and gastric cancer[45]. In contrast to Rhead *et al*[45], no disease association between *vacA* i genotypes and outcome was found in East Asian and Southeast Asian countries. More studies, from other countries, are needed to determine whether this region is a true virulence determinant[57].

The studies from Turkey, Iran and Iraq (non-Semitic countries) had a high prevalence of *vacA* s1 genotype of more than 70%, whereas strains from Semitic countries such as Egypt, Jordan, Saudi Arabia, Kuwait and Israel, had a low prevalence of less than 60%. The prevalence of *vacA* s1 genotype in the non Semitic countries was significantly higher than that in the Semitic countries[41,58].

Reports from Turkey, Iraq and Iran showed that *vacA* m2 was found in around 70% of typed strains. In Egypt, Saudi and Israel, the percentage of *vacA* m2 was between 85% and 92%. 51.1% of Jordanian strains typed *vacA* m2. *vacA* i region was studied in Iran and Iraq only. *vacA* i1 was associated with gastric cancer and gastric ulcer in Iran and Iraq, respectively.

Studies from Iraq, Kuwait, Jordan, Israel and Iran did not show any association between *vacA* s and m genotypes and gastroduodenal diseases[22,23,39,42,59]. In Iraq, an association with gastroduodenal diseases and *vacA* i-region genotype was shown. Studies from Iran and Turkey[44,60] reported a significant relationship between *vacA* s1 genotype and peptic ulcers. The *vacA* m1 genotype was linked to an increased risk for peptic ulcers in Turkey and Saudi Arabia[61,62].

Studies from Iran, Iraq, Jordan, Turkey and Israel have shown a significant association between *cagA* status and *vacA* s1, m1 and/or i1 genotypes[22,39,42,44,45]. In *cagA*-negative strains, most of the *vacA* genotypes were i2 genotypes[58].

Other virulence factors

***dupA*:** Recently, a novel virulence factor *dupA* (duodenal ulcer promoting gene A) (jhp0917-jhp0918) was shown to be associated with duodenal ulceration and increased epithelial cell interleukin-8 secretion[2]. The *dupA* gene is located in the region of the bacterial genome that encodes surface proteins. A significant relationship between *dupA* and duodenal ulcer was found, and the presence of *dupA* was associated with neutrophil infiltration. These findings, however, were not confirmed in a study of Brazilian children and adults[63], thus indicating possible geographic differences. More recently, it has been found that *dupA* was not significantly associated with duodenal ulceration in populations from Belgium, South Africa, China, and the USA, but was significantly associated with gastric cancer development[64]. In the Middle East, *dupA* was studied in Iraq and Iran. *dupA* was found to be associated with duodenal ulcers in Iraq but not Iran[39]. In addition, *dupA*-negative *H. pylori* strains were found to associate with pre-malignant lesions in Iran[65]. No other studies have been conducted in the Middle East. More studies are needed to address the prevalence of *dupA*-negative strains and the association between this gene and clinical outcome.

***iceA*:** *iceA* (induced by contact with epithelium) exists in allelic variants including *iceA*1 and *iceA*2. *iceA*1 only can be induced in the gastric epithelium. The *iceA*1-positive *H. pylori* strains were shown to be associated with peptic ulceration and increased mucosal IL-8 secretion, while a higher prevalence of *iceA*2 strains was found among patients with non-ulcer dyspepsia[66,67]. In Turkey, *iceA*1 was found to be positive in 32.2% of the strains[68]. In Jordan, analyses of virulence genes revealed that *iceA*2 (73.6%) was the predominant genotype[22]. In a study conducted in Saudi, it was shown that all ulcer cases were infected with *iceA*1, while 94.6% of gastritis and 90.9% of normal subjects were infected with *iceA*2[43]. In Israel, *iceA1* was found in 37% and *iceA*2 in 52% of cases. Both *iceA* alleles were found in 11%[42]. More research is needed to study *iceA* and its association with diseases in this region.

HISTOPATHOLOGICAL CHANGES

All strains of *H. pylori* induce a marked inflammation in the gastric mucosa which is characterised by neutrophil, lymphocyte and other inflammatory cell infiltration. While antral-predominant gastritis leads to increased acid production from the uninflamed corpus and predisposes to duodenal ulceration, corpus-predominant gastritis leads to hypochlorhydria and predisposes to gastric ulceration and adenocarcinoma[3]. In studies conducted in Iraq, Turkey and UAE, it was found there is antral-predominant mononuclear cell infiltration[59,69,70]. In a study conducted in Iran, where the gastric cancer rate is very high, it was found that mononuclear cell infiltration was similar throughout the stomach; on average, patients had pangastritis[71].

Gradual loss of gastric glandular tissue as a consequence of long term mucosal destruction is called atrophic gastritis[3]. The tissue damage may involve progressive loss of all specific mucosal cells including the acid pro-

ducing parietal cells, pepsinogen producing chief cells and mucus producing gland cells. When these cell types have shrunk, the protective mucus layer will gradually disappear and acid secretion will cease[5]. Such pathological changes increase the risk of gastric ulceration and development of gastric adenocarcinoma[72]. However, this protects against duodenal ulcers because of low acid secretion[5]. In a study conducted in Turkey, histological evidence of mucosal atrophy was found in 43% of *H. pylori*-infected subjects[72] while in another study in Turkey atrophy was found in 75% of the subjects[73]. In UAE, gastric atrophy in *Helicobacter* associated gastritis was seen in 54% of cases[70]. In a study conducted in Iran, where the gastric cancer rate is much higher than Turkey, histological evidence of mucosal atrophy was found in 39% and 22% of antral and corpus biopsies, respectively[74]. In Iraq, glandular atrophy was found in only one (3%) specimen taken from the antrum. In Kuwait, *H. pylori* were found in 81.7% patients, of which 28.3% had atrophic gastritis and 15.1% intestinal metaplasia[75]. Atrophy was found in 65% and 54% of examined subjects in Jordan and Egypt, respectively[24,76] (Table 1).

In a study conducted in Saudi where a comparison of Sydney scores from younger and older patients was made, no significant differences were seen in the scores of *H. pylori* density, neutrophilic activity, or chronic inflammatory cell infiltration between the two groups. While intestinal metaplasia was not found in any young patient, 22% of older patients had focal metaplastic changes. The atrophic changes were seen in 19% of older patients and one (3%) younger patient[77].

In Turkey, while a significant relationship was found between *cagA* positivity and neutrophil activity and glandular atrophy in antral specimens, corpus neutrophil infiltration was found to be more severe in the *vacA* m1 group than in the v*acA* m2 group[59]. No association between virulence factors and histopathology was found in Iraq[69]. In Iran, *dupA*-negative strains were associated with premalignant histological changes[65].

In most of the studies conducted in the Middle East, histological changes seen in the antral sections (such as neutrophil infiltration of the lamina propria and the glands and the increase in the number of lymphocytes and plasma cells) were on average of mild scores.

DIET IN THE MIDDLE EAST

Diet pattern correlates with gastric diseases. Most populations in the Mediterranean region (including Middle Eastern populations) adhere to the Mediterranean dietary pattern. Mediterranean food has several common features including low consumption of meat and animal products, a high consumption of fish, vegetables, fruit, and cereal, and olive oil as the main source of fat. A Mediterranean diet, particularly olive oil, vegetable and fruit consumption, has been shown to be related to a low risk of cardiovascular disease and several cancers including upper gastrointestinal tract cancer[78,79]. Whole grain is also related to low risk of gastric cancer[78]. In contrast, intake of refined grains by some Mediterranean populations was associated with increased risk of stomach cancer[78]. Overall, the Mediterranean dietary pattern is associated with better health and protects against various chronic diseases. After revision of most recently published papers about diet and food in the Middle East, I did not find any significant differences in the diet pattern amongst the countries of this region[80,81].

Table 2 Smoking prevalence in different countries (%)

	Males	Females
Saudi: Male Adult (30-70 yr and older), 1996-2001	19.1	8.3
Kuwait: Adult, 1996	29.6	1.5
Italy: Adult (15 yr and older), 2002	31.1	22.3
Iraq: Adult (16 yr and older), 1990	40	5
Jordan: Adult, 1999	48	10
Japan: Adult (15 yr and older), 2002	51	10
Iran: Adult (15 yr and older), 1999-2000	22.2	2.1
Egypt: Adult (15 yr and older), 2000	40	18
Spain: Adult (16 yr and older), 2001	39.1	24.6
Turkey: Adult (20 yr and older), 1997-1998	50.9	10.9
Israel: Adult (25-64 yr and older), 1999-2001	38.6	22.1
Costa Rica: Adult (20-49 yr and older), 2001	29	9.7

SMOKING IN THE MIDDLE EAST

The association between smoking and increased risk of gastric cancer has been observed. In an epidemiological study conducted in Portugal it was shown that smoking is associated with gastric intestinal metaplasia[82]. In other studies, it was shown that tobacco can increase the risk of gastric cancer[83], and epidemiological evidence linking smoking and gastric cancer has been found[84]. The prevalence of smoking in Iraq where the gastric cancer rate is very low is twice as the prevalence in Iran where the gastric cancer is very high. On the other hand, the highest prevalence was found in Turkey[85]. The prevalence of smoking in other Middle Eastern countries can be seen in Table 2.

GENERAL CONSIDERATIONS

H. pylori is a causative agent of peptic ulcer disease and an important risk factor of gastric cancer. Despite the high *H. pylori* infection rate in Middle Eastern countries, gastric cancer incidence is low in all countries but Iran. Previous reports have shown that the age of patients at the onset of infection may help predict the disease development process as the early acquisition of infection carries more risk of disease development. As shown above, *H. pylori* infection is acquired early in life in this region. In spite of this, the cancer rate is low.

In the Middle East, the populations can ethnically be divided into two main groups: Semitic populations (Arab and Jew who are southern region inhabitants) and non Semitic populations (Kurd, Turk, and Persian who are northern region inhabitants).

The prevalence of virulence factors in countries inhabited by non Semitic populations (Turkey, Iran and Iraq) is similar to what was found in South Asian and

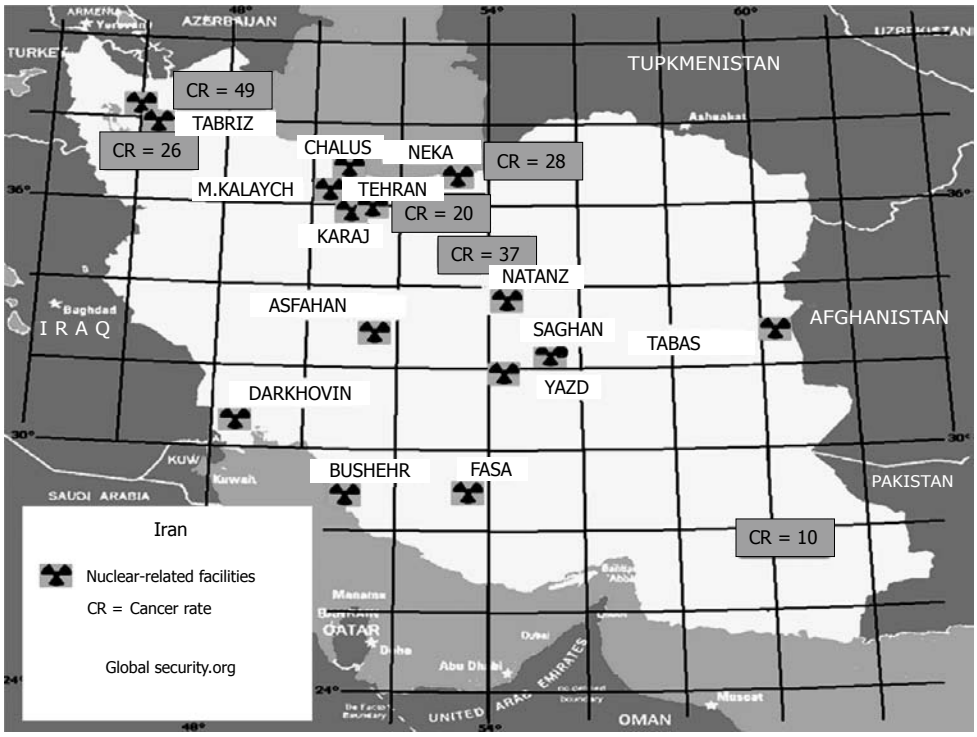

Figure 1 Map showing Iran's nuclear sites and annual incidence of gastric cancer in Iran reported from different cancer registries presented as male age standardized rate per 100 000.

Europe[41] and significantly more than what was found in countries with a Semitic background. This might be because populations which lived in the northern part of the Middle East near Europe and south Asia might correlate with European and south Asian populations. Additionally, this provides more evidence of the ethnical tropism of *H. pylori* infection. It is noticed that virulence factor rates in Saudi *H. pylori* are higher than other Arabic countries. This might be due the fact that Saudi society has been an open society due to continued population movement into this region to perform pilgrimage (Hajj).

Graham *et al*[86] suggested a better strategy for the explanation of the relationship between *H. pylori* and diseases by focusing on underlying patterns of gastritis. This, to a certain extent, is true in this region because all histopathology reports have shown that antral predominant gastritis is the main pattern of gastritis in this region apart from Iran where corpus or pan gastritis is the main pattern. However, if atrophic gastritis, which is precancerous and shown to be associated with cancer, is considered, the high cancer rate in Iran cannot be explained because atrophic gastritis is very high in Turkey, Jordan, UAE, and Egypt with much lower cancer rates. Yet another question to be answered: why this difference in gastritis pattern? Previous reports have blamed environmental factors and diet. For the sake of argument, one would accept the blame but which environmental factor can play such a role in a region which has almost the same environment and traditions. Moreover, as seen above, most Mediterranean countries (including Middle Eastern and South European countries) adhere to a Mediterranean diet pattern and neither diet pattern nor smoking rates seem to explain the difference in cancer rate. The only difference, however, that can be found between Iran and other countries (apart from Israel) is the presence of nuclear facilities in Iran. Examining the map of Iran's nuclear sites, Iranian uranium mines, nuclear reactors, and uranium processing facilities that include three known uranium enrichment plants can be found in Iran's most populous urban areas especially in the northwest and central region where there is a very high gastric cancer rate (Figure 1)[87]. On the other hand, there is no nuclear facility in the southeast where the gastric cancer rate is similar to that found in neighboring countries[87]. In Israel, these facilities are in the desert and relatively isolated areas. But if the theory of the relationship between those facilities and cancer is true, one would expect high rates of other cancers in Iran and this is not true[28].

Host genetics play an essential role in the inflammatory process and in the interactions between the host and *H. pylori* (see review by Kusters *et al*[88]). It has been shown that proinflammatory genetic polymorphisms tend to increase the risk of development of gastric cancer. Hence, would the genetic make-up explain the dilemma of cancer rate in this region? Proinflammatory host genetic facilitates gastric cancer through the development of hypochlorhydric and atrophic gastritis which has been studied and could not have explained the difference in cancer rate. Graham *et al*[86], suggested that host genetics affect individuals and generally cannot explain widespread changes. In addition, the genetic markers we have at present are not sensitive or specific enough to form the basis of a screening strategy[89]. According to Canedo *et al*[89], any genetic association studies should fulfil specific criteria among which there should be no evidence of population admixture. This criterion is almost impossible to fulfil in this region. When looking back through history, you will find that Indo-European races (Kurd and Iranian) are not indigenous. In addition, after the rise of Islam and the Arab conquest of the surrounding countries, there was much

intermixture between Indo-European and Semitic populations. Further evidence for this intermixture is that in a study of human genetics, a close relatedness of Semitic and Indo-Europeans with each other and with neighbouring geographic groups was shown. In the same study it was shown that Semitic North African groups are more distant genetically from Semitic-speaking groups from the Near East and Iran[90]. Hence, avoiding a mixed population is insuperable in this region. However, this does not negate the importance of the host genetic analyses of cytokine polymorphisms affecting mucosal inflammation and gastric acid secretion. Carefully planned projects would provide additional information to identify predictive markers for an individual's risk for gastric atrophy and malignancy.

CONCLUSION

To conclude, there is unexplained variation in the distribution of virulence factors and gastritis patterns in the Middle East. These variations fail to explain the discordance between *H. pylori* infection rates and the variations in gastric cancer prevalence. Although detailed studies are needed to investigate dietary pattern, generally diet is unlikely to contribute because all the countries are following the same Mediterranean pattern. Smoking rates could not explain the variation in cancer rates as we have seen countries with a very high smoking rate but low cancer rate. This might indicate the presence of an enigma similar to or part of that reported (controversially) in Africa and Asia[91,92]. Further detailed investigations and international comparative studies of each risk factor need to be performed to investigate whether this phenomenon represents a true enigma.

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S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3235

World J Gastroenterol 2010 July 14; 16(26): 3235-3238
ISSN 1007-9327 (print)

GUIDELINES FOR BASIC SCIENCE

Two-layer cold storage method for pancreas and islet cell transplantation

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Received: March 19, 2010 Revised: April 19, 2010
Accepted: April 26, 2010
Published online: July 14, 2010

Abstract

The two-layer cold storage method (TLM) was first reported in 1988, consisting of a perfluorochemical (PFC) and initially Euro-Collins' solution, which was later replaced by University of Wisconsin solution (UW). PFC is a biologically inert liquid and acts as an oxygen-supplying agent. A pancreas preserved using the TLM is oxygenated through the PFC and substrates are supplied by the UW solution. This allows the pancreas preserved using the TLM to generate adenosine triphosphate during storage, prolonging the preservation time. In a canine model, the TLM was shown to repair and resuscitate warm ischemically damaged pancreata during preservation, improve pancreas graft survival after transplantation, and also improve the islet yield after isolation. Clinical trials using the TLM in pancreas preservation before whole-pancreas transplantation and islet isolation have shown promising outcomes. We describe the role of the TLM in pancreas and islet transplantation.

Key words: Two-layer method; Pancreas preservation; Pancreas transplantation; Islet transplantation; Perfluorochemical

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Fujino Y. Two-layer cold storage method for pancreas and islet cell transplantation. *World J Gastroenterol* 2010; 16(26): 3235-3238 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3235.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3235

INTRODUCTION

Successful transplantation depends on multiple factors including the prevention of preservation injuries incurred by cold storage and reperfusion[1,2]. A shortage of donor organs has an impact on the development of preservation methods. We developed the two-layer cold storage method (TLM) using perfluorochemicals (PFC) to reduce ischemic injury and maintain cellular integrity during preservation.

In 1966, it was reported that mice could breathe and survive in oxygen-saturated PFC[3]. PFC is a hydrocarbon, comprising a colorless and odorless solution with specific gravity approximately twice that of water. The most interesting property of PFC is a very high capacity for dissolving respiratory and other nonpolar gases[4]. A negligible O_2-binding constant of PFC allows them to release O_2 more effectively than hemoglobin into the surrounding tissue. As a result of this unique property, PFC-based solutions have been examined as oxygen carriers for blood substitutes, myocardial protection, respiratory support[4], and organ preservation before transplantation[5].

In this editorial, the role of the TLM in pancreas and islet transplantation is discussed.

DEVELOPMENT OF TWO-LAYER COLD STORAGE METHOD

The TLM was first reported in 1988 at Kobe University as a pancreas cold storage method[5]. The TLM first consisted of a PFC and initially Euro-Collins' solution (EC), which was later replaced by University of Wiscon-

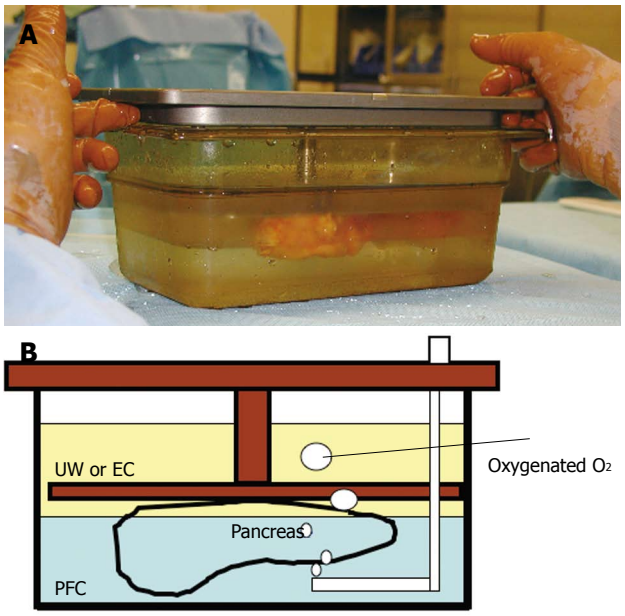

Figure 1 The two-layer method. The pancreas graft is surrounded by UW or EC and floated on the oxygenated PFC. A: The photograph; B: The schema. UW: University of Wisconsin solution; EC: Euro-Collin's solution; PFC: Perfluorochemical.

sin solution (UW)[6]. Because the PFC is lipophilic and has a high density, the PFC and EC or UW separate into layers. This allows for the pancreas graft to float on the oxygenated PFC surrounded by EC or UW (Figure 1).

Before UW was developed, EC was the standard solution for pancreas preservation before whole-pancreas transplantation. EC could preserve human pancreata for up to 10 h[7] and canine pancreata for up to 24 h[5]. The original TLM (EC/ PFC) could preserve canine pancreata for up to 72 h[5]. UW solution could preserve canine pancreata for up to 72 h[6], and the modified TLM (UW/ PFC) could preserve canine pancreata for up to 96 h[6]. Although PFC alone may provide an excellent preservation solution, this solution only preserved canine pancreata for 48 h[8]. Despite evidence for the superiority of the TLM, simple cold storage using UW has become the standard organ preservation method in clinical pancreas transplantation. This is likely to be because UW effectively preserves human pancreata for more than 24 h and the TLM is relatively more complicated compared with UW cold storage.

MECHANISM OF THE TWO-LAYER METHOD

The TLM reduced cold ischemic[6], warm ischemic[9], rewarming ischemic[10], and reperfusion[11] injuries in pancreas and islet transplantation. The mechanisms of the TLM have been vigorously examined using experimental transplantation models. During preservation by the TLM, the pancreas is directly oxygenated through PFC and maintains an oxygen tension at about 60% of the normal physiologic level[12].

During ischemia, tissue adenosine triphosphate (ATP) degrades to hypoxanthine following an increase of xanthine oxidase. Next, reactive oxygen radicals are generated and involved in a complex interaction of immediate cellular damage in ischemia-reperfusion injury[13,14]. On the other hand, tissue ATP is maintained in grafts preserved by the two-layer method and is rapidly recovered after reperfusion, which may be a better prophylaxis and treatment for the first step of ischemia-reperfusion injuries. During preservation by the TLM, pancreas grafts continuously generate ATP for up to 96 h[6]. ATP levels are also enhanced in human pancreas stored using the TLM. This proves the ability of the TLM to oxygenate human pancreas[15,16]. The ATP generated is used to drive a sodium-potassium pump to maintain cell integrity; thus, the TLM prevents pancreas swelling more effectively than UW solution[17]. Furthermore, the TLM improves the viability of the vascular endothelium and microcirculation[18]. Although the mechanisms involved in reducing ischemic reperfusion injury are unclear, one possibility is the induction of heat shock proteins during preservation[11]. ATP is essential for protein synthesis, and we demonstrated that protein synthesis was likely to be involved in the process of postischemic cellular recovery during preservation by the two-layer method[11,19]. These heat shock proteins may work to provide an anti-reperfusion or cell repair property.

The TLM also has an excellent ability to resuscitate pancreas grafts that have suffered from warm ischemic injury and to prolong the preservation time of ischemically damaged pancreata[20]. After 90 min of warm ischemic injury, the canine pancreas grafts lost ATP and were no longer viable. However, when the damaged pancreata were preserved and resuscitated by the TLM for 24 to 48 h at 4℃, the grafts regained tissue ATP and became viable[19]. The resuscitation effect is associated with active protein synthesis, suggesting active cell repair, and may involve heat shock proteins[19]. This suggested the possibility of pancreas transplantation from ischemically damaged or non-heart-beating donors.

Resuscitation by the TLM before pancreas transplantation correlates with the pancreatic tissue ATP levels after the TLM[21]. This is important because it predicts the outcomes of marginal donor grafts before transplantation. Recently, the TLM has shown promise in small intestine[22] and heart preservation[23].

PANCREAS PRESERVATION BEFORE ISLET ISOLATION

The most common exclusion criteria of pancreata for islet isolation are an unstable blood pressure, significant downtime, and high-level vasopressor usage for donor management. All of these criteria are related to warm ischemic injury and can be alleviated using the TLM; thus, the TLM could potentially make the majority of the donor pancreata usable for islet isolation.

Tanioka *et al*[24] first reported islet isolation after preservation by the TLM in a canine pancreas model. After

24 h storage of the canine pancreas in UW solution, islet yields were significantly decreased and the posttransplant outcome was further deteriorated. However, after 24 h of preservation by the TLM, the islet yield and posttransplant outcome were essentially equal to the immediate isolation.

After 60 min of warm ischemia, the islet yield after islet isolation was significantly decreased compared with the islet yield from the pancreas without warm ischemia[25]. When pancreata ischemically damaged for 60 min were preserved using the TLM for 24 h, the level of islet yield became similar to that from pancreata without warm ischemia[26]. Tanaka *et al*[27] also demonstrated that pancreata damaged on 30 min warm ischemia were restored by 3 h TLM preservation. The TLM may facilitate the selective use of non-heart-beating donors as an alternative source for islet transplantation.

In the rat model, the TLM for pancreas preservation prior to islet isolation resulted in an excellent islet function in addition to improved islet yield, which was almost comparable to freshly isolated islets[28].

At the University of Alberta (Edmonton, Canada), it was observed that seven of seven patients with type 1 diabetes receiving allogeneic islet transplants became insulin independent[29]. Two major drawbacks of the Edmonton protocol are that it requires pancreata to have short preservation periods before islet isolation, and almost always requires two or more donor pancreata to cure one diabetic patient.

On the basis of these data, Matsumoto *et al*[30] examined the effect of the TLM on the pancreas before islet isolation using preclinical, nonhuman primate pancreata and human pancreata. In the primate model, storing pancreata in UW solution for 5 h yielded a similar islet number, viability, and *in vitro* function compared with immediate isolation. However, 5 h of TLM preservation significantly increased the islet yield, viability, and *in vitro* function. They also suggested that the TLM maintains or repairs exocrine cell integrity and prevents trypsin activation, which enables effective collagenase delivery and protects islets from enzymatic digestion.

Zhang *et al*[31] also demonstrated that the islet yield from pancreata preserved using the TLM was more than that from pancreata stored in UW solution. Islet viability was significantly higher with the TLM *vs* UW solution. This experiment confirmed the superiority of the TLM-based preservation of pancreata for human islet isolation compared with UW solution. Although the beneficial effect of the TLM is controversial, the overall performance of the TLM could improve the outcome of islet isolation and transplantation[32].

CLINICAL APPLICATION OF THE TWO-LAYER METHOD

The TLM was clinically employed for pancreas transplantation at the University of Minnesota for the first time in 1999[33]. In this first clinical trial involving 10 cases, the TLM had no adverse effect on the recipients after transplantation. Furthermore, the morphologic quality of the human pancreas grafts after reperfusion was excellent compared with the pancreata stored in UW solution. There was no episode of acute rejection of pancreata preserved using the TLM. This is interesting because the immunosuppressive property of PFCs was one of the reasons not to use them as a blood substitute[34]. This immunosuppressive property could be beneficial for allogeneic organ preservation, and the role is under investigation.

Pancreas preservation using the TLM before islet isolation for clinical islet transplantation began at the University of Minnesota[35]. Hering *et al*[35] reported the cure of four patients with islets from a single donor. In this report, cadaver pancreata were subjected to the TLM immediately after retrieval at the procurement site without UW solution storage before islet isolation. Their islet yield was sufficient to cure one patient from one donor. Their experiment suggests that pancreata should be preserved by the TLM immediately at the time of procurement.

CONCLUSION

Irrespective of these promising data, the role of the TLM requires further investigation in pancreas and islet transplantation.

ACKNOWLEDGMENTS

The author would like to thank Dr. Yoshikazu Kuroda for his advice.

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S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3239

World J Gastroenterol 2010 July 14; 16(26): 3239-3248
ISSN 1007-9327 (print)

REVIEW

Interstitial cells of Cajal, the Maestro in health and disease

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Received: April 12, 2010 Revised: May 15, 2010
Accepted: May 22, 2010
Published online: July 14, 2010

Abstract

Interstitial cells of Cajal (ICC) are important players in the symphony of gut motility. They have a very significant physiological role orchestrating the normal peristaltic activity of the digestive system. They are the pacemaker cells in gastrointestinal (GI) muscles. Absence, reduction in number or altered integrity of the ICC network may have a dramatic effect on GI system motility. More understanding of ICC physiology will foster advances in physiology of gut motility which will help in a future breakthrough in the pharmacological interventions to restore normal motor function of GI tract. This mini review describes what is known about the physiologic function and role of ICCs in GI system motility and in a variety of GI system motility disorders.

Key words: Interstitial cells of Cajal; Gastrointestinal motility; Peristalsis

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Mostafa RM, Moustafa YM, Hamdy H. Interstitial cells of Cajal, the Maestro in health and disease. *World J Gastroenterol* 2010; 16(26): 3239-3248 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3239.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3239

INTRODUCTION

Physiology of gut motility has been always a fascinating chapter in gastroenterology. It poses interesting challenges to physiologists. Our understanding of basic gut motility processes is advancing; a major emphasis is placed on elucidating gut regulatory mechanisms. A better appreciation of the importance of the presence of normal function of interstitial cells of Cajal (ICC) transformed this field of research. In 1893, Spanish Nobel Laureate physician and neuropathologist Santiago Ramon y Cajal, was the first to describe cells that are located between the nerve endings and smooth muscle cells in the gastrointestinal (GI) tract. Their location prompted him to call them "interstitial". They are now known as the ICC.

ICC may be considered to be a specialized population of smooth muscle cells. Both arise from common mesenchymal cells[1-3]. However, whereas smooth muscle cells develop an extensive array of contractile elements, ICC have few contractile elements but contain large numbers of mitochondria, an abundance of endoplasmic reticulum and distinct sets of channels in their membrane. The ICC consist of a fusiform cell body with a thin cytoplasm, a large oval nucleus and dendritic-like processes[4]. Two to five primary dendritic processes divide further into secondary and tertiary processes[5]. Many ICC express Kit, a tyrosine kinase receptor (Kit-ir); this allows them to be recognized by their ability to bind antibodies to Kit[4]. Similarly ICC readily react with

antibodies to vimentin whereas nearby smooth muscle cells do not[6]. The presence of ICC is not restricted to the GI tract. They can be found in the bladder[7,8], the ureteropelvic junction[9], the vas deferens[10], the prostate[11], the penis[12,13], the mammary gland, the uterus[14], the pancreas[15], blood vessels[16] such as the portal vein[17] and the vagina[18]. More recently, they have been found in the vermiform appendix in childhood[19]. Some of these cells are thought to have a pacemaker function (such as those in the portal vein, in the lymphatics or prostate) but not those in the arteries, uterus (where the influence is, if any, an inhibitory one) or bladder[20].

The motor activity of the GI tract is critical for life[21]. It is a complex process involving multiple cell types such as enteric neurons that can sense the contents of the GI tract, integrate information and devise a suitable motor pattern, ICC that transduce inputs from enteric motor neurons and generate intrinsic electrical rhythmicity, and smooth muscle cells that can interpret and integrate large arrays of inputs and develop appropriate responses[22]. ICC are a minor component of the tunica muscularis of the GI tract (only about 5% of cells present[23]); however, these cells have very significant physiological roles in GI motility[22].

Many tissues, isolated from different regions of the GI tract, contract rhythmically in the absence of neuronal or hormonal stimulation. When contractions and membrane potential are recorded simultaneously each contraction is seen to be triggered by a long lasting wave of depolarization: because of their low frequency of occurrence and long duration, the waves of depolarization have been termed slow waves[24]. The origin and basis of the generation of slow waves have been debated for many years. It was initially thought that the generation of slow waves reflected some properties of GI smooth muscle cells[25,26], but studies on isolated smooth muscle cells have consistently failed to demonstrate a capability to generate slow wave activity[27]. It has also long been recognized that the generation of slow waves does not rely on the sequential activation of voltage-dependent ion channels as do cardiac pacemaker cells. Rather, many early studies raised the possibility that rhythmical activity relied on the cycling of one or more metabolic processes within cells of the gut wall. Thus Conner and his colleagues proposed that the generation of slow waves involved changes in the activity of the sodium pump[25].

Subsequently Nakayama *et al*[28] suggested an involvement of glycolytic pathways, again assuming that pace making activity originated in smooth muscle cells. Although ICC were first described in the intestine a century ago by Cajal, they were long viewed as an oddity. Their role in the generation of pacemaker activity in the GI tract was suggested on the basis of histological studies[29]. More recently, studies on mutants that lack subpopulations of ICC revealed their role in the generation of rhythmicity[30].

Critically, whereas isolated smooth muscle cells rarely generate spontaneous electrical activity[27], isolated ICC invariably do[31,32].

STRUCTURAL ORGANIZATION AND IDENTIFICATION OF SPECIFIC POPULATIONS OF ICC

The discovery that ICC express *c-Kit*, the proto-oncogene that encodes the receptor tyrosine kinase Kit has offered a simple and reliable immunohistochemical method for determining the structure and distribution of ICC networks[30]. ICC are found throughout the GI tract from the esophagus to the internal anal sphincter[33,34]. Hanani *et al*[35] mentioned that while it is becoming clear that more than one type of ICC exists, based on both morphological and functional data, we still subdivide ICC based on location. Furthermore, Farrugia[36] emphasized the importance of revisiting a classification based solely on location and move towards a classification that is based on function, suggesting a reasonable start, to subdivide ICC into those that have the machinery to, and generate, unitary potential and slow waves and those that do not. Morphological studies now supported by some functional evidence suggest that at least three separate functional groups of ICC exist. In most regions of the GI tract, a network of ICC are located within the intermuscular space at the level of the myenteric plexus (ICC-MY) between the circular and longitudinal muscle layers. ICC-MY are the pacemaker cells in the stomach and small intestine that trigger the generation of slow waves in the tunica muscularis[37].

A second population of ICC (referred to as intramuscular ICC or ICC-IM) are found within the muscle layers of the GI tract and are innervated preferentially by enteric motor nerves[37]. ICC-IM are closely associated with not only enteric motor nerves but also vagal afferent nerves. Vagal afferent nerve fibers, labeled by the injection of neural tracers into the nodose ganglia, can terminate as intramuscular arrays within the musculature and as intraganglionic laminar ending within the myenteric ganglion of the stomach and duodenum. These afferent fibers transmit mechanoreceptive information from the muscle wall[38,39]. Horiguchi *et al*[40] gave histological evidence that a third population of ICC, ICC-SEP, lies within the septa between the circular muscle bundles, and suggested that it may play a role in conducting electrical information from ICC-MY deep into the distant circular muscle bundles, Figure 1 showing functional organization of ICC in the canine gastric antrum[41].

Electrophysiological data are presented which indicate that when the normal pathway from ICC-MY is sectioned, electrical stimulation of the cut ends of the muscle bundles can initiate slow waves over considerable distances. In the absence of stimulation, the muscle bundles isolated from ICC-MY can generate rhythmical activity but do so at low frequencies. Thus a distinct population of ICC, ICC-SEP, exists which can transfer pacemaker depolarization from ICC-MY deep into the distant bundles of circular muscle. Although ICC-SEP have the potential to generate pacemaker activity they are not normally the dominant pacemaker centre. As an analogy with the

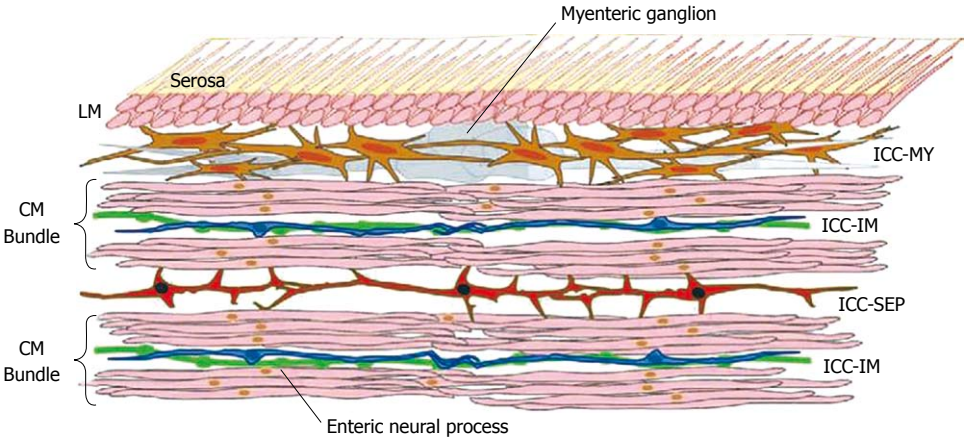

Figure 1 Diagram showing functional organization of ICC in the canine gastric antrum[41]. ICC: Interstitial cells of Cajal.

generation of pacemaker activity in the heart, the plexus of ICC-MY, like the sino-atrial node, is the dominant pacemaker centre. ICC-SEP, like Purkinje fibers, have the potential to generate pacemaker activity, but normally function to convey electrical activity from the dominant pacemaker region to more distant tissues[41].

PHYSIOLOGICAL FUNCTIONS OF ICC

Peristaltic motor activity is a motor pattern orchestrated by complex sequencing of neural excitation and inhibition in cooperation with intrinsic muscular control mechanisms, including those residing in ICC[42]. Peristalsis is defined as waves of contraction propagating along the GI tract for various distances as a means of mixing and propelling its content distally. Both the type of neural activity and the type of intrinsic myogenic control mechanism differ widely throughout the GI tract[42]. Physiological activation of peristalsis will in most cases involve the stretching of a segment of stomach, intestine, or colon and it will occur by neural pathways that contain additional mechanisms to those required for the ascending excitatory reflex[43]. When peristaltic motor activity occurs, in particular in stomach and proximal small intestine, the waves of contraction always have rhythmicity to it. This rhythmicity is determined by electrical slow wave activity in the musculature, referred to as pace maker activity[44].

New reagents, coupled with immunohistochemical techniques and new electrophysiological experimental approaches opened the door to recent progress in identification of the important roles of ICC as pacemakers, in propagation of slow waves and as mediators of inputs from enteric motor neurons[45]. Other functions, such as mechanosensors have also been proposed, but little physiological evidence supporting this function has been published[45].

Laboratory approaches used for ICC study

Isolated ICC have been examined using conventional patch clamp recording techniques. This approach, which has been applied to ICC-MY, allows a description of the specific populations of ion channels present in their membrane[31,32,46] and an analysis of the cellular mechanisms which regulate the channels[47,48]. Simple intracellular recording from smooth muscle cells in isolated segments of GI tissues and isolated segments of urethra, after blocking smooth muscle L-type Ca^{2+} channels, record primarily the activity of the ICC in the tissues. The properties of ICC-MY can be determined *in situ* using sharp electrodes, allowing one to monitor the behavior of populations of interconnected ICC-MY and to determine how pacemaker potentials generate signals in adjacent smooth muscle layers[47,49,50]. A third method used to study the properties of ICC IM involves recording from small isolated segments of circular muscles; if dissected appropriately the preparations are isopotential and contain up to 2000 smooth muscle cells linked to up to 200 ICC-IM. The membrane potential of both smooth muscle cells and ICC-IM, can be varied over a limited range and the effects of nerve stimulation can be analyzed[51-53]. Finally, the use of mutant mice in which specific sets of ICC are either absent of dramatically reduced in numbers has allowed an evaluation of the physiological properties of tissues, with and without different sets of ICC[24].

PATHOPHYSIOLOGY OF ICCs

Many GI motor disorders can be related to changes in number and/or structure and/or density of ICCs[54-57]. These changes can be primary, due to toxin substances, neurotoxins or viral diseases, or secondary as a consequence of neural damage, degraded tissue or treatment effect[54,58].

An absence or reduction in the number of ICCs causes abnormal electrical slow waves causing a decreased contractility of smooth muscle cells resulting in a diminished intestinal transit[56]. Although the density decreases, the slow wave is still present in most affected patients but the frequency and duration are prolonged[54].

COMMON GI MOTILITY PROBLEMS

ICC in the human esophagus and cardia

ICC in human esophagus has a myoid ultrastructure with abundant smooth endoplasmic reticulum, numerous mitochondria, intermediate filaments, scattered caveolae, and discontinuous basal lamina. They are most frequent in the esophageal part of the lower esophageal sphincter (LES)

but rare in the gastric part. They are in close contact with nerve terminals and make specific junctions with smooth muscle cells[59,60].

Achalasia: Achalasia is a disorder of esophageal motility that has been well documented for over 300 years[61]. Achalasia is characterized by relaxation failure of the LES and lack of peristaltic contraction of the esophageal body[62]. The mechanism of LES relaxation is complex, requiring the coordinated interaction of nerves, smooth muscle, ICC and hormones. The LES is a functional and anatomic barrier between the stomach and esophagus. It consists of a thickening of the circular smooth muscle layer of the esophagus at the gastroesophageal junction. It is anatomically asymmetric, and this is reflected in the physiology of the sphincter as demonstrated by ultrasound and pharmacologic manometric studies[63]. The LES is tonically contracted. Initiation of a peristaltic wave in the esophagus is accompanied by a decrease in LES pressure as a result of smooth muscle relaxation. This allows the swallowed bolus to enter the stomach[61].

ICC involvement in achalasia is debated. Electron microscope studies of the muscle coat of the LES in seven patients with achalasia showed that muscle wall components (nerve endings, smooth muscle cells, ICC and connective tissue) were modified. ICC ultrastructure was altered, namely clear cytoplasm, fewer mitochondria, and scarce smooth endoplasmic reticulum[64].

A reduced number of contacts between nerves and ICC were reported. Specific changes in smooth muscle cells were also documented, whereas the nerve endings had abnormal ultrastructure. Alterations in older patients were more pronounced[65]. Since the LES components specifically altered in achalasia are the nerve endings and ICC, they are regarded as principally responsible for abnormal motility[65].

Achalasia is uncommon among the pediatric population. It is usually sporadic and affects mainly teenagers[66]. A rare familial form combining early onset achalasia of cardia, alacrymia (absence of tears), and ACTH insensitivity, are known as Allgrove's syndrome[67,68] or "Triple A" syndrome[69]. These forms are inherited on the autosomal recessive mode[70]. Massive loss of neural elements and neuronal nitric oxide synthase as well as a marked fibrotic process of the muscle layers of the cardia have been observed in "Triple A" syndrome[71]. ICC in the cardia are also markedly diminished or are completely absent while ICC (and neural structures) are preserved in the pylorus[59].

Gastroesophageal reflux: Gastroesophageal reflux (GERD) is a common condition and its prevalence varies in different parts of the World[72]. Typical symptoms of heartburn and acid regurgitation are encountered in 15%-20% of the general population[62]. The major mechanism for GERD is transient relaxation of the LES[73]. The role of the ICC in inhibitory transmission in the LES is still being discussed[62].

In W/Wv mutant mice (lack of ICC) LES pressure was lower than wild-type mice but a normal swallow still induced LES relaxation, arguing against the role of ICC in inhibitory transmission[74]. Another study demonstrated that in W/Wv animals, cholinergic and nitrergic neurotransmission is greatly reduced pleading for the role of ICC in mediating neural inputs[37]. However, enteric neurons, varicose processes, and the ability to release neurotransmitters are not reduced, and smooth muscle cells demonstrate responsiveness to exogenous transmitters[37].

Loss of ICC during development or in pathologic conditions would significantly compromise the ability of GI muscles to generate typical motor reflexes[75].

Esophagitis itself may be at the origin of an alteration of normal function of the Cajal cells: in advanced stages of GERD, inflammatory changes in the esophageal wall will also involve the ICC. That way, the more severe the esophagitis, the more severe is the ICC impairment. This destruction leads to loss of effective contraction of esophagus, maintaining reflux and thus aggravating the symptoms[76].

ICC in the human stomach and pylorus

Gastroparesis: The pathogenesis of gastroparesis is complicated and poorly understood. This lack of understanding remains a major impediment to the development of effective therapies for this condition. Most of the scientific information available on the pathogenesis of gastroparesis has been derived from experimental studies of diabetes in animals. These studies suggest that the disease process can affect nerves (particularly those producing nitric oxide, but also the vagus nerve), ICC and smooth muscle[77]. It is broadly defined as disordered gastric emptying, and is a commonly encountered clinical problem[78]. Delayed gastric emptying can be secondary to muscular, neural, humoral causes or use of anticholinergic and opiate medicines. In the absence of an identified cause, gastroparesis is termed as idiopathic[79]. Gastroparesis has a broad range of clinical presentations ranging from dyspeptic symptoms to nausea, vomiting, abdominal pain, malnutrition, frequent hospitalizations and incapacitation[80], chronic abdominal pain and vomiting leading to dehydration, electrolyte imbalance, nutritional impairment and weight loss[81].

The ICC are fundamental in the generation of gastric slow waves[79]. A decrease in ICC density ranging from 60%-100% depending on the area investigated was demonstrated in histologic studies of the stomach of type 1 diabetic patients[55]. The number of immunopositive cells for c-kit was significantly decreased in the corpus and antrum of the gastroparesis patients compared with control tissues[62]. The loss of intramuscular ICC and associated nerves in the gastric fundus could explain the low basal gastric tone and increased compliance of the stomach. The hypomotility of the antrum can also be explained by the absence of slow wave generation by the ICC[23].

Infantile hypertrophic pyloric stenosis: Infantile hy-

pertrophic pyloric stenosis (IHPS) is common in infants, characterized by marked delayed gastric emptying and hypertrophy of the inner (circular) muscle layer of the pylorus[59]. IHPS has been known for more than a century[82] but it remains a puzzling disorder[83]. The genetic susceptibility to development of IHPS seems to be multifactorial[84]. Hypertrophy of the pyloric musculature develops after birth[85] and produces the characteristic palpable pyloric "olive." The pyloric lumen is however not fully occluded[86] and can be intubated relatively easily[87], suggesting that the obstruction of the gastric outlet in IHPS is not merely due to a mechanical obstruction by the hypertrophied musculature. The extent of muscle hypertrophy appeared to be unrelated to the age or duration of symptoms[88].

Various neurotransmitters[89-91] and the neuronal isoform of NO synthase[92] are reduced or lacking in the hypertrophic musculature. The increased thickness of the pyloric muscular coats appears to be due to hypertrophy, rather than to hyperplasia, of the smooth muscle cells[93]. ICC, identified either by electron microscopy[94] or by Kit-ir[95,96] were consistently lacking in the hypertrophic circular muscle layer. However, Kit-ir cells, similar to Kit-ir ICC observed in controls, were observed in the innermost part of the hypertrophic pylorus and in the antrum, indicating that the lack of Kit-ir is restricted to the hypertophic pyloric musculature[95].

The lack of ICC in IHPS may interfere with the propagation of slow waves and may be, at least partly, involved in antro-pyloric incoordination[59]. Homozygous transgenic mice carrying inactivated genes ("knock-out") coding for the neuronal NO synthase developed hypertrophy of the pylorus[97]. The link between the lack of ICC, the lack of inhibitory nitrergic neurotransmission, and the hypertrophy of the smooth musculature in IHPS remains to be elucidated[59].

Small intestine and colon

Hirschsprung's disease: Hirschsprung's disease (HD) is characterized by the lack of intrinsic enteric nervous system (ENS) in the distal part of the GI tract ("aganglionosis"). The affected segment extends cranially from the anus and encompasses a variable portion of the gut. Functionally, the lack of propulsive movements may lead either to an early obstructive syndrome in infancy or to a severe constipation[98]. Lack of slow wave activity in the aganglionic segment has been identified[99]. Kit immunohistochemistry identified ICC in HD. However, the cellular density of Kit+ ICC appeared markedly reduced in the aganglionic segment[100]. ICC-MP were rather abundant in the (aganglionic) space between the muscle layers. Kit+ ICC were specially scarce in the inner part of the circular musculature and in the submuscular plexus. However, the presence of some ICC-SMP was confirmed by electron microscopy.

In contrast, another study reported a distribution of Kit+ ICC in HD comparable to controls and claimed that Kit1 ICC-MP form "normal" networks in aganglionic segments when studied by confocal microscopy on whole mount preparations[101].

Differences in interpretation may be less significant than it appears as there is an agreement in the literature to acknowledge the presence of a number of interconnecting ICC-MP in aganglionic segments but there is no objective criterion to assess the "normality" of networks. Considering the very close relationships of ICC with intrinsic nerves and glial cells in the normal gut, a normal arrangement of ICC appears quite unlikely in the absence of both intrinsic nerves and glial cells as encountered in aganglionic segments. In the embryonic chicken[1] or mouse[102] gut experimentally deprived of neural crest derivatives, ICC develop in the absence of ENS, confirming the mesenchymal nature of ICC. But it has not been established if ICC fully develop morphologically and functionally in such conditions.

HD is a heterogenous, multigenic disease and reviewing its genetic aspects is beyond the scope of this paper. Several systems regulating neural crest migration have recently been identified[103]. Some genes are expressed by the neural crest, others by the mesenchyme of the gut. Kit has previously been considered as a possible candidate in the search for genes involved in hereditary forms of HD[104] but the absence of linkage between HD and the region of the *Kit* gene has been more recently reported[105]. The genetic defects leading to aganglionosis in the HD patients enrolled in all studies on ICC published so far have not been assessed. Subtle differences may explain the discrepancies observed between studies, and a link between some specific genetic defect leading to aganglionosis and the differentiation of ICC in HD patients cannot be ruled out.

Intestinal neural dysplasia: A clinical condition that resembles HD was first described by Meier-Ruge[106] in 1971 as a malformation of the enteric plexus. In 1983, Fadda *et al*[107] subclassified intestinal neural dysplasia (IND) into two clinically and histologically distinct subtypes. Type A occurs in less than 5% of cases, is characterized by congenital aplasia or hypoplasia of the sympathetic innervations, and presents acutely in the neonatal period with episodes of intestinal obstruction, diarrhea and bloody stools. The clinical picture of Type B resembles HD and is characterized by malformation of the parasympathetic submucous and myenteric plexuses and accounts for over 95% of cases of isolated IND. IND occurring in association with HD is of Type B[108]. IND have been reported to be associated with loss or deficiency of ICC networks[109].

Chronic intestinal pseudo-obstruction: Chronic intestinal pseudoobstruction (CIPO) is characterized by defective GI propulsion together with symptoms and signs of bowel obstruction in the absence of any lesions or mechanical obstacle[110]. It is generally a serious, even life-threatening, condition with frequent need for long-term parenteral nutrition. CIPO can either be restricted to the intestine, can involve other parts of the GI tract, or can be part of

a multisystemic disorder[59]. CIPO can be secondary to a number of identified disorders or can be "idiopathic"[111]. Very little is known about the etiology of idiopathic CIPO. Pathological features of CIPO are pleiomorphic. A number of alterations of the ENS ("neuropathic" forms)[112] and "myopathic" forms, limited to the musculature of the GI tract or involving also the musculature of the urinary system[113,114], have been described.

Slow transit constipation: Functional constipation encompasses a group of functional disorders that exhibit persistent difficult, infrequent, or seemingly incomplete defecation and infrequent, lumpy, or hard stools[115,116]. This symptom is very common and may occur in up to 20% of populations, depending on demographic factors, sampling, and the definitions employed[115,117]. The term constipation is probably better viewed as a sort of semantic umbrella, covering pathophysiologic subtypes, among which 2 major groups may now be identified: slow transit constipation (STC) and pelvic floor dysfunction[118].

STC is thought to have, as a primary defect, slower than normal movement of contents from the cecum to the rectum[119]. This is a very prevalent motility problem, but its mechanisms are unclear[62]. Although STC may not be a congenital disease, the frequent onset in adolescence and strong female predominance suggest that STC could be a result of a sex modified multifactorial disorder of the GI tract with a genetic basis[120].

ICC volume was significantly lower in the STC patien^{+} cross all colonic regions[121]. Expression of *c-kit* mRNA and c-kit protein was significantly decreased in the colon of STC, suggesting that the c-kit signal pathway may play an important role in ICC reduction in STC[122]. Shafik *et al*[123] concluded that a disorder of the ICC, which generate electric activity, may have a role in inducing diminished or absent colonic motor activity, a point that should be further investigated.

TUMORS OF GI TRACT

GI stromal tumors (GISTs) have been recognized as a biologically distinctive tumor type, different from smooth muscle and neural tumors of the GI tract. They constitute the majority of GI mesenchymal tumors[124].

GISTs exhibit considerable phenotypic heterogeneity[125]. Their origin remains unclear, although origin in smooth muscle cells has been proposed[126,127]. CD34-ir is often present in GIST[125,128-130], a property shared with various other solid tumors[131,132].

Kit-ir may be a suitable marker for GIST[133], possibly superior to CD34-ir[134]. Mutations (usually activating) of the proto-oncogene Kit have been identified in GIST[133,135-137]. GIST with Kit mutation appear to have a poorer prognosis[133,135-138]. Therefore Kit mutations may merely be part of the oncogenic process rather than an indication of the origin of these tumors.

Recent studies suggesting that ICC in the human gut were both Kit-ir and CD34-ir raised the idea that Kit+ CD34+ GIST may derive from ICC[128,133,137].

The majority of GISTs occurs in the stomach (60%-70%), small intestine (20%-30%) and only 10% or less in the esophagus, colon and rectum, and they affect mainly middle aged patients. Similar tumors, sometimes known as extra-GIST, may arise in the omentum, mesentery, or retroperitoneum and at least one case of pancreatic tumor was described[139,140]. The presence of ICC in normal pancreas was demonstrated recently[15].

The symptoms may vary from none or slight abdominal discomfort to brisk GI hemorrhage, perforation or obstruction.

Imatinib mesylate, a synthetic tyrosine kinase inhibitor developed for the use in the management of interferon resistant chronic myeloid leukemia, was shown to be effective against a number of other tyrosine kinases including c-kit and platelet derived growth factor and now it is considered to be the drug of choice for metastatic and inoperable GISTs[124,141].

CONCLUSION

Knowledge on the role of ICC in GI disorders is increasing and there is currently overwhelming evidence to support the idea that ICC play important roles in GI motility in laboratory animals. Studies of several animal models have shown that the lack of specific ICC subpopulations produces major disturbances of GI motility. ICC are unique cell types with a central role in the control of gut function. Further studies of ICC may, therefore, lead to a major breakthrough in more understanding of GI physiology which may be considered as a promising target, at least in the long run, for specific pharmacological interventions to restore the normal physiology and motor functions of the GI tract.

ACKNOWLEDGMENTS

This article is dedicated to the memory of Professor Ahmed Shafik, my teacher, guide and guardian. His productive and creative mind, his unabated interest in new fields of research, and his unflagging energy were all qualities which formed part of a great personality. He is perhaps the last encyclopedic mind to be found on the crossroads of research and gastrointestinal tract surgery. May his soul rest in peace and tranquility.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3249

World J Gastroenterol 2010 July 14; 16(26): 3249-3257
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Nardostachys jatamansi extract protects against cytokine-induced β-cell damage and streptozotocin-induced diabetes

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Supported by Grant from the Ministry of Science and Technology/Korea Science and Engineering Foundation through the Diabetes Research Center at Chonbuk National University, R13-2008-005-0000-0, and by a Research Fund of Chonbuk National University in 2009 (to Park BH)
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Received: February 3, 2010 Revised: March 17, 2010
Accepted: March 24, 2010
Published online: July 14, 2010

Abstract

AIM: To investigate the anti-diabetogenic mechanism of *Nardostachys jatamansi* extract (NJE).

METHODS: Mice were injected with streptozotocin *via* a tail vein to induce diabetes. Rat insulinoma RINm5F cells and isolated rat islets were treated with interleukin-1β and interferon-γ to induce cytotoxicity.

RESULTS: Treatment of mice with streptozotocin resulted in hyperglycemia and hypoinsulinemia, which was confirmed by immunohistochemical staining of the islets. The diabetogenic effects of streptozotocin were completely abolished when mice were pretreated with NJE. Inhibition of streptozotocin-induced hyperglycemia by NJE was mediated by suppression of nuclear factor (NF)-κB activation. In addition, NJE protected against cytokine-mediated cytotoxicity. Incubation of RINm5F cells and islets with NJE resulted in a significant reduction in cytokine-induced NF-κB activation and downstream events, inducible nitric oxide synthase expression and nitric oxide production. The protective effect of NJE was further demonstrated by the normal insulin secretion of cytokine-treated islets in response to glucose.

CONCLUSION: NJE provided resistance to pancreatic β-cell damage from cytokine or streptozotocin treatment. The β-cell protective effect of NJE is mediated by suppressing NF-κB activation.

Key words: *Nardostachys jatamansi*; Cytokines; Streptozotocin; Pancreatic β cells; Nuclear factor κB; Nitric oxide; Diabetes mellitus

Peer reviewer: Dr. Shivananda Nayak, PhD, Department of Preclinical Sciences, Biochemistry Unit, Faculty of Medical Sciences, The University of The West Indies, Building 36, EWMSC, Mount Hope, Trinidad and Tobago

Song MY, Bae UJ, Lee BH, Kwon KB, Seo EA, Park SJ, Kim MS, Song HJ, Kwon KS, Park JW, Ryu DG, Park BH. *Nardostachys jatamansi* extract protects against cytokine-induced β-cell damage and streptozotocin-induced diabetes. *World J Gastroenterol* 2010; 16(26): 3249-3257 Available from: URL: http://www.

wjgnet.com/1007-9327/full/v16/i26/3249.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3249

INTRODUCTION

Type 1 diabetes mellitus is an autoimmune disease that causes selective destruction of insulin producing β-cells in the islets of Langerhans. In early-stage disease, infiltration of inflammatory cells into the pancreatic islets can be observed histologically[1]. The inflammatory cells produce and release cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). IL-1β, alone or in combination with TNF-α or IFN-γ, upregulates inducible nitric oxide synthase (iNOS), and produces high levels of nitric oxide (NO) in pancreatic islets[2,3]. NO is produced by the oxidation of L-arginine to L-citruline by NOS, and generation of excess NO can inhibit mitochondrial metabolism, protein modification, and DNA cleavage; any one of which could lead to impaired insulin secretion and β-cell death[4,5]. Streptozotocin (STZ) is a diabetogenic agent that is toxic to pancreatic β-cells and is commonly used in diabetes research[6]. Streptozotocin contains a nitroso moiety and releases NO during its metabolism[7]. In rodents, STZ activates poly-ADP ribose polymerase, depletes cellular NAD and ATP, breaks DNA strands, and initiates β-cell necrosis[8].

NO production is regulated by transcription factors that bind to specific sites in the iNOS promoter. Nuclear factor (NF)-κB, which can be activated by cytokines and STZ, has been implicated as a key signaling mediator in iNOS induction[9,10]. When inactive, NF-κB is located in the cytosol complexed with NF-κB inhibitory factor (IκB). Various inducers cause complex dissociation, presumably *via* IκB phosphorylation. Released NF-κB translocates to the nucleus, where it interacts with recognition sites to mediate gene transcription[11]. We and others have shown that NF-κB-dependent NO production is involved in the dysfunction and destruction of β-cells, which suggests NO involvement in autoimmune diabetes pathogenesis[9,12-15].

Nardostachys jatamansi (*N. jatamansi*) is used in Ayurvedic medicine to treat mental disorders, hyperlipidemia, hypertension, and convulsions[16-18]. Various sesquiterpenes such as lignans and neolignans are present in root extracts of this plant[19]. *N. jatamansi* is suggested to protect cells and tissues through its antioxidative properties[20,21]. We found that *N. jatamansi* extract (NJE) protects against development of acute cerulean-induced pancreatitis[22]. However, as far as we are aware, no studies have reported on the antidiabetic effects of NJE. Therefore, in this study we examined the effect of NJE on cytokine- or STZ-stimulated pancreatic β-cell damage and the resultant development of type 1 diabetes.

MATERIALS AND METHODS

Cell culture and reagents

Rat pancreatic β-cell line RINm5F was from the American Type Culture Collection and were grown at 37℃ in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum and 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. IL-1β and IFN-γ were obtained from R&D Systems (Minneapolis, MN, USA). All reagents were from Sigma (St. Louis, MO, USA), unless otherwise noted.

Preparation of NJE

N. jatamansi was from a standard commercial source (Omni Herb, Seoul, Korea), and its identity was confirmed at the Korean Drug Test Laboratory (Seoul, Korea). Voucher specimens (NO; Oh/wh/nj-43) were deposited at the School of Oriental Medicine Herbarium, Wonkwang University. NJE was prepared by decocting 200 g of dried herbs with 1800 mL boiling distilled water for approximately 2 h. The extract was filtered, freeze-dried and stored at 4℃.

Type 1 diabetes induction

Specific pathogen-free male ICR mice, weighing 25-30 g, were purchased from Orientbio Inc. (Seoungnam, Korea) and housed at our animal facility for 1 wk. All mice were kept under specific pathogen-free conditions with free access to a standard commercial diet and were used at 5-6 wk of age. To induce diabetes, mice were injected *via* the tail vein with 80 mg/kg STZ dissolved in 0.1 mol/L sodium citrate buffer (pH 4.0), prepared within 5 min of administration. Mice were divided into the following groups: (1) non-treated controls; (2) STZ; (3) NJE; and (4) NJE + STZ (n = 5 for each group). Control animals received citrate buffer alone. Group 4 received intraperitoneal injections of 125 mg/kg NJE daily for 3 d before administration of STZ. The day on which STZ was first administered was defined as day 1. At day 5, mice were sacrificed by decapitation without anesthesia and trunk blood was collected in pre-chilled tubes that contained 1 mg/mL EDTA. Plasma glucose was assayed using the glucose oxidase-peroxidase method, and plasma insulin was measured using a radioimmunoassay kit (Linco Research, St. Charles, MO, USA). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Chonbuk National University, Jeonbuk, Korea.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability

The viability of cultured cells was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. RINm5F cells were seeded overnight in clear, flat-bottomed 96-well tissue culture plates at 10^5 cells/well in 100 μL medium. Cells were pretreated with NJE as indicated for 3 h, then IL-1β (1 U/mL) and IFN-γ (100 U/mL) were added for an additional 48 h. Cells were washed twice with PBS, and MTT was added (100 μg/100 μL PBS). After incubation at 37℃ for 1 h, 100 μL DMSO was added to dissolve the formazan crystals, and absorbance was measured at

570 nm using a Spectra MAX PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

NO measurement

Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions. NO production was measured as nitrite concentration in cell-free culture supernatants using a colorimetric assay. Briefly, 5×10^5 RINm5F cells or 30 islet samples were pretreated with the indicated concentrations of NJE for 3 h prior to the addition of IL-1β (1 U/mL) and IFN-γ (100 U/mL). After 24 h, 100 μL aliquots of culture supernatant were incubated with 100 μL of a modified Griess reagent of a 1:1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid, at room temperature for 5 min, and the absorbance was measured at 540 nm using a spectrophotometer (Ultrospec 2100 pro; Amersham Biosciences). NO concentration was determined from a linear standard curve of serial dilutions of sodium nitrite in a working medium.

Whole cell and nuclear protein extracts

Cells, islets, or pancreatic tissues were washed with PBS and lysed in CytoBuster protein extraction buffer (Novagen, Madison, WI, USA). Lysate was centrifuged at 10 000 × *g* for 5 min at 4℃, and the supernatant was used as whole cell protein extract. Cytosolic and nuclear extracts were prepared from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA).

Western blotting analysis

RINm5F cells (5×10^6) or 30 islet samples were homogenized in 100 μL ice-cold lysis buffer (20 mmol/L HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and 20 μg protein separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with 1 μg/mL primary antibody against p50, p65, iNOS, actin, or PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected with horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA, USA).

Electrophoretic mobility shift assay

NF-κB activation was assayed using a gel mobility shift assay with nuclear extracts from control and treated cells. An oligonucleotide that contained the κ-chain binding site (κB, 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3') was used as a probe. The two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Binding reactions that contained labeled oligonucleotide (10 000 cpm), 10 μg nuclear extract protein, and binding buffer (10 mmol/L Tris-HCl, pH 7.6, 500 mmol/L KCl, 10 mmol/L EDTA, 50% glycerol, 100 ng poly (dI·dC), 1 mmol/L dithiothreitol) in a final volume of 20 μL were incubated for 30 min at room temperature. Reactions were separated on 4% polyacrylamide gels in 0.5 × Tris-borate buffer, and the gels were dried and visualized by autoradiography. Specificity of the DNA/NF-κB interaction was demonstrated by competitive assays with 50-fold excess unlabeled oligonucleotide.

RNA isolation and real-time RT-PCR

RNA was isolated from RINm5F cells or islets using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was precipitated with isopropanol and dissolved in DEPC-treated distilled water. Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen), and first-strand cDNA was generated using random hexamer primer in a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Specific primers for iNOS were designed using primer express software (Applied Biosystems): iNOS (accession No. NM_012611), 5'-TGTGCTAATGCGGAAGGTCAT-3' (forward), and 5'-CGACTTTCCTGTCTCAGTAGCAAA-3' (reverse). Control 18S rRNA was purchased from Applied Biosystems and was used as the invariant control. Real-time RT-PCR mixtures consisted of 10 ng reverse transcribed total RNA, 167 nmol/L forward and reverse primers, and 2 × PCR Master Mix in a final volume of 10 μL. Reactions were carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All experiments were performed in triplicate.

Glucose-stimulated insulin secretion assay

Pancreatic islets were isolated from 250-300 g male Sprague-Dawley rats, using the collagenase digestion method[23]. Islets were cultured for 24 h with IL-1β and IFN-γ in the presence or absence of NJE, then washed three times in Krebs-Ringer bicarbonate buffer (25 mmol/L HEPES, 115 mmol/L NaCl, 24 mmol/L $NaHCO_3$, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 0.1% bovine serum albumin, pH 7.4), which contained 3 mmol/L D-glucose. Insulin secretion assays were performed with either 5.5 or 20 mmol/L D-glucose. All experiments were performed in triplicate.

Immunohistochemistry

Immunohistochemical staining was performed with the DAKO Envision system (DAKO, Carpinteria, CA, USA), which used dextran polymers conjugated with horseradish peroxidase to avoid contamination with endogenous biotin. Pancreases were removed and immediately placed in fixative (10% formalin solution in 0.1 mol/L PBS). Histological sections of 4 μm were cut from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization, tissue sections were treated using a microwave antigen retrieval procedure in 0.01 mol/L sodium citrate buffer. After blocking endogenous peroxidase, the sections were incubated with Protein Block Serum-Free (DAKO) to block nonspecific staining, then with anti-insulin antibody (Santa Cruz Biotechnology). Peroxidase activity was detected with 3-amino-9-ethylcarbazole.

Statistical analysis

Statistical analyses were performed using ANOVA and

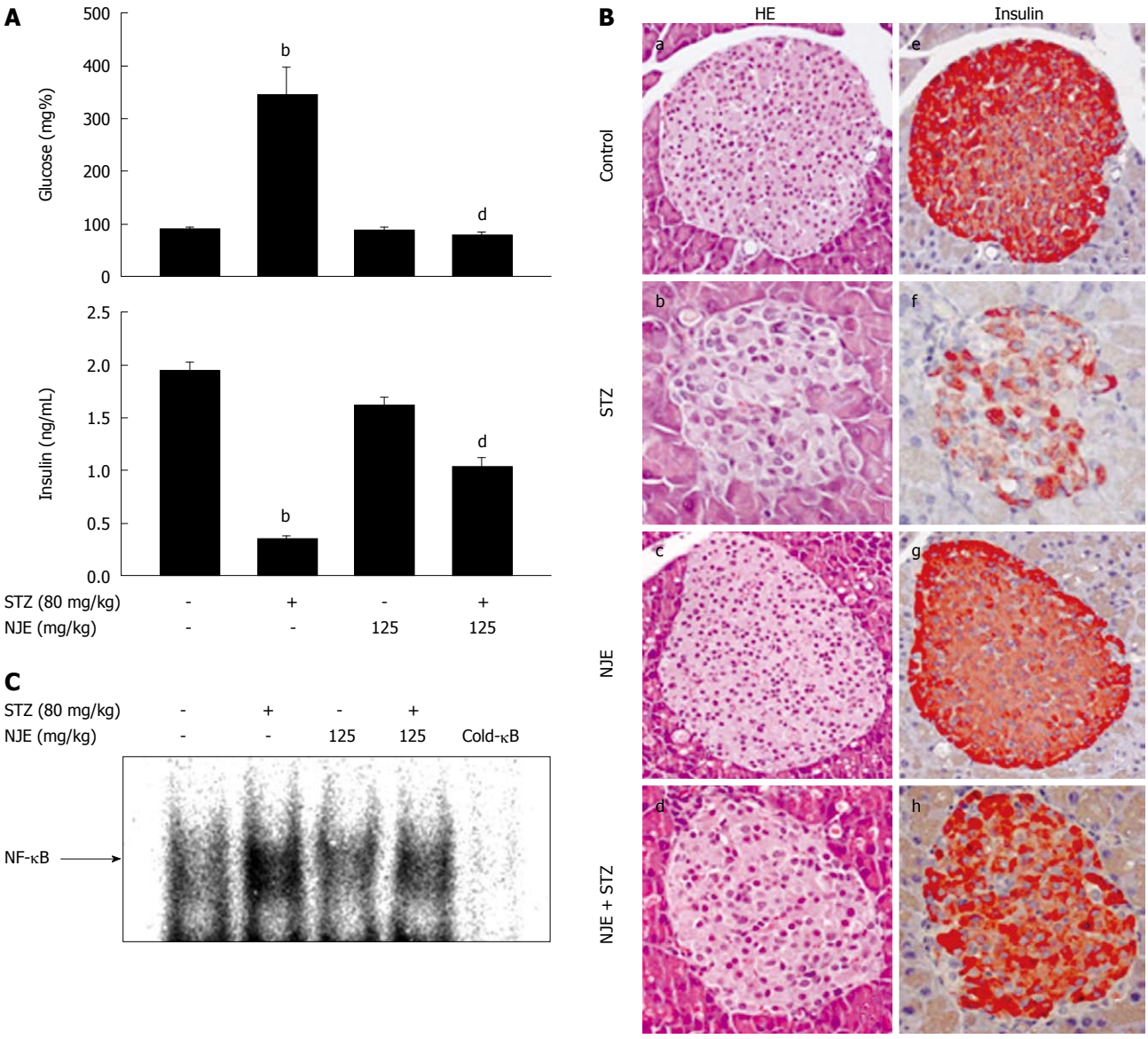

Figure 1 NJE protects islets from STZ-induced destruction. A: ICR mice were intraperitoneally injected daily with NJE at 125 mg/kg for 3 d and then injected with STZ (80 mg/kg) intravenously. Levels of fasting glucose and insulin were determined; B: Pancreases were obtained from normal controls (a, e), and STZ-injected (b, f), NJE-injected (c, g), and NJE and STZ-injected mice (d, h). Islets and adjoining exocrine regions were counterstained with HE (a-d). Islets were labeled with insulin antibody and peroxidase-labeled anti-rabbit IgG (e-h); C: Nuclear extracts from pancreatic tissues were prepared 30 min after STZ injection, and NF-κB DNA binding was analyzed using electrophoretic mobility shift assay. $^{b}P < 0.01$ *vs* untreated control; $^{d}P < 0.01$ *vs* STZ-injected group. NJE: *Nardostachys jatamansi* extract; STZ: Streptozotocin; NF-κB: Nuclear factor κB.

Duncan's tests. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Anti-diabetic effect of NJE in mice

Mice injected with STZ gradually became hyperglycemic, with an increased incidence of diabetes observed starting at day 3. At day 5, the average blood glucose level of mice injected with STZ was 344 ± 52.8 mg/dL. Mice that were pretreated with NJE were fully resistant to diabetes development (Figure 1A), and treatment with NJE alone did not affect blood glucose concentration. In addition, the mean plasma insulin level at day 5 in the STZ group decreased by 84.2% compared with the control (from 1.9 ± 0.1 to 0.3 ± 0.1 ng/mL), while the severity of hypoinsulinemia was attenuated in mice pretreated with NJE (Figure 1A). These results indicate that NJE is protective against STZ-induced diabetes.

The preventative effect of NJE on STZ-induced diabetes was histologically examined. Pancreatic tissues at 5 d after STZ administration, with or without NJE pretreatment, were subjected to hematoxylin and eosin (HE) staining and immunohistochemistry. STZ-treated mice showed degenerative and necrotic changes and islet shrinkage (Figure 1B, b), as well as weak insulin-reactivity in a few β-cells (Figure 1B, f). However, tissues from STZ-treated mice pretreated with NJE showed round, nearly normal, and clearly defined islets that were strongly positive for insulin (Figure 1B, d and h).

To elucidate the antidiabetogenic mechanism of NJE, we examined its effect on STZ-induced NF-κB activation. Figure 1C is a representative electrophoretic mobility shift assay (EMSA) that shows the ^{32}P-DNA/NF-κB complex formed with nuclear extracts from the pancreas 30 min after STZ administration. The findings were similar to those of our previous study, with STZ treatment resulting in increased NF-κB binding to DNA[10]. This complex was not detected in pancreatic nuclear extracts from NJE-pretreated mice. Taken together, these results show that NJE inhibits NF-κB activation and prevents type 1 diabetes development in mice.

NJE prevented cytokine-mediated cell death in RINm5F cells

We investigated the antidiabetogenic effect of NJE at the cellular level. Untreated RINm5F cells or cells pretreated with NJE for 3 h were exposed to cytokine for 48 h, and viability was assessed using an MTT assay. Treatment with cytokine significantly reduced cell viability to 39.8% ± 0.6% of the controls (Figure 2A). Conversely, NJE increased the viability of cytokine-treated RINm5F cells in a concentration-dependent manner. Treatment with NJE alone did not affect cell viability at the concentrations used in this study (data not shown).

NO production was also evaluated. In 24 h, control RINm5F cells generated 9.8 ± 0.5 μmol/L nitrite, while cytokine-treated cells generated 34.2 ± 2.7 μmol/L (Figure 2B). A concentration-dependent reduction in cytokine-mediated nitrite production was observed in RINm5F cells treated with cytokine plus NJE. Near complete inhibition of nitrite production was observed in cells that were pretreated with 100 μg/mL NJE.

To investigate the regulatory effects of NJE on NO production, we examined the effects of NJE on cytokine-induced iNOS mRNA and protein expression, using real-time RT-PCR and Western blotting. Cytokine increased iNOS mRNA and protein levels (Figure 2C). However, when cells were treated with NJE prior to cytokine treatment, mRNA and protein levels decreased in a concentration-dependent manner. Treatment with 100 μg/mL NJE completely blocked iNOS expression.

NF-κB was implicated in STZ toxicity (Figure 1C). Therefore, the effect of NJE on the cytokine-stimulated translocation of NF-κB from the cytosol to the nucleus in RINm5F cells was examined. Nuclear extracts from cytokine-stimulated RINm5F cells showed increased NF-κB binding activity (Figure 3A, lane 2), as well as increased nuclear levels of p65 and p50 subunits (Figure 3B), compared to those of unstimulated cells. In contrast, cytokine-induced NF-κB activation was markedly suppressed by NJE pretreatment, which suggested that NJE inhibited iNOS expression by suppressing NF-κB activation. We previously have reported that IκBα, but not IκBβ, is the major participant in cytokine-induced NF-κB activation[23]. Therefore, we investigated IκBα levels in the cytosol following cytokine treatment (Figure 3B). Cytokine-treated RINm5F cells showed a decreased level of IκBα

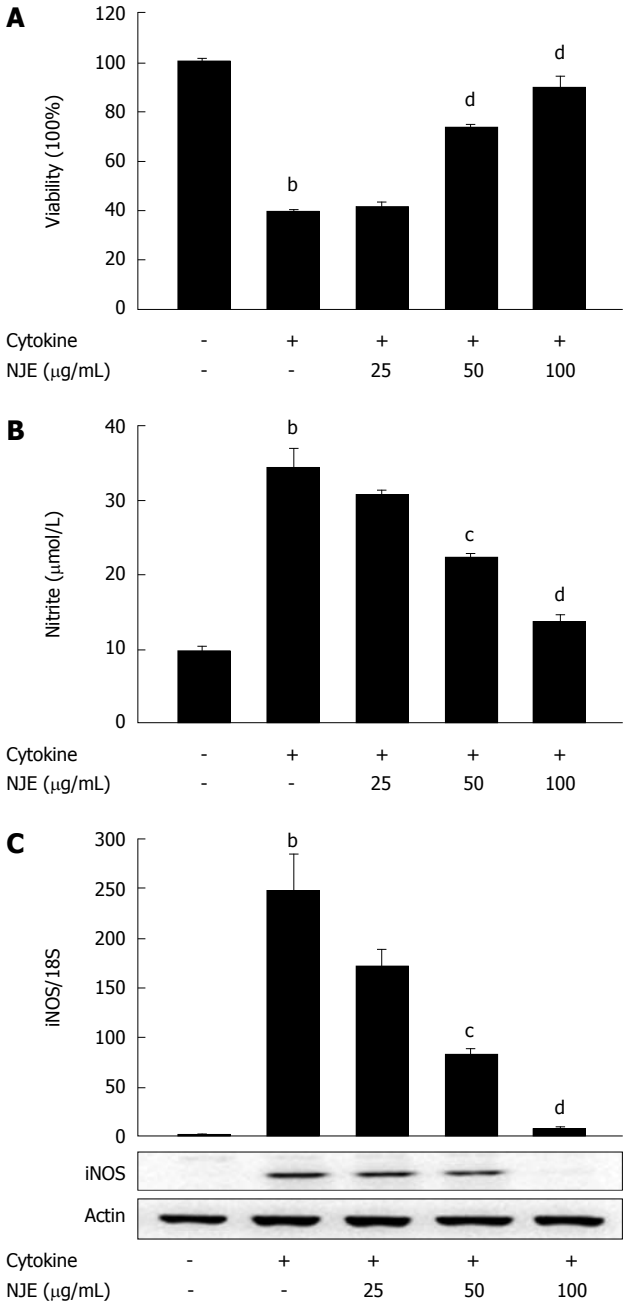

Figure 2 NJE prevents cytokine-induced cell death in RINm5F cells. A: RINm5F cells were pretreated with NJE for 3 h, and IL-1β (1 U/mL) and IFN-γ (100 U/mL) were added for 48 h. Cell viability was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; B and C: RINm5F cells were pretreated with NJE for 3 h, and IL-1β and IFN-γ were added. Following 24 h of incubation, the level of nitrite production and iNOS mRNA and protein expression were determined. Each value is the mean ± SE of three independent experiments. $^{b}P < 0.01$ *vs* untreated controls; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* cytokine. IL-1β: Interleukin 1β; IFN-γ: Interferon γ.

protein in the cytosol compared to a similar fraction from unstimulated cells; however, increased IκBα degradation as a result of cytokine treatment was markedly suppressed by pretreatment with NJE.

NJE suppressed the cytokine-induced NF-κB pathway and preserved glucose-stimulated insulin secretion in rat islets

We further assayed the preventive effects of NJE using rat

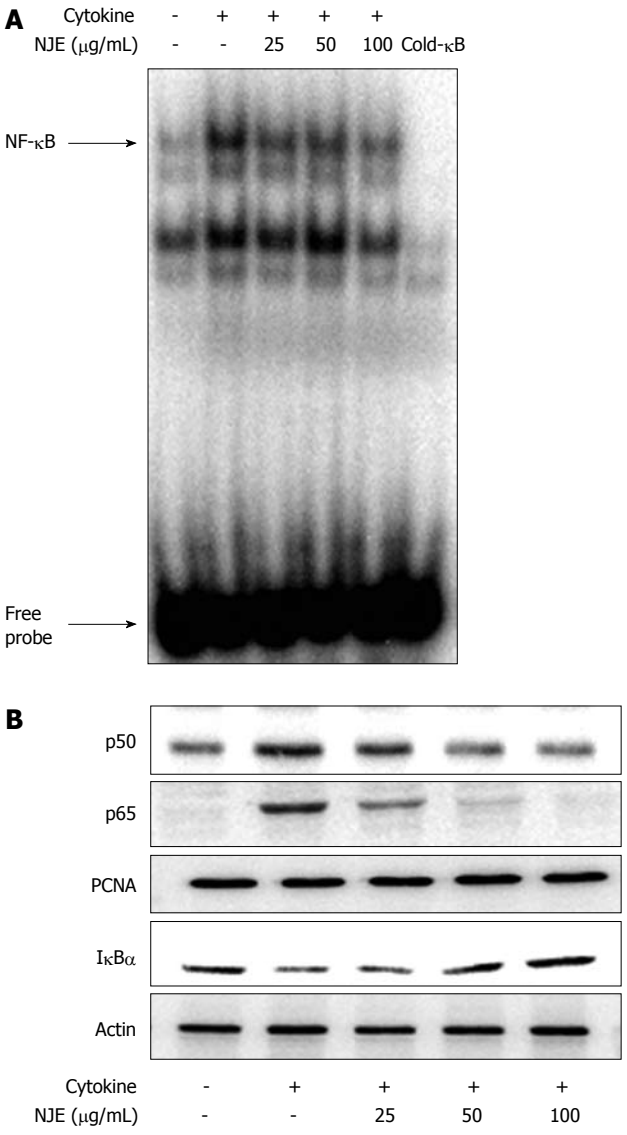

Figure 3 NJE inhibits cytokine-induced NF-κB activation in RINm5F cells. RINm5F cells were pretreated with NJE for 3 h, and IL-1β (1 U/mL) and IFN-γ (100 U/mL) were added. After 30 min, NF-κB DNA binding was analyzed by EMSA (A), and translocation of p65 and p50 to the nucleus and IκBα degradation in the cytosol (B) were determined by Western blotting. β-actin and PCNA were used as loading controls for cytosolic and nuclear proteins, respectively.

pancreatic islets isolated from male Sprague-Dawley rats. Incubation of rat islets with cytokine for 24 h resulted in a 2.8-fold increase in NO production (Figure 4A). Real time RT-PCR and Western blotting revealed that iNOS mRNA and protein levels were markedly increased by cytokines (Figure 4A). Consistent with results from RINm5F cells, pretreatment of islets with NJE abolished the cytokine effects and reduced NO production and iNOS expression to the level of those of control islets. Additionally, treatment with cytokine increased NF-κB DNA binding activity in islets (Figure 4B), and pretreatment of islets with NJE completely abolished these effects. To add functional data, NJE protection against cytokine-induced impairment of glucose-stimulated insulin secretion (GSIS) was evaluated. After 24 h of cytokine exposure, insulin secretion was assayed in response to 20 mmol/L glucose. Control islets secreted 3.4 ± 0.4 ng/mL insulin, while cytokine-treated islets secreted significantly less, at 1.2 ± 0.2 ng/mL ($P < 0.01$) (Figure 4C). However, pretreatment with NJE blocked the cytokine effect and maintained islet cell insulin secretion to levels similar to those of the controls. In addition, treatment with NJE alone did not affect insulin secretion in response to glucose (data not shown).

DISCUSSION

In this study, we present a mode of action for NJE protection against development of type 1 diabetes. Intraperitoneal administration of NJE prevented diabetes development after STZ and preserved β-cell mass. In addition, NJE protected β-cells from cytokine toxic challenge in RINm5F cells and islets.

We demonstrated that NJE prevented STZ-induced diabetes in mice. STZ destroys islet cells through several mechanisms, including the production of reactive oxygen species (ROS)[24], activation of pancreatic NF-κB[25], and induction of pronounced immune and inflammatory responses[26]. STZ-treated mice showed marked islet destruction and relatively small numbers of insulin-positive β-cells, while NJE pre-treated mice showed well-defined islets and strong insulin staining. An EMSA revealed increased NF-κB binding activity in pancreatic nuclear extracts derived from STZ-treated hyperglycemic diabetic mice. However, pretreatment with NJE prevented NF-κB activation, which resulted in the maintenance of plasma glucose and insulin levels in the normal range. NF-κB participates in the transcriptional regulation of pro-inflammatory genes, and their activation results in the production of pro-inflammatory mediators[27,28]. Therefore, NF-κB might be a key regulator in local cytokine response pathways in STZ-mediated β-cell destruction. Alternatively, the NJE anti-diabetic effect could be related to its anti-oxidative properties. Several studies have confirmed that NJE is an ROS scavenger[20,21], and that ROS induces NF-κB activation[29,30], which suggests that ROS scavenging by NJE suppresses STZ-induced NF-κB activation. Taken together, these results suggest that manipulation of NF-κB activity by NJE in pancreatic β-cells allows these cells to withstand and survive STZ-mediated immune attack.

NJE not only protected against STZ-induced diabetes, but also protected RINm5F cells and rat islets against cytokine toxicity. IL-1β has been implicated in early events in β-cell destruction. Suppression of IL-1β production or inhibition of its interaction with corresponding cellular receptors significantly inhibits IL-1β-mediated deleterious effects on β-cells[31,32]. IL-1β exerts its main effects through the NF-κB pathway[33-35]. IFN-γ alone does not stimulate iNOS expression in rodent or human islets, but it reduces the concentration of IL-1β required to induce iNOS expression in rat islets, and a combination of IL-1β and IFN-γ is required to induce iNOS expression and β-cell dysfunction in mouse and human islets[36]. In addition to cytokines, different intracellular pathways that lead to β-cell death (e.g. oxidative stress, chemical generation

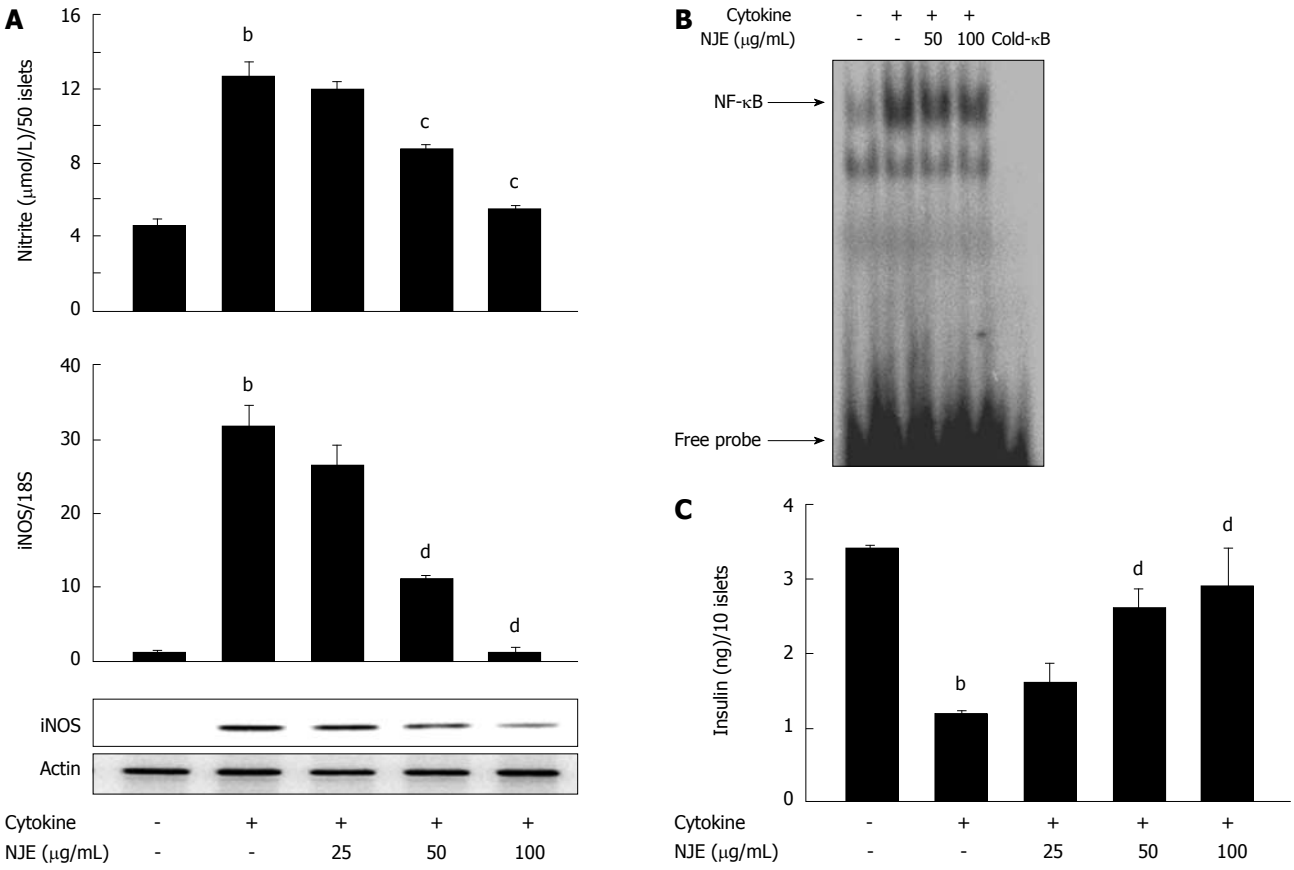

Figure 4 NJE inhibits cytokine-induced activation of NF-κB and maintains glucose-stimulated insulin secretion in rat islets. Rat islets were treated with IL-1β (1 U/mL) and IFN-γ (100 U/mL) with or without 3 h pretreatment with NJE. Nitrite production and iNOS mRNA and protein expression (A) were determined after 24 h, and NF-κB DNA binding (B) was determined 1 h later; C: Rat islets (10 islets/500 μL) were treated with IL-1β (1 U/mL) and IFN-γ (100 U/mL) with or without 3 h pretreatment with NJE. Following 24 h incubation, glucose-stimulated insulin secretion was quantified. The results of triplicate samples are expressed as the mean ± SE. $^{b}P < 0.01$ *vs* untreated controls; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* cytokine.

of NO, mitogen-activated protein kinase activation, JAK-STAT activation, and endoplasmic reticulum stress) partially converge at NF-κB[12,14,37,38]. Hypothetically, this gives us, employing only one approach, the ability to block NF-κB signaling, to save β-cell mass and thereby prevent diabetes development. In this model, NJE completely inhibits NO production in IL-1β- and IFN-γ-stimulated RINm5F cells and islets, through suppression of NF-κB-dependent iNOS expression, thereby protecting RINm5F cells and islets from IL-1β and IFN-γ cytotoxicity. In addition to increased cell viability, we observed the preservation of insulin secretion in NJE pretreated rat islets. The molecular mechanism by which NJE inhibits NF-κB activation by IL-1β and IFN-γ appears to involve both inhibition of IκBα degradation and translocation of p65 and p50 into the nucleus.

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables. Several flavonoids have been shown to inhibit the expression of NF-κB-dependent cytokines, iNOS, and *cyclooxygenase-2* genes[39]. Therefore, it would be interesting to analyze flavonoid composition in NJE extracts.

In summary, this study is believed to be the first to demonstrate that NJE has a β-cell protective effect. Specifically, NJE protected β-cells from cytokine-induced injury *in vitro* and counteracted the development of type 1 diabetes in response to STZ *in vivo*. This β-cell protective effect might be mediated, at least in part, by suppressing NF-κB activation. NJE did not cause serious side effects in mice, therefore, it could be a therapeutic alternative for rescuing β-cells in cases of ongoing β-cell destruction.

COMMENTS

Background

Type 1 diabetes mellitus is an autoimmune disease that causes selective destruction of insulin-producing β-cells in the islets of Langerhans. Once those cells are destroyed, they do not ever produce insulin again. Type 1 diabetes affects younger individuals and requires lifelong insulin treatment. Without treatment, the blood glucose rises to levels which can cause hyperglycemia. Type 1 diabetes cannot be prevented. There is no practical way to predict who will develop the disease because most people who develop it are otherwise healthy. Therefore, the best way to control type 1 diabetes is understanding the disease better and finding a therapeutic regimen to preserve functional β-cell damage.

Research frontiers

Cytokines such as interleukin (IL)-1β and interferon (IFN)-γ, which are released during islet inflammation, are believed to participate in β-cell damage during the development of autoimmune type 1 diabetes. Evidence has suggested that activation of nuclear factor (NF)-κB in response to cytokines is an important component of the signal that triggers β-cell death. For this reason, NF-κB has been targeted for preventing type 1 diabetes development.

Innovations and breakthroughs

Nardostachys jatamansi is used in Ayurvedic medicine to treat mental disorders, hyperlipidemia, hypertension, and convulsions. *N. jatamansi* has been suggested to protect cells and tissues through its antioxidative properties. However, no studies to date have reported the antidiabetic effects of *N. jatamansi*. In this study, the authors observed that *N. jatamansi* extract (NJE) had antidiabetic effects in *in vitro* and *in vivo* models of diabetes.

Applications

NJE did not cause serious side effects *in vivo*, therefore, it might be a therapeutic alternative for rescuing β-cells in cases of ongoing β-cell destruction. NJE-treated islets can also be used to increase islet survival in an allograft transplantation model.

Terminology

IL-1β and IFN-γ are cytokines that are secreted from infiltrated inflammatory cells into the pancreatic islets. Streptozotocin is a diabetogenic drug and particularly toxic to pancreatic β-cells.

Peer review

The article is written well, however, there are many major queries which have to be answered by the authors. Authors have to report on the phytochemical constituents of the extract (at least qualitative analysis).

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3258

World J Gastroenterol 2010 July 14; 16(26): 3258-3266
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

CD74 is a survival receptor on colon epithelial cells

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Supported by The Israel Science Foundation (Morasha), the Minerva foundation, the Israel Cancer Association, and the Moross institute
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Received: November 10, 2009 Revised: January 24, 2010
Accepted: January 31, 2010
Published online: July 14, 2010

Abstract

AIM: To investigate the expression and function of CD74 in normal murine colon epithelial cells (CEC) and colon carcinoma cells.

METHODS: Expression of CD74 mRNA and protein were measured by reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and fluorescence-activated cell sorter (FACS). The effect of migration inhibitory factor (MIF) on the survival of normal CEC from C57BL/6, NOD/SCID, and CD74 deficient mice both *in vitro* and *in vivo*, and on the CT26 carcinoma cell line was analyzed by (quantitative) qRT-PCR, RT-PCR, Western blotting and FACS.

RESULTS: CD74 was found to be expressed on normal CEC. Stimulation of CD74 by MIF induced a signaling cascade leading to up-regulation of Bcl-2 expression, resulting in a significant increased survival of CEC. CD74 was also expressed on the CT26 colon carcinoma cell line and its stimulation by MIF resulted in enhanced cell survival, up-regulation of Akt phosphorylation and Bcl-2 expression.

CONCLUSION: CD74 is expressed on CEC and colon carcinoma cells and serves as a survival receptor in these cells. These results may have implications on colorectal cancer research.

Key words: CD74; Migration inhibitory factor; Colon epithelial cells

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Maharshak N, Cohen S, Lantner F, Hart G, Leng L, Bucala R, Shachar I. CD74 is a survival receptor on colon epithelial cells. *World J Gastroenterol* 2010; 16(26): 3258-3266 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3258.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3258

INTRODUCTION

CD74 (Invariant chain; Ii) is a type Ⅱ integral membrane protein, which acts as a chaperone for major histocompatibility complex (MHC) class Ⅱ protein expression[1]. It is a non-polymorphic type Ⅱ integral membrane protein; the mouse protein is comprised of a 30 amino acid (aa) N-terminal cytoplasmic tail, followed by a single 24 aa transmembrane region and an approximately 150 aa long lumenal domain. The CD74 chain was initially thought to function mainly as an MHC class Ⅱ chaperone, which promotes endoplasmic reticulum (ER) exit of MHC class Ⅱ molecules, directs them to endocytic compartments,

prevents peptide binding in the ER, and contributes to peptide editing in the MHC class Ⅱ compartment[1]. A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the surface of antigen presenting cells (APCs), including monocytes and B cells. In addition to APCs, other cells of the gastrointestinal tract, such as epithelial cells, express class Ⅱ MHC proteins and CD74 and act as APCs[2]. The role of CD74 in the epithelium is not fully defined. It was formerly accepted that the functions of CD74 in these cells have been correlated with its role in antigen processing and presentation in conventional APCs. However, CD74 may play additional roles in epithelial cells. CD74 expression on normal human colon epithelial cells (CEC) is still controversial, as the presence of this molecule was demonstrated using immunohistochemistry by some groups[3], but not by others[4,5]. On human colonic epithelial cell lines it was found to be expressed only after interferon-γ treatment[6]. In mice, CD74 expression on CEC is also not clear, although CD74 was not demonstrated on normal murine CEC[7]. In one study, CLIP (class Ⅱ invariant chain peptide) was shown to be expressed in a complex with MHC class Ⅱ, suggesting that CD74 is expressed on these cells as well[8]. Recently, it was demonstrated that CD74 is expressed on CEC of *APC*$^{Min/+}$ mice. However, these mice bear a point mutation in the murine homolog of the *APC* gene and develop multiple intestinal adenomas[9]. In humans, on the contrary, there was no CD74 expression on normal CEC, but it was expressed on CEC of sporadic colorectal adenomas[10].

It was shown previously that macrophage migration inhibitory factor (MIF) binds to the CD74 extracellular domain, a process that results in the initiation of a signaling pathway in a CD44 dependent manner[11-13].

In our previous studies, we showed that CD74 expressed on B cells is directly involved in shaping the B cell repertoire[14-16] through a pathway leading to the activation of transcription mediated by the nuclear factor-κB (NF-κB) p65/RelA homodimer and its co-activator, TAFII105[17]. We demonstrated that CD74 stimulation with anti-CD74 antibody or MIF leads to NF-κB activation, enabling entry of the stimulated B cells into the S phase, an increase in DNA synthesis, cell division, and augmented expression of anti-apoptotic proteins. These findings indicated that surface CD74 functions as a survival receptor[13,18,19].

In addition, CD74 is expressed at high levels from an early stage of the B cell leukemia, B-CLL. The activation of CD74 on human B-CLL cells by MIF, initiates a signaling cascade that contributes to tumor progression. This pathway induces NF-κB activation, resulting in the secretion of interleukin 8, which in turn promotes cell survival. Blocking of this pathway leads to decreased cell survival. Thus, CD74 expressed on the surface of B-CLL cells plays a critical role in regulating the survival of these malignant cells[20].

MHC class Ⅱ expression was initially thought to be limited to a restricted set of cells collectively known as APCs. However, in addition to conventional APCs, other cell types, including mucosal epithelial cells, were subsequently reported to express class Ⅱ MHC molecules and to present antigens[21].

Surface expression of newly synthesized CD74 is followed by rapid internalization to the endosomal pathway. Experiments that investigate cell surface CD74 are complicated by the fact that CD74 remains on the cell surface for a very short time. The surface half-life of CD74 was calculated to be less than 10 min[22,23].

In this article, we followed CD74 expression in colonic intestinal epithelial cells in the mouse. We show that CD74 is expressed on CEC derived from C57BL/6 and on the CT26 colon carcinoma cell line and serves as a survival receptor on these cells. This finding may suggest a role for CD74 in colon cancer development.

MATERIALS AND METHODS

Mice

C57BL/6, C57BL/6 CD74 deficient[24], C57BL/6 MIF deficient[25], CD44 deficient NOD/SCID mice (Jackson Lab), were used. All animals were used at 6-10 wk of age.

All animal procedures were approved by the Animal Research Committee at the Weizmann Institute of Science.

Intraperitoneal MIF administration

Recombinant murine MIF was purified from an expression system as previously described and contaminating endotoxin removed by C8 chromatography[26]. Mice were injected daily ip with MIF (400 ng) or with PBS, as indicated. Mice were sacrificed after 3.5 or 24 h and CEC were isolated.

Isolation of CECs

CEC were isolated using a modification of the method described previously[27]. Briefly, mice were sacrificed, colons were immediately removed and washed with phosphate buffered saline (PBS) until all content was removed. Colons were inverted and washed gently with Roswell Park Memorial Institute solution. Mucus was removed by incubation for 10 min in 1 mmol/L DTT. Specimens were treated with Dispase Ⅱ (Roche Diagnostics; 3 mg/mL) in DMEM for 30 min (vortexing every 5 min) at 37℃. CEC were isolated from the remaining tissue by passage through a metal filter. In order to purify CEC, cells were centrifuged on a discontinuous Percoll gradient for 30 min, 2000 r/min. Cells found on the top 0%-30% gradient are CEC[28]. Isolated cells were washed in PBS. CEC isolated in this fashion contained over 90% viable cells as determined by Trypan blue exclusion. A total of 92%-95% of the cells stained with the anti-epithelial cell marker anti-pan cytokeratin-26 (FITC conjugated) (Sigma-Aldrich). The remaining contaminating cells represented $CD3^+$ and B_220^+ cells.

CT26 cell line

CT26 murine colon carcinoma cells were grown as monolayer cultures in DMEM-10%, fetal bovine serum (FBS) (Invitrogen) supplemented with 100 IU/mL penicillin and

100 μg/mL streptomycin. Cells were maintained in a 37℃ incubator with 5% CO_2-humidified air.

Stimulation of cells by MIF

CECs (3-6 × 10^6 cells/well) isolated from CD57BL/6 mice, were cultured in 12-well plates at 37℃ in DMEM medium supplemented with 10% FCS, 2 mmol/L glutamate, 300 U/mL penicillin, 300 μg/mL streptomycin, with or without 400 ng/mL of MIF for 17 h.

1.5 × 10^6 cells (CT26 cell line) were plated in complete medium into dishes of 6-well cell culture plates, and were allowed to adhere for 24 h. After washing the cells twice with PBS, they were incubated with MIF (400 ng/mL) for 3 or 6 h (as indicated in the text) in serum-free medium.

RNA isolation and reverse transcription

Total RNA was isolated from cells using the Tri Reagent Kit (MRC), according to the manufacturer's instructions. Reverse transcription was carried out using Superscript Ⅱ RT (Gibco-BRL).

Primers that were used in polymerase chain reaction (PCR) reactions: Bcl-2: 5'-CACCGAACACTTGATTCTG, 3'-AGATCTCTGGTTGGGATTC; Cyclin E: 5'-GAAAATCAGACCACCCAGAGCC, 3'-GAAATGATACAAAGCAGAAGCAGCG; CD74: 5'-GGAGTACCCGCAGCTGAAGGGG, 3'-GAAGATAGGTCTTCCATGTCCAGTG; HPRT: 5'-GAGGGTAGGCTGGCCTATGCCT, 3'-GTTGGATACAGGCCAGACTTTGTTG.

Real time PCR

Levels of mRNA of Actin, Bcl-2 and Cyclin E, were analyzed by quantitative real-time reverse transcriptase (RT)-PCR using a Light-Cycler instrument (Roche Diagnostics, Mannheim, Germany). Total RNA was isolated from cells using the Tri Reagent Kit (MRC). Reverse transcription was carried out using Superscript Ⅱ RT (Gibco-BRL). The reaction volume (10 mL) contained 3 mmol/L $MgCl_2$, LightCycler HotStart DNA SYBR Green I mix (Roche Diagnostics), specific primer pairs, and 2.5 mL of cDNA. Conditions for PCR were as follows: 10 min at 95℃ followed by 60 cycles of 15 s at 95℃, 15 s at 60℃, and 15 s at 72℃. PCR was performed in triplicates. Primer sequences were as follows: Bcl-2: 5'-GCTACCGTCGTGACTT-3', 5'-GCCGGTTCAGGTACTC-3'; Cyclin E: 5'-GTAACATAAGCAAACTG-3', 5'-TTCTTCTGGATTGGCTAA-3'; Actin: 5'-CAGTAACAGTCCGCCT-3', 5'-GTGACGTTGACATCCG-3'; β-actin levels were used to normalize samples for calculation of the relative expression levels of the genes.

Western blotting analysis

To detect whether CD74 protein is expressed by CEC and to examine levels of Bcl-2 protein, cells were lysed in RIPA lysis buffer (10 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 5 mmol/L EDTA) containing complete protease inhibitor cocktail [10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 μg/mL chymostatin (Roche), 1 mmol/L PMSF (Sigma), and 20 mmol/L N-etheyl-melamide (Sigma)], for 30 min on ice and then centrifuged at 14000 *g* at 4℃ to remove cell debris. Lysates were separated by 12% (w/v) SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane and probed with anti-CD74 (FL-293; Santa Cruz) or anti-Bcl-2 (C-2; Santa Cruz), followed by horseradish peroxidase-conjugated anti-mouse (Jackson Laboratories).

To detect changes in Akt phosphorylation, stimulated cells were lysed in buffer containing: 25 mmol/L Tris, pH 7.4; 2 mmol/L vanadate; 75 mmol/L glycophosphate, pH 7.2; 2 mmol/L EDTA; 2 mmol/L EGTA; 10 mmol/L NaPPi; and 0.5% NP-40 in the presence of protease inhibitors. Lysates were separated by 10% (w/v) SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti-p-Akt antibody (Cell Signaling Technology) followed by peroxidase-conjugated anti-mouse (Jackson Labs). The membrane was then stripped and reprobed with anti-tubulin antibody (Sigma), followed by peroxidase-conjugated anti-mouse (Jackson Laboratories).

Immunofluorescence and flow cytometry

Staining of IECs was performed using anti-CD74 (Santa Cruz), anti pan-cytokeratin (Sigma-Aldrich).

Cell survival assays

Annexin and PI staining: Cells were centrifuged, washed, and stained with annexin (BD Biosciences) and propidium iodide (PI) for 15 min at room temperature. The extent of annexin and PI staining was analyzed by FACS. Unstained cells were classified as living cells; annexin stained cells are apoptotic, and PI stained cells were considered necrotic.

FLICA staining: Analysis of apoptosis. To detect earlier stages of apoptosis, intracellular caspase 3 and 7 activity was analyzed using the FLICA (Fluorochrome Inhibitors of Caspases) Apoptosis Detection kit from Immunochemistry Technologies (Bloomington, MN). The kit contains carboxyfluorescein-labeled valylalanylaspartic acid fluoromethyl ketone, which tightly binds activated caspases. Cells were harvested and incubated with a FLICA solution in the complete medium at 37℃ for 1 h. After washing twice cells were analyzed by FACS.

Statistical analysis

Results are represented as averages of several experiments (as indicated) ± SE. Comparison between groups was done by Student's *t*-test.

RESULTS

CD74 is expressed on mouse colonic epithelial cells

To determine whether CD74 is expressed in normal CEC, we first analyzed its mRNA and protein levels in control and CD74 deficient (CD74-/-) CEC. As shown in Figure 1, CD74 mRNA (Figure 1A) and protein (Figure 1B) were detected in CEC, while they were not observed in the CD74 deficient cells. Isolation of CEC from C57BL/6

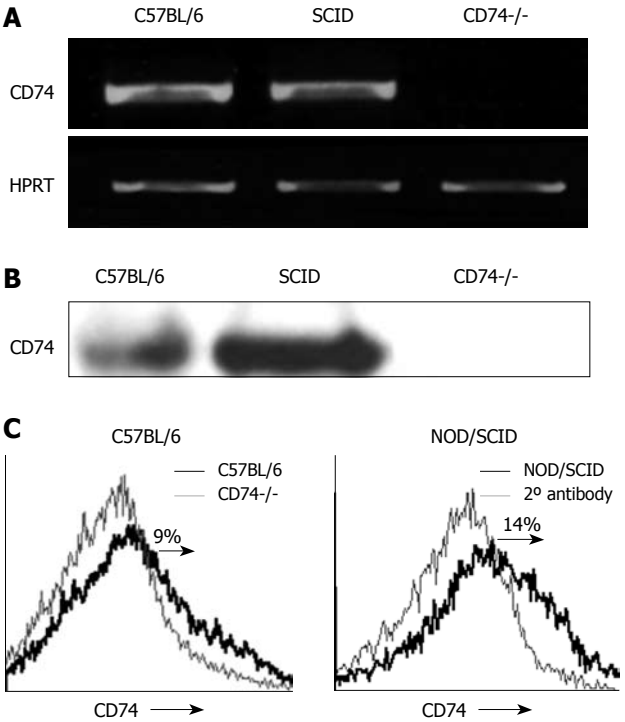

Figure 1 CD74 is expressed on naive CEC isolated from C57BL/6 mice. CEC were isolated from C57BL/6, NOD/SCID and CD74-/- mice. A: RNA was purified and CD74 or HPRT mRNA were analyzed by RT-PCR. The results presented are representative of four separate experiments; B: Cells were lysed and proteins were analyzed by Western blotting; C: Cells were stained with anti cytokeratin and anti CD74 antibodies, and analyzed by FACS. Histograms show CD74 expression on cytokeratin positive cells. CEC: Colon epithelial cells; RT-PCR: Reverse transcriptase-polymerase chain reaction; FACS: Fluorescence-activated cell sorter.

resulted in a population that was more than 90% pure, containing less than 5% B cells (data not shown), which expressed relatively high levels CD74. To eliminate contamination by B cell CD74, we analyzed CD74 mRNA and protein levels in CEC isolated from NOD/SCID mice, which lack B and T cells. As demonstrated in Figure 1, CD74 mRNA (Figure 1A) and protein (Figure 1B) were detected in CEC derived from NOD/SCID mice as well, showing that normal CEC express CD74. We next analyzed, by FACS analysis, CD74 cell surface expression on normal CEC. As shown in Figure 1C, CD74 is specifically expressed on normal CEC derived from C57BL/6 or NOD/SCID mice. Thus, CD74 is expressed on normal CEC.

CD74 serves as a survival receptor on CEC

We have previously shown that CD74 stimulation results in augmented expression of anti-apoptotic proteins, resulting in induction of cell survival[13,18]. In order to examine whether CD74 serves as a survival receptor on CEC, we followed the downstream cascade initiated by MIF in CEC. CEC from C57BL/6 mice were incubated *in vitro* for 17 h with MIF or with PBS, and the percentage of live and apoptotic cells in each group was analyzed using annexin-PI staining. As shown in Figure 2, MIF stimulation resulted in elevation of the proportion of live CEC (42%, $n = 4$, $P = 0.003$) and reduction in the apoptotic population.

To further show the *in vivo* role of MIF and CD74 in CEC survival, MIF was injected ip to control C57BL/6 and NOD/SCID mice and Bcl-2 mRNA and protein levels were determined. As can be seen in Figure 3A and B, a significant elevation in Bcl-2 mRNA and protein levels were detected following MIF stimulation compared to PBS-treated cells. The MIF-induced cascade was CD74 dependent, since in its absence, MIF was not able to increase Bcl-2 levels.

Since CEC survive poorly *in vitro*, and following 17 h incubation a large proportion of the cells are dead, we followed CEC survival immediately after their isolation. Thus, 24 h after MIF injection to C57BL/6, NOD/SCID and CD74-/- mice, cells were isolated and their cell death was analyzed using FLICA, which detects early stages of apoptosis by analyzing intracellular caspase 3 and 7 activity. The percentage of apoptotic cells was significantly higher in the PBS treated cells compared with the MIF treated cells derived from C57BL/6 and NOD/SCID mice (Figure 3C), while the CD74-/- cells were insensitive to MIF treatment. Results of four experiments are summarized in Figure 3D.

It has been recently shown that CD44 forms a complex with CD74 which regulates MIF-induced B cell survival[11,13]. CD44 expressed on CEC was previously characterized as a negative regulator of apoptosis as well[29]. Accordingly, CD44 deficient cells (CD44-/-) demonstrated an elevated basal apoptotic rate compared to control mice. In addition, there was no increase in CEC survival upon MIF stimulation (Figure 3C and D), suggesting that CD44 is a crucial component in the CD74-induced survival cascade.

Cell cycle progression is regulated by cyclin dependent kinases (Cdks). Cdks are constitutively expressed during the cell cycle and are activated upon specific cyclin binding. Different cyclins are differentially expressed during various stages of the cell cycle. This transient expression activates Cdks and regulates cell cycle progression. To further determine whether CD74 regulates cell entry to the S-phase, we followed cyclin E, which is expressed upon S-phase initiation. MIF (400 ng) was injected to C57BL/6 and NOD/SCID mice. 3.5 h later, CEC were isolated and their cyclin E mRNA levels were analyzed. As demonstrated in Figure 4A and B, cyclin E mRNA levels were upregulated in both C57BL/6 and NOD/SCID derived cells. These results demonstrate that following CD74 stimulation, CEC cells synthesize DNA, enter S phase, and probably divide.

Taken together, our results demonstrate that CD74 serves as a survival receptor on CEC. MIF binding to CD74 induces a signaling cascade resulting in Bcl-2 and cyclin E expression and survival in normal CEC.

In order to evaluate whether our finding may have implications on malignant cells, we further assessed the expression and function of CD74 in the colon carcinoma cell line CT26. We found that CD74 is expressed on the CT26 cell line (Figure 5A) and MIF stimulation reduces their apoptosis (Figure 5B and C), by upregulating Bcl-2 mRNA (Figure 5D and E), protein (Figure 5F) and cyclin E (Figure 5G) mRNA levels.

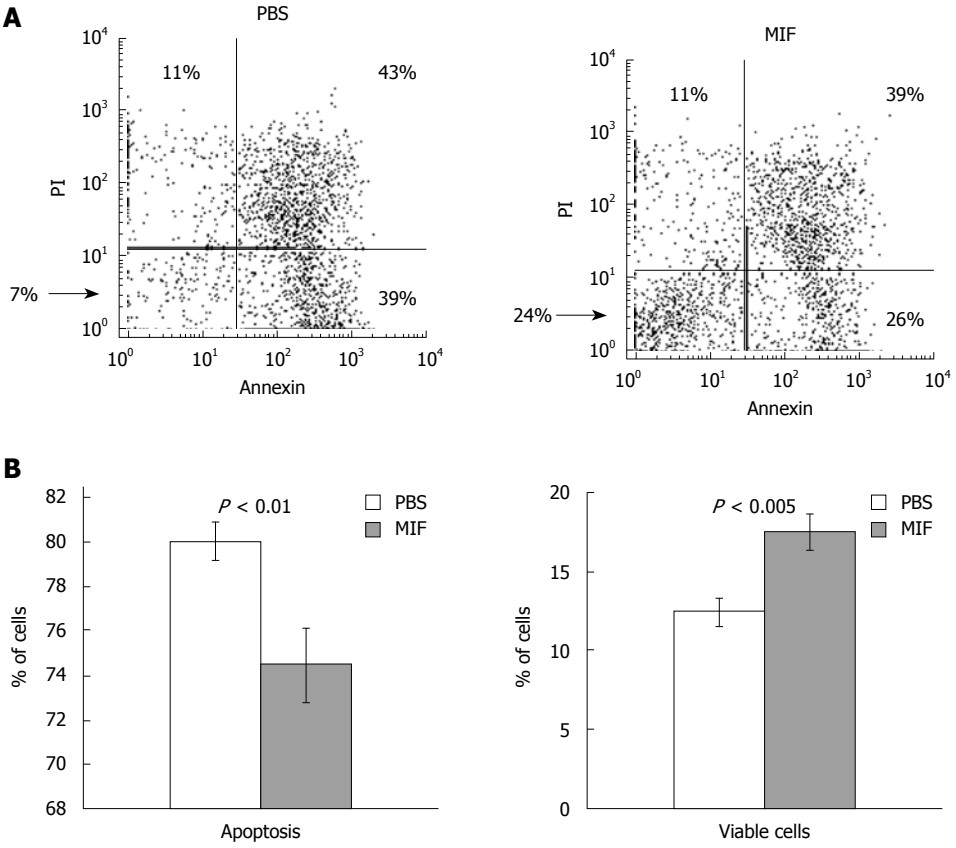

Figure 2 ***In vitro*** **stimulation with MIF induces CEC survival.** CEC from C57BL/6 mice were incubated in the presence or absence of MIF (400 ng/mL). A: After 17 h, cells were collected and analyzed for survival by annexin and propidium iodide staining; B: Graphs summarizing the results of four independent experiments. MIF: Migration inhibitory factor; PBS: Phosphate buffered saline.

We have previously shown that in B cells the CD74 induced survival cascade involves Akt phosphorylation[18]. In intestinal epithelial cells, it has been shown that phosphorylation of Akt enhances cell survival as well[30] and is closely associated with CRC[31,32]. We therefore analyzed Akt phosphorylation in CT26 cells. As shown in Figure 5H, MIF elevated Akt phosphorylation 1 min following stimulation.

Together, our results suggest that MIF and CD74 regulate colon tumor cell proliferation and survival.

DISCUSSION

The importance of CD74 as a survival receptor on B cells, and its role in the pathogenesis of certain malignancies is a subject of intense study. In the colon, CD74 is expressed on malignant cells, and its expression correlates with tumor grade[5]. Recent evidence suggests that up-regulation of CD74 and MIF on human colon adenomas is in correlation with dysplasia of the epithelial cells[10]. However, contradictory results regarding the presence of CD74 on normal human CEC were published through the years. In one report, the authors were able to detect the presence of CD74 by using immunohistochemistry, whereas, in a more recent study, using similar methodology, CD74 was not detected. In this article, we show CD74 expression in mouse normal CEC at the levels of mRNA, protein, and cell surface expression. Furthermore, we found that stimulation of CD74 by its natural ligand, MIF, results in increased survival of CEC. This can be at least partially attributed to enhanced expression of the survival gene, Bcl-2, which was found to be up-regulated in MIF-stimulated CEC. These findings suggest a role for CD74 on normal CEC.

Demonstration of CD74 on CEC is complicated by two main phenomena. First, CD74 has a very short half-life on these cells, and secondly, CEC are difficult to handle. These cells typically undergo apoptosis and/or necrosis, shortly after isolation[33]. Therefore, experiments involving incubation of these cells for more than a few hours usually result in death of most of the cells. In addition, isolates of CEC are never free of contaminating cells, specifically B cells from the lamina propria that also express CD74. In order to overcome these limitations, we performed our experiments shortly after CEC isolation. In experiments where we tested stimulation by MIF, we used mainly *in vivo* stimulation by ip injections of MIF for various amounts of time. To avoid contamination with B cells, we also repeated most of the experiments in NOD/SCID mice, which lack B cells. Our results show that CD74 is expressed on normal CEC cells.

Due to the difficulties discussed above, we used multiple models in order to demonstrate the positive effect CD74 has on CEC survival. In these experiments, we analyzed by FACS the percentage of living and apoptotic CEC. We demonstrated that stimulation by MIF either

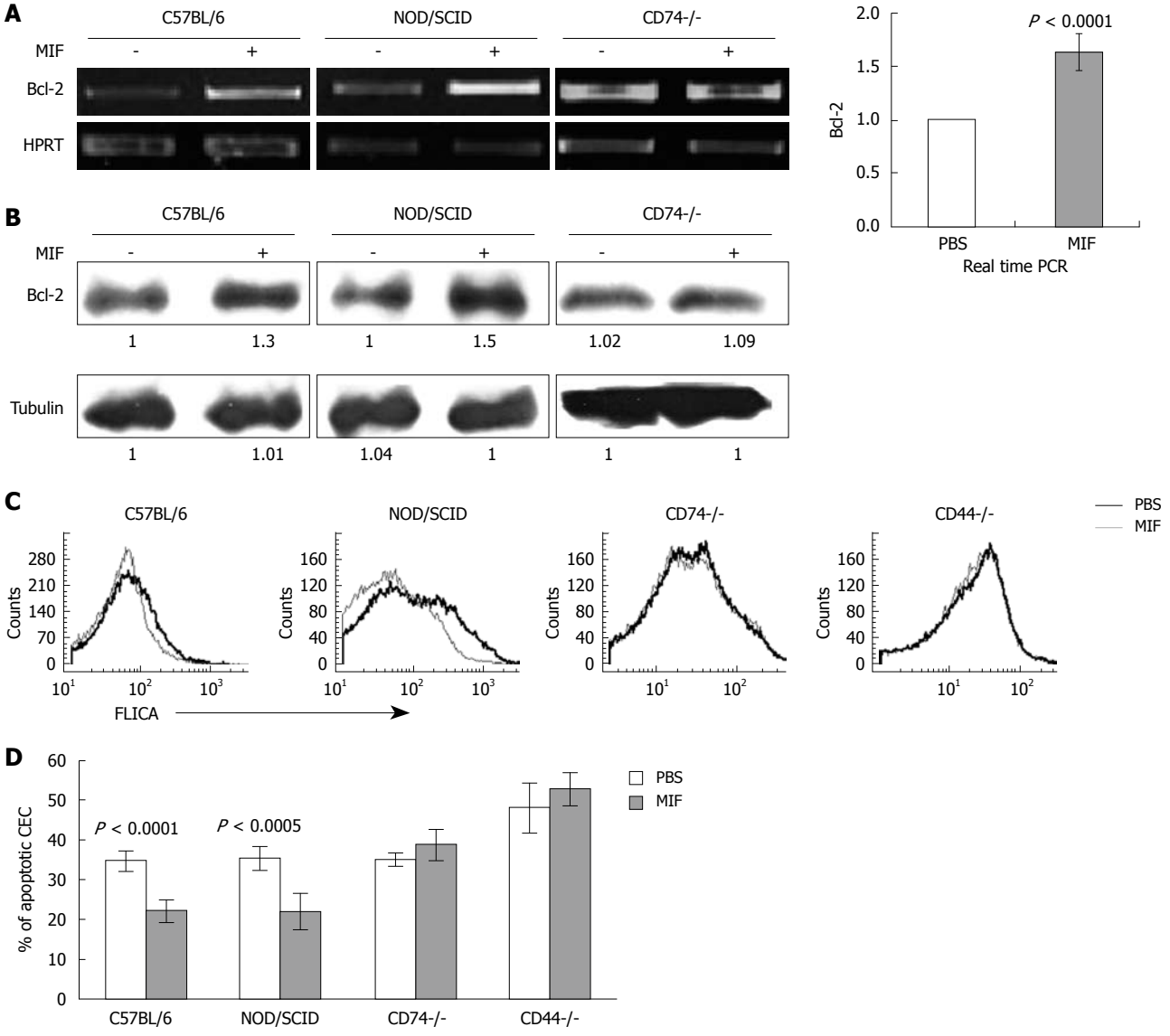

Figure 3 MIF stimulation elevates Bcl-2 and survival in CEC and their *in vivo*. CEC were isolated from CD74-/-, C57BL/6 and from NOD/SCID mice that were i.p injected with MIF (400 ng) or PBS 3.5 h before sacrifice. A: CEC were isolated, and RNA was purified; Bcl-2 or HPRT mRNA were analyzed by RT-PCR. The results presented are representative of four different experiments. The graph is a summary of four separate experiments of quantitative real time PCR using primers for Bcl-2 in CEC isolated from C57BL/6; B: Cells were lysed and Bcl-2 protein expression was analyzed by Western blotting analysis; C, D: C57BL/6, NOD/SCID, CD44-/- and CD74-/- mice were injected with MIF (400 ng) or PBS. After 24 h, mice were sacrificed and their CEC were isolated and stained with FLICA. C: Histograms show FLICA staining in MIF injected (grey line) compared to PBS injected (black line) CEC; D: Graph representing the average of five different experiments, demonstrating decreased apoptosis CEC in MIF treated C57BL/6 and NOD/SCID mice, compared to PBS treated animals.

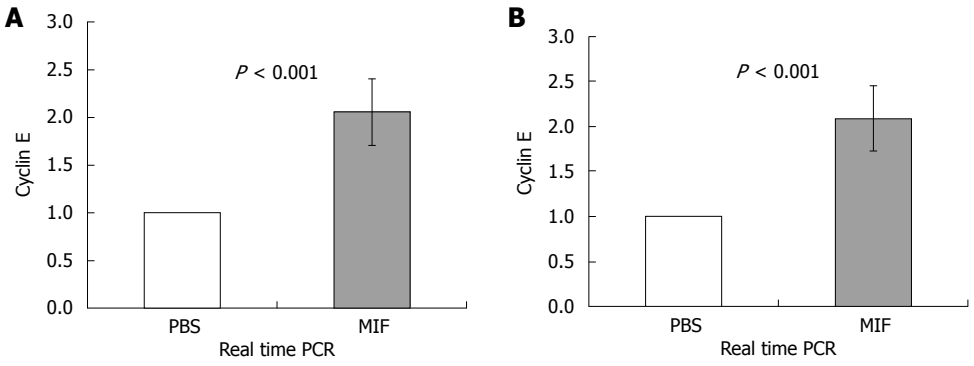

Figure 4 MIF induces cyclin E expression. CEC were isolated from C57BL/6 (A) and NOD/SCID (B) mice that were i.p injected with MIF (400 ng) or PBS 3.5 h prior to isolation of CEC. A, B: Quantitative real time PCR was performed using primers for cyclin E and β-actin. β-actin levels were used to normalize samples for calculation of the relative expression levels of cyclin E. Results are expressed as a fold of change in cyclin E expression at stimulated cells compared to non stimulated cells, which was defined as 1. Results shown are summary of four separate experiments.

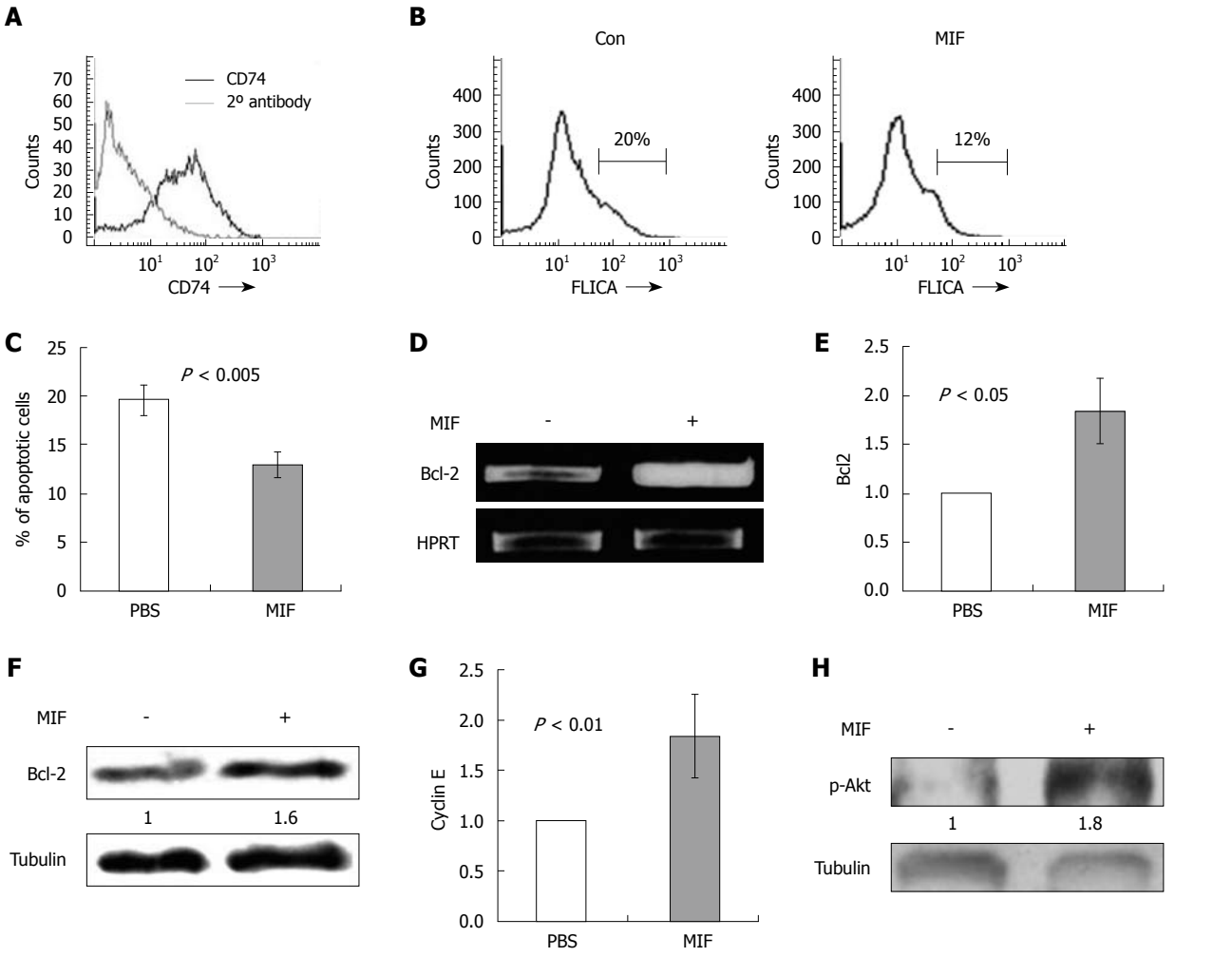

Figure 5 CD74 is expressed on CT26 cells and regulates their survival. A: CT26 cells were stained with anti CD74 antibodies, and analyzed by FACS. Histogram shows CD74 expression on these cells; B: Serum-starved CT26 cells were incubated in the presence or absence of MIF (400 ng/mL) for 6 h. Histograms show FLICA staining for apoptotic cells in both groups; C: Graph representing the average of five different experiments, demonstrating decreased apoptosis in MIF treated compared to untreated CT26 cell line; D: Serum-starved CT26 cells were either kept untreated or treated with MIF (400 ng/mL) for 3 h and levels of Bcl-2 mRNA were analyzed by RT-PCR. The results presented are representative of five different experiments; E: Quantitative real time PCR was performed using primers for Bcl-2. β-actin levels were used to normalize samples for calculation of the relative expression of Bcl2 level. Results are expressed as a fold of change in Bcl-2 expression at stimulated cells compared to non stimulated cells, which was defined as 1. Results shown are a summary of four separate experiments; F: Following 6 h of starvation the CT26 cells were lysed and Bcl-2 protein expression was analyzed by Western blotting analysis; G: Quantitative real time PCR was performed using primers for cyclin E and β-actin. β-actin levels were used to normalize samples for calculation of the relative expression levels of cyclin E. Results are expressed as a fold of change in cyclin E expression at stimulated cells compared to non stimulated cells, which was defined as 1. Results shown are a summary of four separate experiments; H: CT26 cells were incubated in the presence or absence of MIF (400 ng/mL) for 1 min. Cells were fast-frozen and lysed as described in Methods. Lysates were separated on 10% (wt/vol) SDS/PAGE and blotted with anti-p-Akt antibody (results presented are representative of three separate experiments) followed by HRP-conjugated anti-mouse antibodies. The membranes were then stripped and blotted with anti-tubulin.

by ip injection *in vivo* or by incubation of isolated CEC with MIF *in vitro*, resulted in elevated CEC survival and decreased apoptosis. These results strengthen our hypothesis that CD74 is a survival receptor on CEC and suggests that MIF is the natural ligand of CD74 on CEC, as described for B lymphocytes[13,18,19].

MIF is expressed throughout the human gastrointestinal tract, and has previously been implicated in control of apoptosis of non-epithelial and epithelial cells[34,35]. MIF induces Bcl-2 expression and cell survival. It was previously shown that CD74 forms a complex with CD44 in normal[11,12] and tumor cells[36]. We show here that CD44 is crucial for the MIF/CD74 induced survival cascade, since stimulation of CD44 deficient cells by MIF did not affect cell death.

Evidence for a role of CD74 in colorectal cancer was suggested by its expression on carcinoma cell lines of the colon[37], and by the observation that in human colorectal carcinomas, the grade of the tumor correlated with the level of CD74 expression on the transformed CEC[5]. Interestingly, both MIF and CD74 have been associated with tumor progression and metastasis. It was reported that MIF mRNA is over-expressed in various tumors[38,39] and MIF has also been associated with the growth of malignant cells[40]. Numerous studies have demonstrated the overexpression of CD74 in various cancers[20,41-46]. CD74 expression may also serve as a prognostic factor in many of these cancers, with higher relative expression of CD74 behaving as a marker of tumor progression[47]. Our findings further support a possible role for CD74 in

CRC. Additionally, CD44, which we showed essential in the MIF-CD74 survival cascade, is overexpressed in CRC, and has an anti-apoptotic effect on these cells, and probably involved in disease progression and metastases[29,48]. Moreover, we show that CD74 is expressed on the CT26 cell line and its stimulation by MIF enhances Akt phosphorylation, cyclin E and Bcl-2 expression resulting in their survival.

Our work clearly shows that CD74 is expressed on mouse intestinal epithelial cells, and serves as a survival receptor on these cells. Our results further show that CD74 may regulate survival of colorectal carcinoma tumor cells. Since we found that CD74 is a survival receptor on CEC, any change in gene expression that causes enhanced expression of CD74 or MIF may result in increased risk of colorectal cancer. Thus, CD74 may be found in the future to serve not only as a marker of colorectal cancer, but also as a therapeutic target.

COMMENTS

Background

CD74 is a protein that is expressed in and on some of the cells of the immune system, such as B lymphocytes and antigen presenting cells. This protein is known for its function in helping the immune cells to process and present foreign bodies. During recent years it was found that CD74 serves additionally as a survival receptor on cells of the immune system, and that its stimulation by its natural ligand - migration inhibitory factor (MIF) - prevents apoptosis (self destruction) of the cells.

Research frontiers

CD74 was found to be markedly expressed on numerous tumors - hematologic as well as epithelial; in some of these tumors it can serve as a tumor marker and its level of expression may be related to the prognosis. Moreover, activation of CD74 on B lymphocytes in B cell leukaemia, results in tumor progression, and blocking this pathway leads to decreased cell survival, and thus may be applicable as a therapeutic intervention. Whether CD74 is expressed on colon intestinal epithelial cells is controversial, although it was shown to be expressed on epithelial cells of colorectal adenomas.

Innovations and breakthroughs

The authors' work clearly shows that CD74 is expressed on mouse intestinal epithelial cells and serves as a novel survival receptor on these cells. They show that CD74 may regulate survival of colorectal carcinoma as well. Since they found that CD74 is a survival receptor on colonic epithelial cells, any change in gene expression that causes enhanced expression of CD74 or MIF may result in increased risk of colorectal cancer.

Applications

As CD74 is a survival receptor on colon epithelial cells and on malignant cell lines from mice, CD74 may be found in the future to serve not only as a marker of colorectal cancer, but also as a therapeutic target. Future research should focus on the role of CD74 in colorectal cancer models in animals and on human tissues.

Peer review

This paper is well written and documents expression on CD74 receptor mRNA and protein in colon epithelial cells. The study is very well done and straightforward. The experiments are of good quality.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3267

World J Gastroenterol 2010 July 14; 16(26): 3267-3278
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions

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Supported by The Grant of Medicine and Health Key Projects of Zhejiang Province, Science and Technology Fund of Ministry of Health of the People's Republic of China, No. WKJ2007-2-037; Shaoxing Key Project for Science and Technology, No. 2007A23008; the Natural Science Foundation of Zhejiang Province, China, No. Y2090337
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Received: March 23, 2010 Revised: April 26, 2010
Accepted: May 3, 2010
Published online: July 14, 2010

Abstract

AIM: To identify the key cytokines involved in hepatic differentiation of mouse bone marrow mesenchymal stem cells (mBM-MSCs) under liver-injury conditions.

METHODS: Abdominal injection of CCl_4 was adopted to duplicate a mouse acute liver injury model. Global gene expression analysis was performed to evaluate the potential genes involved in hepatic commitment under liver-injury conditions. The cytokines involved in hepatic differentiation of mBM-MSCs was functionally examined by depletion experiment using specific antibodies, followed by rescue experiment and direct inducing assay. The hepatic differentiation was characterized by the expression of hepatic lineage genes and proteins, as well as functional features.

RESULTS: Cytokines potentially participating in hepatic fate commitment under liver-injury conditions were initially measured by microarray. Among the up-regulated genes determined, 18 cytokines known to closely relate to liver growth, repair and development, were selected for further identification. The fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM) were finally found to be involved in hepatic differentiation of mBM-MSCs under liver-injury conditions. Hepatic differentiation could be dramatically decreased after removing FGF-4, HGF and OSM from the liver-injury conditioned medium, and could be rescued by supplementing these cytokines. The FGF-4, HGF and OSM play different roles in the hepatic differentiation of mBM-MSCs, in which FGF-4 and HGF are essential for the initiation of hepatic differentiation, while OSM is critical for the maturation of hepatocytes.

CONCLUSION: FGF-4, HGF and OSM are the key cytokines involved in the liver-injury conditioned medium for the hepatic differentiation of mBM-MSCs.

Key words: Hepatic differentiation; Mouse bone marrow mesenchymal stem cells; Inducing cytokines; Fibroblast growth factor-4; Hepatocyte growth factor; Oncostatin M

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Dong XJ, Zhang H, Pan RL, Xiang LX, Shao JZ. Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions. *World J Gastroenterol* 2010; 16(26): 3267-3278 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3267.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3267

INTRODUCTION

It is generally believed that the bone marrow mesenchymal stem cells (BM-MSCs) provide an appropriate hematopoietic microenvironment that exerts regulatory effects on the self-renewal and differentiation of hematopoietic stem/progenitor cells. They are capable of differentiating into mesoderm cell lineages, including osteoblasts, chondrocytes and adipocytes[1-3]. However, emerging new findings suggest that BM-MSCs are able to give rise to a more broad range of cells, including hepatocytes, neurons, epithelial cells and keratinocytes[4-8]. This plasticity of BM-MSCs has attracted much attention to their *in vivo* new functions under either metabolic or pathologic conditions, and their clinical therapy for tissue repair. In fact, several studies in animal models have suggested that endogenous MSCs may "naturally" be involved in wound healing and tissue regeneration, and the engrafted exogenous MSCs have beneficial effects in tissue repair, including that of bone, myocardial tissue, skin, kidney and liver[9-19]. These may encourage further studies on the new insight into MSCs biology and the mechanisms underlying MSCs differentiation, which are still poorly understood at present.

Recently, by an *in vivo* tracing technology, we have demonstrated that BM-MSCs could be recruited from the bone marrow into peripheral blood, and toward into the wounded sites in response to the injured-liver signals, which indicated a close relationship between BM-MSCs and liver repair[20]. Moreover, we have also found that the engrafted exogenous BM-MSCs could be recruited to the injured liver, and were able to differentiate into multiple hepatic-lineage cells, which greatly improved the wound healing, providing further insight into the relationship between BM-MSCs and injured liver[20]. Our previous reports also support the idea that the liver-injury conditioned culture medium can induce the differentiation of BM-MSCs into functional hepatic cells in an *in vitro* experiment[4]. These observations indicated that the hepatic differentiation of BM-MSCs may be induced by the cytokines secreted from the injured liver cells, since no cellular interactions existed in such cell-free cultural medium. However, which cytokines direct hepatic fate specification of BM-MSCs still remains unclear. In the present study, we identified the key cytokines that play a crucial role in the differentiation of mBM-MSCs in the liver-injury conditioned medium. We hope our finding will benefit the better understanding of the novel mechanisms underlying BM-MSCs involved liver repair and regeneration, and help improve the cytokine-based hepatic inducing strategy and provide a rich cellular resource from BM-MSCs for cytotherapy of acute liver diseases.

MATERIALS AND METHODS

Experimental animals

Eight to ten-week-old male ICR mice obtained from the Laboratory Animal Unit of Zhejiang Academy of Medical Sciences (Hangzhou, China) were used in the experiments. Animals were housed under specified pathogen-free conditions. All animal experiments were done in accordance with a legal regulation, which includes approval by a local ethical committee.

Isolation and culture of bone marrow MSCs

The mouse bone marrow MSCs (mBM-MSCs) were prepared as described previously[4]. Briefly, the bone marrow was extruded by clipping of the epiphysial ends of the bones and flushing with IMDM (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Hyclone, Rockville, MD), 1% penicillin/streptomycin (Medium A). After 3 d, non-adherent cells and debris were removed, and the adherent cells were cultured continuously. At near confluence, the cells were replated at 5×10^4 cells/cm^2. Osteogenic, chondrogenic and adipogenic differentiations were examined for functional identification[5].

Preparation of acute liver-injury mouse model

The acute liver-injury mouse model was prepared according to the method described previously[21]. Briefly, the mice were treated with CCl_4 (1.0 mL/kg body weight of a 10% solution in mineral oil injected intraperitoneally) twice a day and then sacrificed by cervical vertebrae luxation on the 24th h after the last injection.

Hepatocyte isolation and preparation of conditioned medium

The hepatocytes were isolated by the two-step collagenase perfusion from healthy mice (as control) or liver-injury mouse model prepared by the method described above. Briefly, donor animals received 25 U heparin (Sigma) prior to cell isolation. After cannulation of the portal vein, the liver was perfused with a calcium-free buffer solution, 3 mL/min at 37℃ for 10 min. Then, the liver was perfused with 0.025% collagenase Ⅳ (Invitrogen, Carlsbad, CA), 2 mL/min at 37℃ for 15 min. The perfused liver was resected, and the cells were released by gentle shaking and collected in 20 mL IMDM. The supernatant cell suspension was filtered using a 200 mm nylon mesh and the filtrate was washed twice with PBS by centrifugation at 50 × *g* for 45 s to remove cell debris, damaged cells, and non-parenchymal cells. After washing, the hepatocytes were cultured in medium A at 5×10^4 cells/cm^2. Forty-eight hours later, the supernatant was collected and passed through a 0.25 mm filter. The filtrate was finally defined as hepatocyte-injury conditioned medium and stored in aliquots at -20℃ for future use.

Induction of hepatogenic differentiation of mBM-MSCs in conditioned medium

The mBM-MSCs of passage 3 were inoculated in differentiation medium at 5×10^4 cells/cm^2 on culture flasks. The differentiation medium consisted of 50% fresh IMDM medium (Medium A) and 50% conditioned medium. As a negative control, mBM-MSCs were cultured in medium A only. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. Cultures were maintained by medium exchange every 3 d. The cell morphology was observed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss Inc., Jena, Germany).

Gene expression analysis by real-time polymerase chain reaction

The expressions of hepatic linage genes [α-fetoprotein (AFP), albumin (ALB), hepatocyte nuclear factor 3β (HNF3β), tyrosine aminotransferase (TAT)] and cytokine genes [fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM)] involved in hepatic differentiation and commitment were analyzed by real-time polymerase chain reaction (PCR). For this, total RNA was extracted from undifferentiated control or differentiated cells, normal or injured liver cells, respectively, using NucleoSpin® RNAIIKits, and then 5 μg of which was reversely transcribed into cDNA with SuperScript Ⅲ first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transcripts normalized to β-actin were measured by real-time PCR using Mastercyclerep realplex system and Real-Time Detection software (Eppendorf, Germany), in which the standard double-stranded DNA dye (SYBR Grean Ⅰ) was used. Gene-specific primers were designed using the Primer Premier software (Table 1).

Protein expression analysis by ELISA

The expressions of hepatic linage proteins (AFP and ALB) in differentiated hepatocytes and cytokine proteins (FGF-4, HGF and OSM) in injured liver cells were further analyzed by ELISA. The total proteins from differentiated cells or undifferentiated control cells, normal or injured liver cells were extracted using T-PER (a protein extraction reagent) (Qiagen, Germany) according to the manufacturer's manual, and were coated on a 96-well polystyrene plate at 4℃ overnight. The wells were washed with PBST (500 μL Tween 20/L PBS) and then blocked with 0.5% BSA for 1 h at 37℃. The hepatic linage proteins (AFP and ALB) or cytokines (FGF-4, HGF and OSM) were detected using the polyclonal antibodies, including rabbit anti-mouse HGF and FGF4, goat anti-mouse OSM (abcam, UK) rabbit anti-AFP and goat anti-ALB (Biodesign, Saco, Maine, USA) after washing with PBST for 3 times. The secondary antibodies, including goat anti-rabbit and rabbit anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA), labeled with horseradish peroxidase (HRP) were added at a proper dilution. Finally, the TMB was added, and the observation density was detected by a microplate reader at 450 nm after 2 mol/L sulphuric acid was added to stop the reaction.

Table 1 Primers and annealing temperatures used for PCR

Gene	Sequence (5′-3′)	Annealing (℃)	Product (bp)
AFP-F	CACTGCTGCAACTCTTCGTA	52	300
AFP-R	CTTTGGACCCTCTTCTGTGA		
HNF3b-F	GACCTCTTCCCTTTCTACCG	51	551
HNF3b-R	TTGAAGGCGTAATGGTGC		
ALB-F	TCTTCGTCTCCGGCTCTG	55	475
ALB-R	CTGGCAACTTCATGCAAAT		
TAT-F	CTTCAGTCCTGGATGTTCGC	55	619
TAT-R	CAGGGATTGGACGGGTTGTT		
FGF4-F	CTGGTGGCTCACAGGACAATAA GAT	62	483
FGF4-R	GCTGGCTGAAGAAACAGGTAAT AGGT		
HGF-F	GTGCCAACAGGTGTATCAG	62	399
HGF-R	TGTCACAGACTTCGTAGCG		
OSM-F	CTCACGGTCCACTACAACAC	62	123
OSM-R	GAGCCATCGTCCCATTCC		
β-actin-F	TTCCTTCTTGGGTATGGAAT	55	200
β-actin-R	GAGCAATGATCTTGATCTTC		

PCR: Polymerase chain reaction; AFP: α-fetoprotein; HNF: Hepatocyte nuclear factor; ALB: Albumin; TAT: Tyrosine aminotransferase; FGF-4: Fibroblast growth factor-4; HGF: Hepatocyte growth factor; OSM: Oncostatin M.

Periodic acid-Schiff stain for glycogen

The hepatic differentiation was functionally determined by glycogen storage. For this, the culture dishes containing differentiated cells were fixed in 95% alcohol for 10 min. Samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized (d) H_2O, treated with Schiff's reagent for 15 min, and rinsed in dH_2O for 5 min. Finally, the preparations were assessed under light microscope, and the positive rate of differentiated cells was counted as previously described[4].

Evaluation of urea synthesis

The hepatic differentiation was functionally determined by urea synthesis. The mBM-MSCs were plated at 5×10^4 cells/cm^2 on collagen coated six-well plates in differentiation medium or control medium. After washing extensively with PBS, cells differentiated at days 0, 4, 8, 16, 20 and 21 were incubated in 2 mL of serum-free Hanks' buffered salt solution containing 5 mmol/L NH_4Cl for 2 h at 37℃. After incubation, the urea concentrations in the supernatant were measured according to the method described previously[22].

Global evaluation of cytokines potentially involved in injured liver commitment

A global gene expression analysis was performed by microarray to identify the potential cytokines responsible for hepatic commitment. Total RNA and complementary DNA were prepared from CCl_4-treated and untreated mouse livers. An Illumina Mouse WG-6 v2.0 BeadChip

(Illumina, San Diego, CA, USA) was used to generate expression profiles of more than 48 000 transcripts with 500 ng of labelled cDNA for each sample, following manufacturer's recommended protocols. A randomized design was used to minimize chip effects. Four individuals were replicated in the two batches. Expression intensity was measured with an average of 30 beads for each transcript. The BeadChips were imaged with an Illumina BeadArray Reader. The raw intensities were extracted with the Gene Expression Module in Illumina's BeadStudio software. Expression intensities were log2 transformed and median-centered by subtracting the mean value of each array from each intensity value.

Cytokine identification in depletion experiment

The cytokines involved in hepatic specification in liver-injury conditioned medium were initially identified in a depletion experiment. The liver-injury conditioned medium was incubated with a number of different cytokine antibodies (anti-HGF, -FGF-4, -OSM, -FGF-3, -FGF-10, -FGF-12, -FGF-13, -FGF-14, -FGF-15, -FGF-17, -FGF-18, -FGF-20, -FGF-21, -bNGF, -IGF-1, -TGF-β1, -TGF-β2, and -TGF-β3, abcam and Biodesign) at different concentrations (1:100, 1:200 and 1:500) overnight at 4℃ under agitation. Then, the cytokines were removed by affinity co-immunoprecipitation. The protein A-coupled sepharose beads were prepared according to the manufacturer's instructions (Abcam), and were added into the antibody pretreated liver-injury conditioned medium. After being incubated at 4℃ for 4 h, the samples were centrifuged at 10 000 × *g* for 5 min. Supernatants were collected for hepatic differentiation induction assay. The hepatic differentiation was examined based on the hepatic lineage gene and protein expressions and functional characterizations as described above. In parallel, a non-specific rabbit antibody (IgG isotype) was used in control groups.

Cytokine identification in rescue experiment

To confirm the observation from depletion experiment that HGF, FGF-4 and OSM may be involved in hepatic differentiation in liver-injury conditioned medium, a rescue experiment was performed by adding back these three cytokines into the co-immunoprecipitated liver-injury conditioned medium. The liver-injury conditioned medium was pretreated with anti-HGF, anti-FGF-4 and anti-OSM, followed by protein A-coupled Sepharose beads as described above, and mouse FGF-4 (0, 1.25, 2.5, and 5 μg/mL), HGF (0, 2.5, 5 and 10 μg/mL) and OSM (0, 1.25, 2.5 and 5 μg/mL) (R&D Systems, Abingdon, UK) were added back to this medium to rescue its liver-inducing activity. The hepatic differentiation was examined based on the hepatic lineage gene and protein expressions and functional characterizations as described above.

Induction of hepatic differentiation by FGF-4, HGF and OSM

To further investigate the role of FGF-4, HGF and OSM in hepatic differentiation, an *in vitro* hepatic induction assay was conducted using different combinations of FGF-4, HGF and OSM. The mBM-MSCs were inoculated in medium A with different combinations of 10 ng/mL FGF-4, 20 ng/mL HGF and 10 ng/mL OSM[21] at 5×10^4 cells/cm^2 on culture flasks. As a negative control, the mBM-MSCs were cultured in medium A without FGF-4, HGF and OSM. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. After 72 h, non-adherent cells and debris were removed and the adherent cells were cultured continuously. Cultures were maintained by medium exchange every 3 d. The hepatic differentiation was examined based on the hepatic lineage gene and protein expressions and functional characterizations as described above.

Statistical analysis

Statistical analysis was performed using the SPSS version 16.0, and data were expressed as mean ± SD. Differences between the values were determined by paired-samples *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of cytokines in response to liver injury

To evaluate the possible cytokines that participate in the hepatic differentiation of BM-MSCs under liver-injury conditions, the gene expression levels of cytokines/proteins in injured liver were initially examined at different injury time-points (12, 24 and 48 h) by microarray analysis. The results showed that more than 1200 genes were significantly up- or down-regulated during these time periods, and most of them were closely related to hepatocyte detoxification and metabolisms (data not shown). Totally, 40 cytokines/chemokines or their corresponding receptors closely associated with cellular growth, differentiation and migration were found to be significantly up-regulated (2-62 folds), among which 18 cytokines, including FGF-3, FGF-4, FGF-10, FGF-12, FGF-13, FGF-14, FGF-15, FGF-17, FGF-18, FGF-20, FGF-21, HGF, OSM, b-NGF, IGF-2, TGFβ-1, TGFβ-2 and TGFβ-3, were largely contributed to the hepatic growth and development. Therefore, these cytokines were considered to be potentially involved in the hepatic differentiation of mBM-MSCs under liver-injury conditions, and to be the candidates for further identification.

Cytokine identification in depletion experiment

Based on the microarray analysis as described above, the cytokine candidates were removed from the conditioned medium by a co-immunoprecipitation strategy using different combinations of specific antibodies which fall into several groups, designated as E1 (anti-FGF-3 and -FGF-4), E2 (anti-FGF-10, -FGF-12 and -FGF-13), E3 (anti-FGF-14, -FGF-15 and -FGF-17), E4 (anti-FGF-18, -FGF-20 and -FGF-21), E5 (anti-HGF, -OSM and -bNGF) and E6 (anti-IGF-1, -TGF-β1, -TGF-β2 and -TGF-β3). The conditioned medium from each group

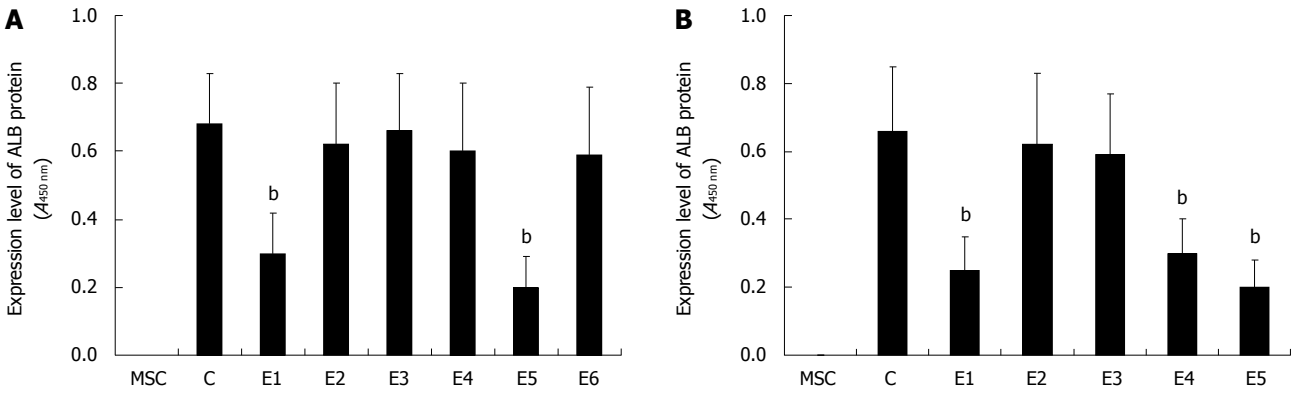


Figure 1 Analysis of albumin synthesis in cells for 20 d by ELISA ($A_{450\ nm}$). MSC group: Control MSCs cultured in medium A with PBS; C group: MSCs induced with liver-injury conditioned medium with nonspecific IgG; (A) E1-E6 groups: MSCs induced with liver-injury conditioned medium and different combinations of specific antibodies (E1: Anti-FGF-3 + anti-FGF-4; E2: Anti-FGF-10 + anti-FGF-12 + anti-FGF13; E3: Anti-FGF-14 + anti-FGF-15 + anti-FGF-17; E4: Anti-FGF-18 + anti-FGF-20 + anti-FGF-21; E5: Anti-HGF + anti-OSM + anti-bNGF; E6: Anti-IGF-1 + anti-TGF-β1 + anti-TGF-β2 + anti-TGF-β3); (B) E1-E5 groups: MSCs induced with liver-injury conditioned medium and different specific antibody lonely (E1: Anti-FGF-4; E2: Anti-FGF-3; E3: Anti-bNGF; E4: Anti-HGF; E5: Anti-OSM). $^{b}P < 0.01$ *vs* C group. ELISA: Enzyme-linked immunosorbent assay; MSC: Mesenchymal stem cells; PBS: Phosphate buffered saline; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; OSM: Oncostatin M.

was used for further hepatic differentiation. The results showed that, after being induced for 20 d, the ALB in differentiated cells in E1 and E5 groups was more significantly down-regulated ($P < 0.01$) than that in the control group. However, no significant down-regulations of ALB could be observed in other experimental groups (E2, E3, E4 and E6), suggesting that FGF-3, FGF-4, HGF, OSM and bNGF in the conditioned medium examined in E1 and E5 groups may play essential roles in the hepatic differentiation of mBM-MSCs (Figure 1A). In an attempt to verify this observation, the FGF-3, FGF-4, HGF, OSM and bNGF were removed selectively: E1, anti-FGF-4; E2, anti-FGF-3; E3, anti-bNGF; E4, anti-HGF; and E5, anti-OSM. The hepatic differentiation of mBM-MSCs in each group was detected and the results showed that the ALB in differentiated cells in E1, E4 and E5 groups was significantly down-regulated ($P < 0.01$) compared with that in the control group. However, no significant down-regulations of ALB could be observed in E2 and E3 groups (Figure 1B). These results indicated that FGF-4, HGF and OSM are key factors involved in the hepatic differentiation of mBM-MSCs. This observation seems in accordance with some previous reports showing that FGF-4, HGF and OSM played important roles in liver healing, regeneration and initiation of development[23-26].

Expression analysis of FGF-4, HGF and OSM during liver injury

To obtain a deep insight into the role of FGF-4, HGF and OSM in liver injury, a kinetic expression analysis of these cytokines during liver injury was performed. The results showed that the expression of HGF mRNA was significantly up-regulated at 6 h after injury ($P < 0.05$), peaked at 12 h ($P < 0.01$) and kept in high level up to 72 h (Figure 2A). Similarly, the expressions of FGF-4 and OSM mRNAs were importantly up-regulated at 12 h ($P < 0.01$) after injury, peaked in 24-48 h ($P < 0.01$) and also kept in high levels up to 72 h (Figure 2B and C). Accordingly, the expression changes of HGF, FGF-4 and OSM proteins were generally identical with their mRNA expressions (Figure 2D-F).

Detection of FGF-4, HGF and OSM in culture medium

To further investigate the existence and occurrence of FGF-4, HGF and OSM in liver-injury conditioned medium, the kinetic secretion of FGF-4, HGF and OSM into the cultured medium was determined. The results showed that the concentrations of HGF and OSM proteins were dramatically up-regulated at 12 h after culture, peaked at 24 or 48 h and kept in high levels up to 72 h (Figure 3A and C); while the FGF-4 was significantly up-regulated at 24 h and peaked at 48 h (Figure 3B). Notably, it showed that FGF-4, HGF and OSM protein expressions could also be detected in the cell culture without undergoing liver injury (Figure 3), possibly due to the fact that isolation of cells from liver itself is a tissue "anatomy" which may result in the "injured signal" to stimulate the cells to secrete cytokines.

Effects of FGF-4, HGF and OSM in hepatic differentiation

To investigate the effects of each cytokine in the hepatic differentiation, a number of depletion experiments were conducted using different combinations of antibodies. The results showed that after treatment with anti-FGF-4 and anti-HGF independently, the hepatic differentiation induced by the conditioned medium was significantly ($P < 0.01$) down-regulated with the increased use of antibody (E1: 1:500, E2: 1:200, E3: 1:100) as determined by the expression of both the early liver-specific marker (HNF3β and AFP) and the late liver-specific marker (ALB and TAT) at mRNA or protein levels, and by the functional [Periodic acid-Schiff (PAS) and urea production] analyses (Figure 4A-H). However, after treatment with anti-OSM alone, the expressions of HNF3β and AFP in cells were not significantly decreased (Figure 4A and B), while the expressions of ALB and TAT were dramatically ($P < 0.01$)

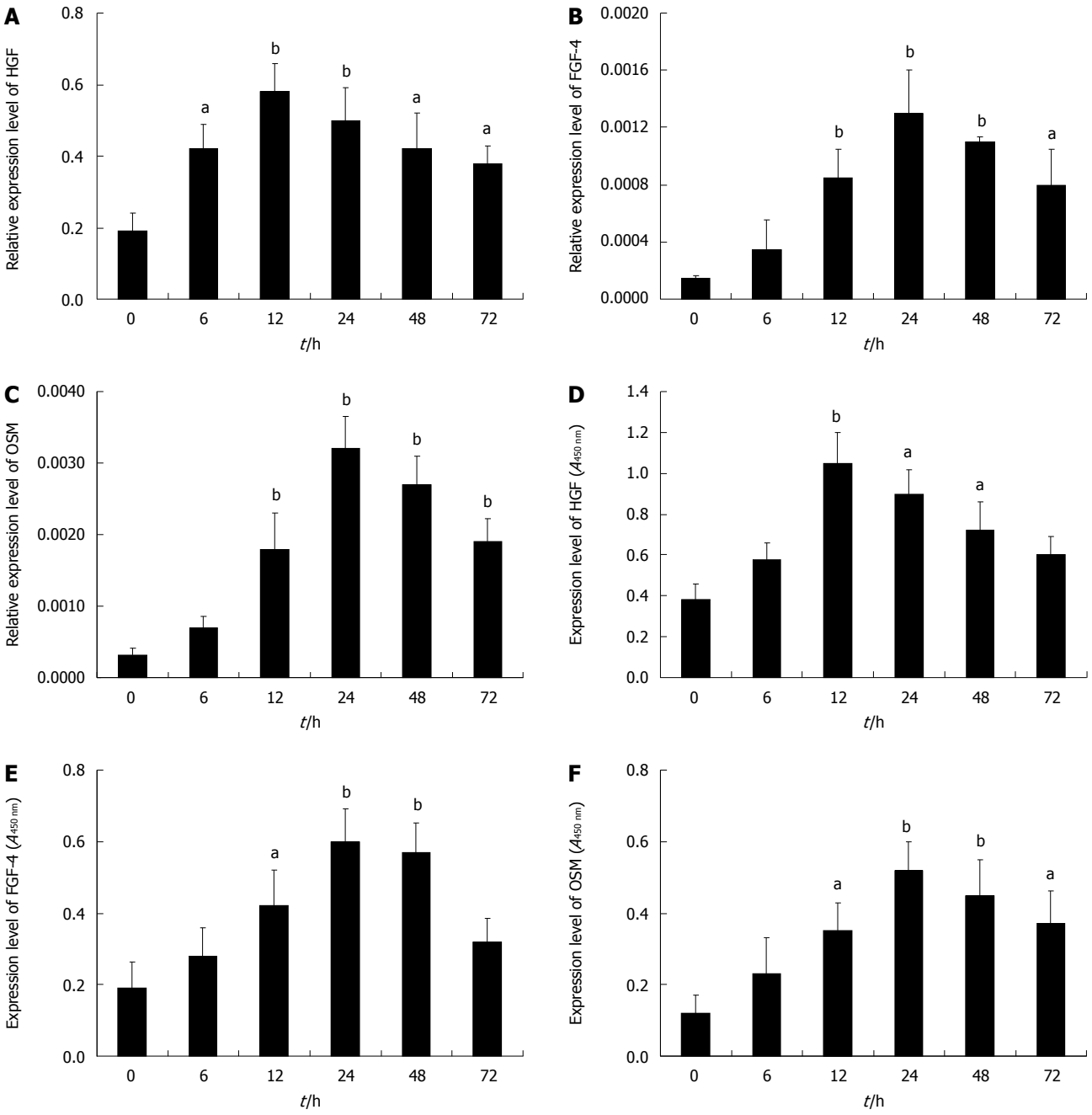


Figure 2 Expression analyses of FGF-4, HGF and OSM during liver injury. A-C: The kinetic expression of FGF-4, HGF and OSM mRNAs detected by real-time PCR during liver injury; D-F: The kinetic expression of FGF-4, HGF and OSM proteins detected by ELISA during liver injury. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* 0 h.

restrained at both mRNA and protein levels (Figure 4C, D and F). Accordingly, the PAS and urea production were also dramatically ($P < 0.01$) decreased in anti-OSM treatment groups (Figure 4G and H). These results suggested that FGF-4, HGF and OSM may play different roles in the hepatic differentiation of mBM-MSCs. Among these factors, FGF-4 and HGF may be essential for the initiation of early hepatic differentiation, while OSM may be critical for the maturation of hepatocytes.

Functional evaluation of FGF-4, HGF and OSM in rescue experiment

In order to find further evidence on the role of FGF-4, HGF and OSM in the conditioned medium in hepatic differentiation, we performed a rescue experiment in which three recombinant cytokines were added back to the cytokine-removed medium treated with anti-FGF-4 (1:100), anti-HGF (1:100) and anti-OSM (1:100). The results showed that administration of FGF-4, HGF and OSM into this cytokine-removed medium could significantly rescue the hepatic differentiation with the increased concentrations of the cytokines as determined by the expression of ALB protein and urea synthesis (E1: 0 ng/mL FGF-4 + 0 ng/mL HGF + 0 ng/mL OSM; E2: 1.25 ng/mL FGF-4 + 2.5 ng/mL HGF + 1.25 ng/mL OSM; E3: 2.5 ng/mL FGF-4 + 5 ng/mL HGF + 2.5 ng/mL OSM;

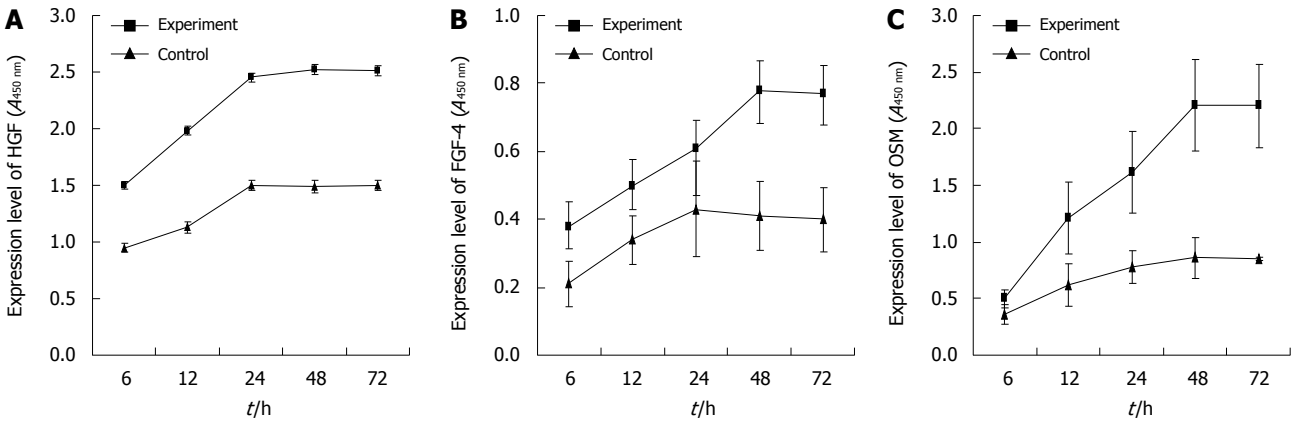


Figure 3 Detection of kinetic secretions of HGF (A), FGF-4 (B) and OSM (C) proteins in liver-injury conditioned medium. Experiment: Hepatocyte-injury conditioned medium; Control: Normal-hepatocyte conditioned medium.

E4: 5 ng/mL FGF-4 + 10 ng/mL HGF + 5 ng/mL OSM) (Figure 5A and B). These results provided solid support that FGF-4, HGF and OSM are the key cytokines that contribute to the induction of hepatic differentiation in the liver-injury conditioned medium.

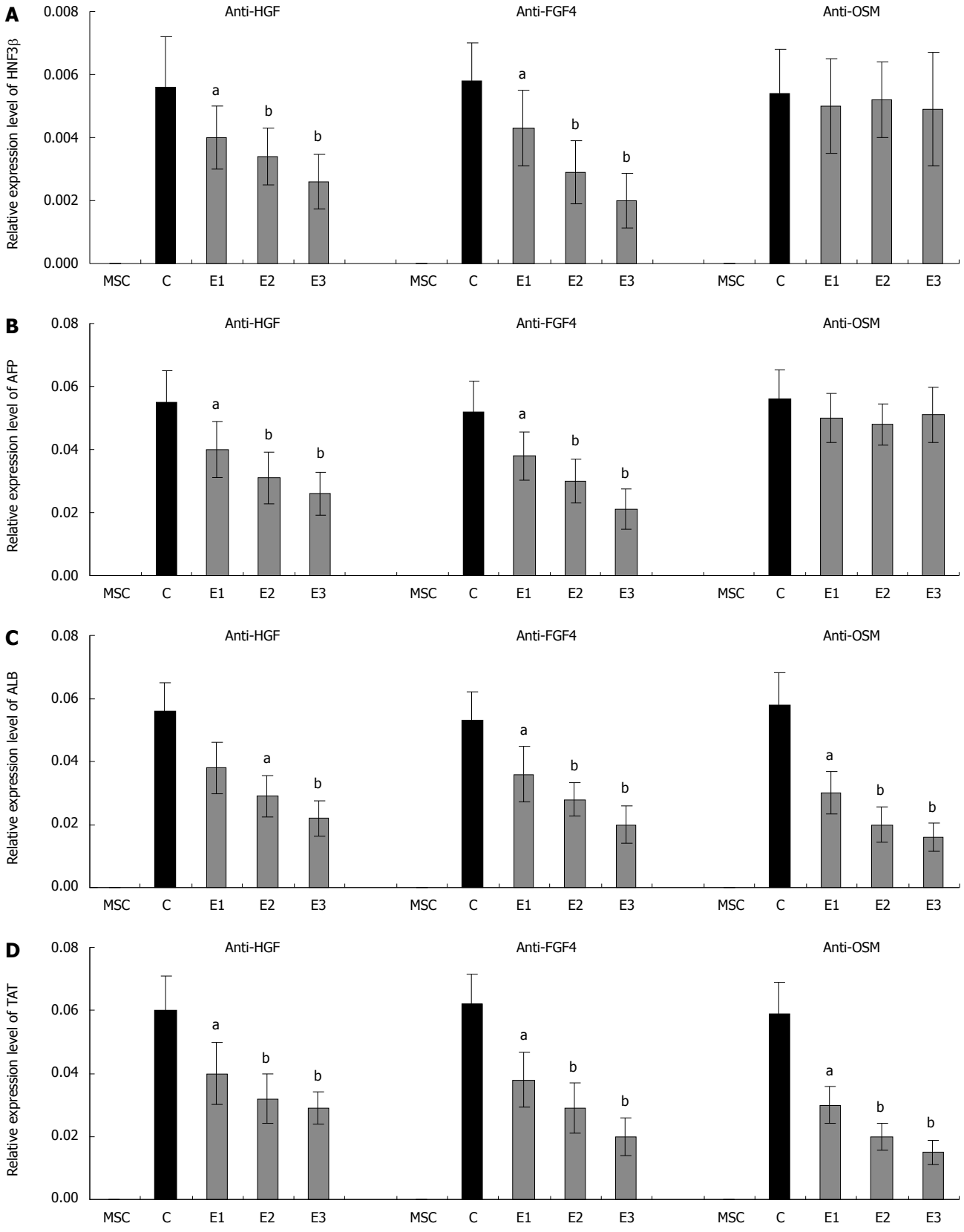
Hepatic differentiation induced directly by FGF-4, HGF and OSM

Based on the observations from the above experiments that FGF-4, HGF and OSM are crucial for hepatic differentiation in the conditioned medium, we performed a further hepatic differentiation experiment induced directly by FGF-4, HGF and OSM in different combinations. The results showed that FGF-4, HGF and OSM may have synergistic effects on the hepatic differentiation of mBM-MSCs, indicating that the three cytokines may play different roles in the induction of hepatic differentiation. As shown in Figure 6, after induced with a factor alone or combination of the two, the hepatic differentiation of mBM-MSCs was lowly detectable as determined by the synthesis of both AFP (early hepatic differentiation marker) and ALB (late hepatic differentiation marker) in differentiated cells. In the group induced with FGF-4 (10 ng/mL) and HGF (20 ng/mL, E4), the concentration of AFP was highly detectable, while ALB was lowly detectable, suggesting that FGF-4 and HGF have a synergistic effect on the initiation of early hepatic differentiation. In contrast, both of the AFP and ALB proteins could be highly detected in group E5 in which the mBM-MSCs were induced by the combination of these three factors. This indicated that FGF-4, HGF and OSM had a synergistic effect in the differentiation of functional hapatocytes from mBM-MSCs. Furthermore, FGF-4 and HGF exhibited a cooperative effect on the early hepatic differentiation of mBM-MSCs, while OSM is essential to the maturation of hepatocytes in the late hepatic differentiation.

DISCUSSION

Mesenchymal stem cells (MSCs) have emerged as a promising resource of functional hepatocytes for treatment of liver diseases because of its plasticity of multiple cell lineages. To date, many inducing systems for hepatic differentiation from MSCs have been developed[22,27,28]. However, the rate of hepatocyte-like cells differentiated from MSCs is still very low and the mechanisms are also not well known. It is undoubted that exposition of MSCs to the inducing systems, resembling the conditions in liver development, injury and regeneration, could acquire a more efficient differentiation[29]. Our previous studies have shown that mBM-MSCs could be induced to differentiate into hepatic cells by conditioned culture medium of hepatocytes[4]. Thus identification of the exact cytokines involved in liver-injury conditions for the mBM-MSCs differentiation needed further studies. This study provides such investigation. Eighteen cytokines closely related to liver growth, repair and development were chosen as candidates from numerous up-regulated cytokine genes by microarray. It was found that three cytokines (FGF-4, HGF and OSM) may play a crucial role in the conditioned medium-induced hepatic differentiation, since hepatic differentiation was dramatically decreased after removing FGF-4, HGF and OSM from the conditioned medium. Therefore, the present study provided a direct basis on the selection of cytokines for hepatic differentiation. However, besides these three key cytokines, some other factors involved in the liver injury need to be identified for improving the cytokine-based inducing system.

FGF-4, HGF and OSM play important roles in liver regeneration, healing, initiation and development. FGF-4 was considered to be one of the most important fibroblast growth factor family members that can irritate the proliferation of mesodermal and endodermal cells and improve development of fetal liver[30]. HGF was found to be essential for the development of several epithelial organs and was one of the most well characterized cytokine for the stimulation of DNA synthesis in primary hepatocyte cultures, and for liver development[31]. The OSM, however, is a member of the interleukin-6 family produced by hematopoietic cells and induces differentiation of fetal hepatic cells, conferring various metabolic activities of


A
Anti-HGF
Anti-FGF4
Anti-OSM
Relative expression level of HNF3β
0.008
0.006
0.004
0.002
0.000
B
Relative expression level of AFP
0.08
0.06
0.04
0.02
0.00
C
Relative expression level of ALB
D
Relative expression level of TAT
a
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MSC
C
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E2
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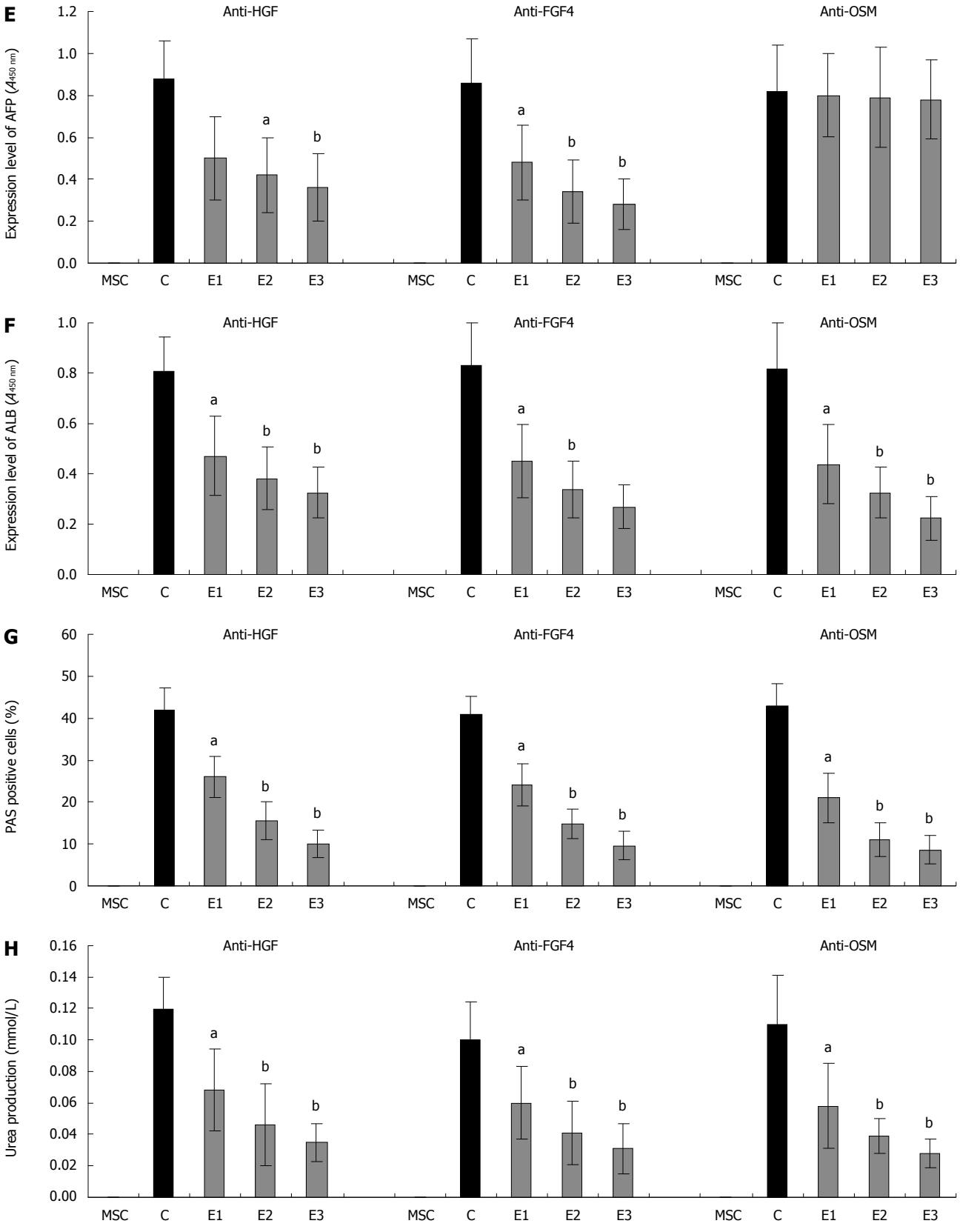

Figure 4 Effects of FGF-4, HGF and OSM in hepatic differentiation. A number of depletion experiments using different combination of antibodies were conducted, and the expression of both liver-specific markers and function was analyzed. A-D: The expression of both the early liver-specific markers (HNF3β and AFP) 10 d after induction and the late liver-specific markers 20 d after induction (ALB and TAT) at mRNA levels detected by real-time PCR; E, F: The expression of AFP 10 d after induction and ALB 20 d after induction at protein levels detected by ELISA; G: Analysis of intracellular glycogen accumulation by PAS staining on day 20 after induction; H: Urea production by differentiated cells on day 20 after induction. MSC group: Control MSCs cultured in medium A with PBS; C group: MSCs induced with liver-injury conditioned medium with non-specific IgG; E1-E3 groups: MSCs induced with liver-injury conditioned medium and different concentrations of specific antibodies (E1: 1:500; E2: 1:200; E3:1:100). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* C group.

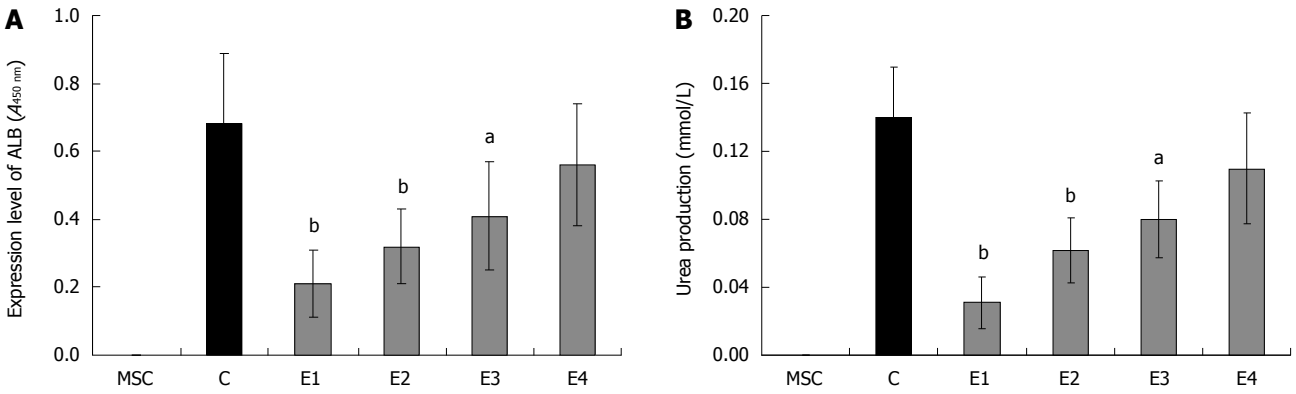

Figure 5 ALB and urea production detection on day 20 after induction in a rescue experiment in which FGF-4, HGF and OSM were added back to the cytokine-removed medium treated with anti-FGF-4 (1:100), anti-HGF (1:100) and anti-OSM (1:100). A: Analysis of albumin secretion by ELISA; B: Urea production was measured using a colorimetric assay kit. MSC group: Control MSCs cultured in medium A with PBS; C group: MSCs induced with liver-injury conditioned medium only; E1-E4: MSCs induced with liver-injury conditioned medium including anti-FGF-4 (1:100), anti-HGF (1:100) and anti-OSM (1:100), as well as different concentrations of the cytokines (E1: 0 ng/mL FGF-4 + 0 ng/mL HGF + 0 ng/mL OSM; E2: 1.25 ng/mL FGF-4 + 2.5 ng/mL HGF + 1.25 ng/mL OSM; E3: 2.5 ng/mL FGF-4 + 5 ng/mL HGF + 2.5 ng/mL OSM; E4: 5 ng/mL FGF-4 + 10 ng/mL HGF + 5 ng/mL OSM). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* C group.

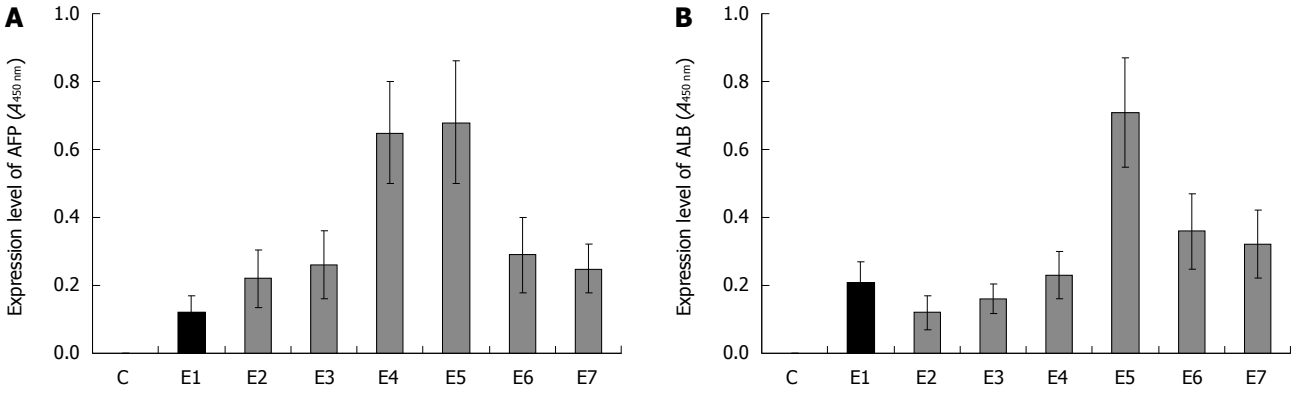

Figure 6 Hepatic differentiation induced directly by different combinations of FGF-4, HGF and OSM. A: Analysis of AFP synthesis by ELISA on day 10; B: Analysis of albumin synthesis by ELISA on day 20. C group: MSCs induced with medium A and PBS; E1-E7: MSCs induced with different combinations of FGF-4, HGF and OSM (E1: OSM 10 ng/mL; E2: HGF 20 ng/mL; E3: FGF-4 10 ng/mL; E4: HGF 20 ng/mL + FGF-4 10 ng/mL; E5: HGF 20 ng/mL + FGF-4 10 ng/mL + OSM 10 ng/mL; E6: FGF-4 10 ng/mL + OSM 10 ng/mL; E7: HGF 20 ng/mL + OSM 10 ng/mL).

adult liver[32]. These three factors participate in different liver developmental stages. Thus, we further examined the exact roles of FGF-4, HGF and OSM in the hepatic differentiation from mBM-MSCs. It clearly showed that after removing FGF-4 and HGF from the conditioned medium by their antibodies, either the early or the late hepatic differentiation induced by the conditioned medium could be significantly down-regulated, while after removing OSM from the conditioned medium, only the late hepatic differentiation was down-regulated. It suggested that FGF-4, HGF and OSM also play different roles in the hepatic differentiation of mBM-MSCs, and FGF-4 and HGF are essential for the initiation of hepatic differentiation, while OSM is critical for the maturation of hepatocytes.

In conclusion, the present study analyzed the potential factors in injured liver for hepatic differentiation from mBM-MSCs. It was found that FGF-4, HGF and OSM might be the key cytokines. They played different roles during hepatic differentiation, which is similar to their functions in liver development. Hopefully, our study would not only provide evidence of cytokine selection for hepatic differentiation, but also benefit the exploration of the molecular mechanisms underlying the differentiation of BM-MSCs into hepatocytes.

COMMENTS

Background

Acute liver failure is a severe liver disease with a mortality of 60%-90%. The only therapeutic option, orthotopic liver transplantation, is limited because of the shortage of suitable donor organs. Mesenchymal stem cells (MSCs), known for their capacity to proliferate indefinitely and differentiate into almost all types of cells, including hepatocytes, have provided the hope of cellular replacement therapy for liver failure.

Research frontiers

Mouse liver-injury conditioned culture medium dramatically facilitated the differentiation of mouse bone marrow MSCs (mBM-MSCs) into functional hepatic cells. However, which cytokines direct hepatic fate specification of mBM-MSCs still remains unclear. In this study, the authors demonstrate that fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM) may play crucial roles in the differentiation of mBM-MSCs in the liver-injury conditioned medium.

Innovations and breakthroughs

In the present study, the authors reported the identification of cytokines involved in hepatic fate specification of mBM-MSCs in the liver-injury conditioned medium. By removing cytokines from conditioned medium and adding back cytokines into the "cytokine-removed" conditioned medium, it was demonstrated that FGF-4, HGF and OSM may play crucial roles in the conditioned medium-induced hepatic differentiation. Furthermore, different combinations of FGF-4, HGF and OSM were used to induce hepatic differentiation, and the result showed that FGF-4 and HGF had a cooperative effect on the early hepatic differentiation of mBM-MSCs, while OSM was essential to the maturation of hepatocytes in the late hepatic differentiation. This is the first study to report that FGF-4, HGF and OSM FGF-4, HGF and OSM not only play crucial roles in the hepatic differentiation of mBM-MSCs, but also have profound synergistic effect on the hepatic differentiation of mBM-MSCs pro and con. This *in vitro* study would contribute to the improvement of hepatic cell resource for cell-based therapies for acute and chronic end-stage liver diseases and provide a model for more detailed characterization on the molecular mechanisms underlying the differentiation of BM-MSCs into hepatocytes.

Applications

This study may benefit not only the better understanding of novel mechanisms underlying BM-MSCs involved in liver repair and regeneration, but also the improvement of cytokine-based hepatic inducing strategy, in which a rich cellular resource for cytotherapy of acute liver diseases with BM-MSCs would be provided.

Terminology

FGF-4 is one of the most important fibroblast growth factor family members that can irritate the proliferation of mesodermal and endodermal cells and improve development of fetal liver; HGF is one of the most well characterized cytokines for the stimulation of DNA synthesis in primary hepatocyte cultures and liver development; OSM is a member of the interleukin-6 family which is produced by hematopoietic cells and induces differentiation of fetal hepatic cells, conferring various metabolic activities of adult liver.

Peer review

The authors corroborate that 3 cytokines (HGF, FGF4 and OSM) are fundamental for directing the differentiation of BM-MSCs towards hepatocytes. The work is interesting and could be helpful for developing effective inducing systems of hepatic differentiation from BM-MSCs.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3279

World J Gastroenterol 2010 July 14; 16(26): 3279-3286
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Randomized controlled trial of minimally invasive surgery using acellular dermal matrix for complex anorectal fistula

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Received: May 13, 2010 Revised: May 31, 2010
Accepted: June 7, 2010
Published online: July 14, 2010

Abstract

AIM: To compare the efficacy and safety of acellular dermal matrix (ADM) bioprosthetic material and endorectal advancement flap (ERAF) in treatment of complex anorectal fistula.

METHODS: Ninety consecutive patients with complex anorectal fistulae admitted to Anorectal Surgical Department of First Affiliated Hospital, Xinjiang Medical University from March 2008 to July 2009, were enrolled in this study. Complex anorectal fistula was diagnosed following its clinical, radiographic, or endoscopic diagnostic criteria. Under spinal anesthesia, patients underwent identification and irrigation of the fistula tracts using hydrogen peroxide. ADM was securely sutured at the secondary opening to the primary opening using absorbable suture. Outcomes of ADM and ERAF closure were compared in terms of success rate, fecal incontinence rate, anorectal deformity rate, postoperative pain time, closure time and life quality score. Success was defined as closure of all external openings, absence of drainage without further intervention, and absence of abscess formation. Follow-up examination was performed 2 d, 2, 4, 6, 12 wk, and 5 mo after surgery, respectively.

RESULTS: No patient was lost to follow-up. The overall success rate was 82.22% (37/45) 5.7 mo after surgery. ADM dislodgement occured in 5 patients (11.11%), abscess formation was found in 1 patient, and fistula recurred in 2 patients. Of the 13 patients with recurrent fistula using ERAF, 5 (11.11%) received surgical drainage because of abscess formation. The success rate, postoperative pain time and closure time of ADM were significantly higher than those of ERAF ($P < 0.05$). However, no difference was observed in fecal incontinence rate and anorectal deformity rate after treatment with ADM and ERAF.

CONCLUSION: Closure of fistula tract opening with ADM is an effective procedure for complex anorectal fistula. ADM should be considered a first line treatment for patients with complex anorectal fistula.

Key words: Acellular dermal matrix; Surgery; Transsphincteric complex fistula

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A ba-bai-ke-re MMTJ, Wen H, Huang HG, Chu H, Lu M, Chang ZS, Ai EHT, Fan K. Randomized controlled trial of minimally invasive surgery using acellular dermal matrix for complex anorectal fistula. *World J Gastroenterol* 2010; 16(26): 3279-3286 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3279.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3279

INTRODUCTION

Anal fistula, an abnormal communication between the anal or rectal lumen and perianal skin, is a common condition in general population, and occurs in 5.6 per 100 000 women and in 12.3 per 100 000 men[1], predominantly in the third and fourth decades of life[2]. It is believed that infection of the intersphincteric glands is the initiating event in anorectal fistula, in a process known as the cryptoglandular hyposis[3]. It is also commonly believed that surgery is the only way to cure it. Up to now, its treatment is diverse due to lack of standard treatment. Incorrect diagnosis and treatment are the important reason of anorectal surgery failure[4]. Traditional surgical procedures include fistulotomy, endorectal advancement flap (ERAF), loose-seton placement, and fibrin glue installation. Anal fistula is described according to the level at which it transgresses the anal sphincter. If the internal opening begins above the anal sphincter, the fistula is described as "high" or transphincteric. Traditional surgery for transsphincteric anal fistula often requires staged operations with fistulotomy and seton insertion. The surgery usually results in large and deep wounds which can take months to heal. Moreover, risk of fecal incontinence is inevitable because part of the anal sphincter is divided during surgery. The success rate of these techniques is disappointed. Fistulotomy invariably requires at least some division of the sphincter muscle with risk of incontinence[4], thus leading to a high recurrence and fecal incontinence. It has been reported that the recurrence rate of ERAF for transsphincteric anal fistula is 0%-63%[5]. Although fibrin glue is an alternative to fistulotomy, its long-term closure rate is low[6-11]. The liquid consistency of fibrin glue is not ideal for closing anorectal fistula, because it is easily extruded from the fistula tract due to the increased pressure[12]. Meta analysis indicates that the healing rate of fibrin glue is not significantly different from that of other sphincter saving procedures for fistula[13]. Complete closure of primary opening and sphincter preserving are the key to successful anorectal fistula surgery. An alternative strategy is to obliterate the fistula tract.

Using a biological substance to close complex anorectal fistula has attracted attention in recent years. A biological anal fistula plug can securely close the primary opening, thus enabling the surgeon to eradicate the fistula tract with a minimal damage to the sphincter. We report our experience with the management of complex anorectal fistulae using acellular dermal matrix (ADM) which is similar to acellular extracellular matrix (AEM). It is a newly developed biomaterial from pigs that have a collagen structure almost identical to that of humans. During manufacturing of the ADM, living cells are removed by special processes to ensure that no transmittable diseases are present in the tissue.

MATERIALS AND METHODS

Study design

Protocol synopsis for this trial and supporting CONSORT checklist were used as supporting information (Figure 1). ADM trial was a single-center, randomized, prospective, single blinded, controlled trial.

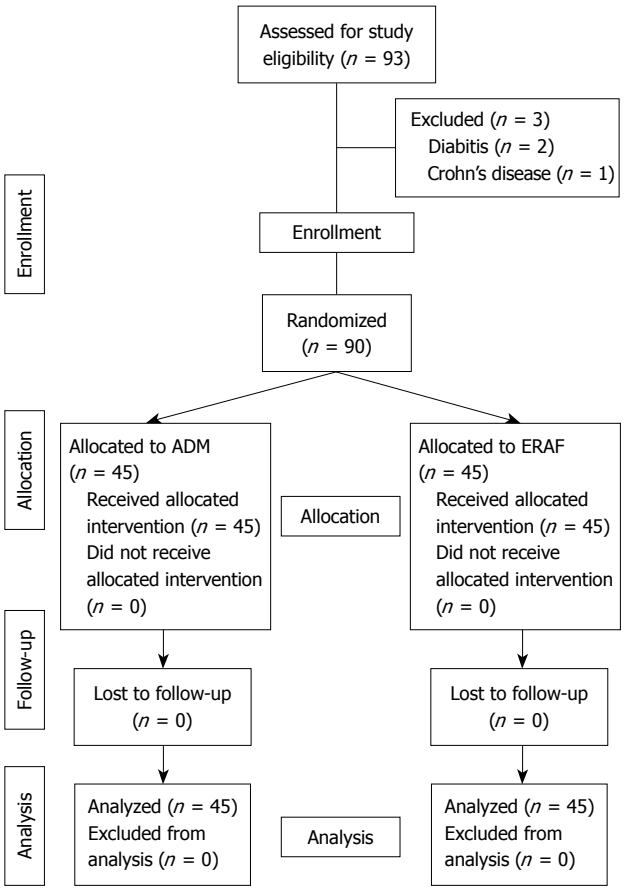

Figure 1 CONSORT diagram. ADM: Acellular dermal matrix; ERAF: Endorectal advancement flap.

Inclusion/exclusion criteria

Patients at the age of 12-60 years with 2-6 cm long intrasphincteric and transsphincteric anorectal complex fistulae identified with a fistula probe during surgery, who gave their informed consent, were included in this trial. Patients with no internal opening found during surgery, and those with positive human immunodeficiency virus, Crohn's disease, malignant cause, tuberculosis, hydradenitis suppurativa, severe cardiovascular state, diabetes, pregnancy, and sepsis were excluded.

Patients

Ninety consecutive patients with complex anorectal fistula, admitted to First Affiliated Hospital of Xinjiang Medical University from March 2008 and July 2009, were randomized into ADM group or ERAF group. Patients were blinded for ADM or ERAF. Demographic data (age and gender of the patients), smoking history, course of disease, fistula types and follow-up time of each patient were recorded (Table 1).

Randomization

Randomization was performed during surgery after the internal opening of fistula was identified. Computer-

Table 1 Demographic data about the patients included in this study

Characteristic	ADM (n = 45)	ERAF (n = 45)	P value
Gender (male/female)	24/21	25/20	NS
Age (SD, range)	18-59 (44.8)	17-61 (45.1)	NS
Smoking history, n (%)	14 (31)	11 (24)	NS
Course of disease (SD, range, mo)	3.0-7.1 (4.6)	2.8-6.9 (5.1)	NS
Type of fistula (intrasphincteric/transsphincteric)	19/26	17/28	NS
Median follow-up time	5.7 (5.1-6.4)	6.1 (5.9-6.5)	NS

ADM: Acellular dermal matrix; ERAF: Endorectal advancement flap; NS: Not significant.

generated random codes were used to produce envelopes containing the information about "ADM" or "ERAF". These envelopes were prepared by a statistician who was not involved in treatment of patients or in other work specific to the study. Computer randomization was completed at Medical Statistical Center, First Affiliated Hospital of Xinjiang Medical University.

Ethics

The trial, conducted in accordance with the Declaration of Helsinki and "good clinical practice" guidelines, local regulations, and China government laws, was approved by the Medical Ethics Committee of First Affiliated Hospital of Xinjiang Medical University. Prior to the randomization, all patients who gave their informed consent were required to comment on the informed consent before the trial.

Sample size and power

Before the trial, sample size was calculated using SPSS software 13.0 version. Armstrong reported that the success rate of anal fistula plug is 87% for the closure of fistula opening[14] and van der Hagen *et al*[5] showed that the success rate of ERAF is 37% for the closure of fistula opening, indicating that to increase the success rate from 40% to 80%, at least 44 patients in each group had to be randomized to achieve a power of 90%.

Surgical technique

All patients underwent a proctology at surgery for configuration of the fistula passage. All patients who were given prophylactic broad-spectrum antibiotics underwent preoperative regular test, endoscopy, anorectal ultrasound and mechanical bowel preparation before surgery. Anesthesia was induced with broad-spectrum parenteral antibiotics and 500 mg of intravenous metronidazole. Patients were placed in the prone jackknife position under spinal or general anesthesia. Primary opening was located using a fistula probe or by injecting hydrogen peroxide into the fistula tract. All fistula tracts were irrigated with a hydrogen peroxide solution. A fistula probe was passed through the fistula tract from the secondary opening and pulled out through the primary opening. After the fistula tract was identified and cleaned with curettage and hydrogen peroxide, ADM materials were prepared according to the length and lumen of the fistula tract, inserted into the clean fistula tracts, and pulled into a position using a silk suture passed through the fistula tract and secured to the tip of ADM, then *via* the secondary opening until it fitted snugly into the primary opening. Excess ADM material was trimmed with the secondary and primary openings flushed and secured to the mucosa and internal sphincter with a 3-0 vicryl suture. All data were recorded and analyzed by the same statistic member who did not attend the intervention.

Rectal advancement flap was done for the control group according to the following techniques. In brief, the primary opening was excised followed by mobilization of the mucosa, submucosa, and a small amount of muscular fibers from the internal sphincter complex. A rectal flap with a 2-3 cm broad base was mobilized. The rectal flap was mobilized sufficiently to cover the internal opening with overlap. Hemostasis was performed to prevent a hematoma under the flap. The fistula tract was curetted. The internal opening was not closed before the flap was advanced over the primary opening. Finally, the flap was sutured to the distal anal canal. All patients were not given analgesics after surgery. Patients were followed up at the discretion of the operating surgeon. Per-operative management (including daily activities, diet) of the two groups was identical.

ADM used in this study was an absorbable J-I type (J. Y. Life Tissue Engineering Co., Ltd., China). It is a complex collagen structure manufactured from the submucosa of porcine small intestine. According to the information about the J-I ADM product, its manufacturing is completely similar to Surgisis AFP™ (Cook Surgical Inc., Bloomington, Indiana, USA). These biologically absorbable xenografts including ADM, AEM, Surgisis or other kinds of anal fistula plug consist of an acellular scaffold similar to the human extracellular matrix[14]. In general, integration into the implant begins within a few days after such materials are placed into the fistula tract through penetrating capillaries.

Postoperative care and follow-up

All patients were hospitalized with a clear liquid diet and bed rest for 48 h. Activity was restricted to minimal. Patients were required to have a warm Sitz bath, 3 times a day. All patients were given intravenous broad-spectrum antibiotics and metronidazole for 3 d after surgery. Stool softeners were used for 10 d. No activity restriction was requested after discharge. Follow-up examination was performed in the outpatient department 2 d, 2, 4, 6, 12 wk, and 5 mo after surgery, respectively. The primary endpoints of this trial were fistula closure rate. Success was defined as closure of all external openings, absence of drainage without further intervention, and no abscess formation. The presence of one persistent fistula tract was considered surgical failure. Outcomes of ADM and ERAF closure were compared in terms of success rate, fecal incontinence rate, anorectal deformity rate, postoperative pain time, closure time and life quality score.

Continence was evaluated before and after opera-

Table 2 Vaizey score system

	Never	Rare	Some-times	Each week	Everyday
Frequency of incontinence	0	1	2	3	4
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Alteration in lifestyle				No	Yes
Need to wear a pad or plug				0	2
Use of constipating medication				0	2
Lack of ability to defer defecation for 15 min				0	4

Table 3 Life quality scale system[16]

Physical
P1 It is difficult for me to get out and do things like going to a movie or to church
P2 I avoid travelling
P3 Whenever I am away from home, I try and stay near a toilet as much as possible
P4 I can't hold on to my bowel motion long enough to get to the bathroom
P5 I try to prevent bowel accidents by staying very near a bathroom
P6 I cut down on how much I eat before I go out
P7 Whenever I go somewhere new, I make sure I know where the toilets are
Social
S1 I avoid visiting my friends
S2 I avoid staying the night away from home
S3 It is important to plan my daily activities around my bowel habit
S4 I leak stool without even noticing it
S5 I can't do many things I want to do
S6 I have sex less often than I would like
S7 I feel different from other people
S8 I avoid travelling by plane or public transport
S9 I avoid going out to eat
S10 I am afraid to have sex
Emotional
E1 I am afraid to go out
E2 I worry about not being able to get to the toilet in time
E3 I feel unhealthy
E4 I feel ashamed
E5 I worry about bowel accidents
E6 I feel depressed
E7 I worry about the smell
E8 I enjoy life less
E9 The possibility of bowel accidents is always on my mind
E10 During the past month have you felt so sad, discouraged, hopeless, or had so many
Problems that you wondered whether anything was worthwhile?
Overall sense of well-being
In general, would you say your health is excellent/very good/good/fair/poor?

tion using the Wexner score and Vaizey scale. The Vaizey scale consists of 3 items about the type (gas, fluid, solid) and frequency of incontinence (Table 2)[15]. The secondary endpoints were healing time, postoperative pain time, postoperative deformity rate, incontinence rate and quality of life. Patients after operation were asked to grade their pain on a visual analogue scale (VAS: 0 = no pain; 10 = worst imaginable pain) at different time points during the follow-up. Fecal incontinence was evaluated according to the Williams grade. Quality of life was evaluated using the life quality scale system (Table 3)[16].

Statistical analysis

Statistical analysis was performed using SPSS software 13.0 version. Recurrence rate, fecal incontinence rate and anal deformity rate associated with each intervention option were assessed by χ^2 test or Fisher's exact test. Healing time and postoperative pain time were calculated by Wilcoxon's test or log rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Three patients were excluded from this trial because of diabetes and Crohn's disease. All patients completed the follow-up during which their data were collected. No patient was lost to follow-up. No significant difference was found in the characteristics of patients including age, sex, classification of fistula and median follow-up time between the two groups. The median follow-up time of ADM and ERAF groups was 5.7 mo (range 5.1-6.4 mo) and 6.1 mo (range 5.9-6.5 mo), respectively ($P = 0.12$). No severe adverse effect occurred in the patients.

The fistula recurred in 2 (4.45%) and 13 (28.89%) of the 45 patients in the ADM and ERAF groups, respectively ($P = 0.0047$), and was healed in 37 (82.22%) and 29 (64.44%) of the 45 patients in ADM and ERAF groups, respectively. Early extrusion of ADM occured in 4 patients, and late extrusion in 1 patient. The overall fistula healing rate was 82.22% (37/45) in ADM group. Five and 1 patients received drainage surgery in ERAF and ADM groups, respectively. The life quality score was higher, the fistula healing time and postoperative pain time were shorter in the ADM group than in the ERAF group ($P < 0.05$, Tables 4-7). The recurrence rate of fistula was significantly lower in the ADM group than in the ERAF group. However, no significant difference was observed in incontinence and anal deformity rate between the two groups. In order to clarify the effect of classification on the results, the efficacy and complication rate of intrasphincteric and transsphincteric fistulae were compared (Tables 5 and 6). The recurrence rate of transsphincteric fistula was significantly lower in the ADM group than in the ERAF group. However, no significant difference was observed in the incontinence and anal deformity rate between the two groups.

DISCUSSION

Surgery is considered the predominant and only procedure for anorectal fistula. Although fistulotomy is a simple procedure for fistula, it is not indicated for transsphincteric fistulae because of prohibitive risk of incontinence. Seton cutting, which can slowly divide the sphincter and prevent recurrent abscess formation, is considered a more efficient procedure for trassphincteric fistula, but can cause incontinence of solid stool in up to 25% of patients[17]. ERAF has become a treatment of choice for most fistulae. However, recent reports sug-

Table 4 Recurrence, fecal incontinence, anal deformity, postoperative pain time, and healing time of patients with ADM or ERAF *n* (%)

	ADM	ERAF	*P* value
n	45	45	
Recurrence	2 (4.45)	13 (28.89)	0.0047
Fecal incontinence	1 (2.22)	4 (8.89)	0.3574
Anal deformity	0 (0)	3 (6.67)	0.2402
Postoperative pain time (d)	1.5 ± 0.5	7.5 ± 1.8	0.0000
Healing time (d)	7.5 ± 3.5	24.5 ± 5.5	0.0000

Table 5 Recurrence, fecal incontinence, anal deformity, postoperative pain time, and healing time of patients with intrasphincteric fistula, ADM or ERAF *n* (%)

	Intra ADM	Intra ERAF	*P* value
n	19	17	
Recurrence	0 (0.00)	3 (17.65)	0.1907
Fecal incontinence	0 (0.00)	0 (0.00)	
Anal deformity	0 (0.00)	0 (0.00)	
Postoperative pain time (d)	1.2 ± 0.4	5.5 ± 1.9	0.0000
Healing time (d)	7.1 ± 3.4	24.6 ± 5.4	0.0000

Table 6 Recurrence, fecal incontinence, anal deformity, postoperative pain time, and healing time of patients with transsphincteric fistula, ADM or ERAF *n* (%)

	Trans ADM	Trans ERAF	*P* value
n	26	28	
Recurrence	2 (7.69)	10 (35.71)	0.0318
Fecal incontinence	1 (3.85)	4 (14.29)	0.9390
Anal deformity	0 (0.00)	3 (10.71)	0.2615
Postoperative pain time (d)	1.5 ± 0.6	8.6 ± 1.4	0.0000
Healing time (d)	7.6 ± 3.6	25.9 ± 5.7	0.0000

Table 7 Life quality score in different groups

Group	*n*	Life quality score	*P* value
ADM	45	85.9 ± 5.3	
Intra ADM	19	87.6 ± 6.5	
Trans ADM	26	83.5 ± 5.7	
ERAF	45	65.3 ± 8.9	0.0000
Intra ERAF	17	64.3 ± 5.1	0.0000[1]
Trans ERAF	28	65.9 ± 7.8	0.0000[2]

[1]Represents comparison between intrasphincteric fistulae using ADM and ERAF; [2]Represents comparison between transsphincteric fistulae using ADM and ERAF.

gest that the recurrence rate of fistula is 36%-45% after ERAF[5,18,19]. ERAF is also technically difficult, and carries a risk of rectal bleeding and incontinence. In our study, hemorrhage occurred in 2 (4.44%) and incontinence occurred in 4 (14.29%) patients in the ERAF group, which are lower than the reported findings[20]. Over the last few years, fibrin glue has been widely described as a better treatment of choice with no side effects, such as pain and incontinence[6,7,9,12,21,22]. However, it was reported that the healing rate of fistula is 78% and 40%-54%, respectively, after the use of fibrin glue[7,23,24], which is not improved even after antibiotics are added or mucosal advancement flap is supplemented[25].

Continence and healing are the two treatment goals to be achieved, but they often conflict with each other. Another sphincter-preserving surgical method is to use bioabsorbable material for anal fistula, which was first reported by Johnson *et al*[14] in 2006 with a success rate of 87%, and O'Connor *et al*[26] reported that the short-term success rate of bioabsorbable material is 80% for anal fistula, indicating that bioabsorbable material can be used in treatment of fistula due to its inherent resistance to infection. Placement of ADM, a minimally invasive procedure for fistula, is an attractive treatment of choice for fistula. Pocrine ADM is a regenerative tissue matrix isolated from decellularized intestines. Similar to Surgisis or other prosthetic meshes, ADM has also been shown to resist infection[27,28]. In our study, the short-term success rate of ADM was 82.22% (37/45) for complex anorectal fistula, which is consistent with the reported findings[26]. In order to compare and evaluate its efficacy, we searched most of the original effects of bioabsorbable material on anorectal fistula across the world (Table 8). Christoforidis *et al*[36] and Ky *et al*[40] showed that the success rate of biologically absorbable substance for complex anorectal fistulas is lower than that reported by Song *et al*[39].

The possible reason why our success rate was much lower than the reported findings[39] is that all the procedures were performed by 4 surgeons. Another possible explanation for this difference may be the selection bias of patients. Contrary to our results, however, Zubaidi *et al*[32] found that trassphincteric fistula is more likely to heal after plug placement. Ky *et al*[40] and Ellis[42] have reported a high success rate of plug placement for trassphincteric fistula. We focused on fistula channel debridement and complete drainage during surgery in order to prevent abscess formation as previously described[31]. Because more granulation tissues in the fistula tract can function as a barrier to cellular infiltration into ADM, it is difficult to imagine that simple irrigation with hydrogen peroxide without thorough curettage of the tract can clean the tract and allow incorporation of ADM material into its surroundings. We believe that the low success rate in the study by Safar *et al*[37] is due to inadequate debridement and curettage of granulation tissue. Complete debridement, curettage of the tract, and hydrogen peroxide irrigation may be important for the surgery to achieve a greater success rate. This point differs from that of Schwandner *et al*[31]. In our study, 5 patients (11.11%) experienced ADM dislodgement, which may be due to the poor ADM fixation to the sphincter in the primary opening. Technical failure associated with plug-falling out is an important reason for surgery failure[31].

In our study, the success rate of ADM for complex fistula was 82.22% (37/45), indicating that placement of ADM is a safe, beneficial, minimally invasive procedure for fistula, and can protect the anal function.

Lawes *et al*[41] proposed to combine anal fistula plug and advancement flap for fistula. However, we hold that selection of patients, complete debridement, tract prepa-

Table 8 Publications on anal fistula plug in the World

Author	Publication form	Total patients (*n*)	Crohn's patients (*n*)	Follow-up median (mo)	Success rate (%)
Adamina *et al*[29], 2010	Article	12	0	12.6 (10-14)	50.0
Schwandner *et al*[30], 2009	Article	60	0	12	62.0
Schwandner *et al*[31], 2009	Article	36	0	9	75.0
Zubaidi *et al*[32], 2009	Article	23	0	12	83.0
Ortiz *et al*[33], 2009	Article	15	0	12	20.0
Chung W *et al*[34], 2009	Article	65	0	12	59.3
Wang *et al*[35], 2009	Article	29	0	18 (9.1-26.8)	34.0
Christoforidis *et al*[36], 2009	Article	37	4	14 (6-22)	32.0
Safar *et al*[37], 2009	Article	35	4	4	14.0
Christoforidis *et al*[38], 2008	Article	49	4	6.5	43.0
Song *et al*[39], 2008	Article	30	0	6.3	100.0
Ky *et al*[40], 2008	Article	45	14	6.5 (3-13)	84.0
Lawes *et al*[41], 2008	Article	20	0	7.4	24.0
Ellis[42], 2007	Article	17	5	6	88.0
van Koperen *et al*[43], 2007	Article	17	1	7	41.0
Ky *et al*[44], 2007	Abstract	37	8	3	84.0
Abbas *et al*[45], 2007	Abstract	17	0	7.4	24.0
Bohe *et al*[46], 2007	Abstract	32	7	6	65.0
Lenisa *et al*[47], 2007	Abstract	27	0	11	63.0
Thekkinkattil *et al*[48], 2007	Abstract	40	0	6	40.0
Poirier *et al*[49], 2006	Abstract	27	0	5	59.0
Champagne *et al*[50], 2006	Article	46	0	12	83.0

ration and plug fixation are closely associated with the final success rate.

It is essential to use antibiotics before surgery. It was reported that anorectal abscess is not formed in patients after treatment with AEM[39] or Surgisis[50]. However, early infection occurred in our study. Although use of Surgisis is not associated with perianal sepsis[14], it has been shown that the incidence of severe perianal sepsis is 23%[41]. Although the sepsis rate of ADM (1/45) was significantly lower than that of ERAF (5/45) in our study, more evidence is needed for the evaluation of ADM. Safar *et al*[37] suggested that the success rate of plug for fistula is not associated with the preoperative bowel preparation, but we performed mechanical bowel preparation before surgery to avoid possible constipation or infection after surgery.

In our study, ADM could obviously shorten the postoperative pain time and the fistula healing time, but not the deformity and incontinence rate of transsphincteric fistula. Transsphincteric fistula recurred in 2 patients, suggesting that it is necessary to perform multicenter randomized controlled trial to evaluate the efficiency of ADM on transsphincteric complex fistula.

A significant number of patients in ERAF group in this study had long-term continence disturbances in forms of gas and liquid stool incontinence, which may be associated with ERAF or fistulotomy, abscess drainage, and other anorectal treatment modalities.

An adequate follow-up time is essential in a comparative study of ADM and ERAF for complex fistula. Only one study is available with a long follow-up time[33]. The follow-up time in our study is similar to that in previous studies[42,43,46]. The success rate of ADM for fistula (82.22%) was associated with the ADM itself and the adequate follow-up time in our study. Zubaidi *et al*[32] showed that a longer follow-up time and over an 80% success rate of biologically absorbable substance for fistula provide better evidence for the use of ADM. Song *et al*[39] reported convincing results based on 6.3 mo follow-up time. However, Wang *et al*[35] showed that the success rate of biologically absorbable substance for fistula is only 34% after a follow-up time of 18 mo.

In conclusion, closure of the fistula tract primary opening using ADM is an effective and acceptable procedure for complex anorectal fistula. ADM is a a safe, beneficial, minimally invasive procedure for fistula, and can protect anal function. ADM dislodgement is associated with the closure techniques. Further longer-term multicenter randomized controlled clinical trials are needed to evaluate its efficacy on complex anorectal fistula.

ACKNOWLEDGMENTS

The authors thank Professor Wei-Li Yan, Department of Epidemiology and Biostatistics, School of Public Health, Xinjiang Medical University, for her help and excellent technical and methodological support to this randomized trial.

COMMENTS

Background

Anal fistula is an abnormal chronic communication between the anal or rectal lumen and perianal skin. Surgery usually results in large and deep wounds which can take months to heal. Moreover, risk of fecal incontinence is inevitable because part of the anal sphincter is divided during surgery. The success rate of different techniques is disappointed. Using a biological substance to close complex anorectal fistula is attractive.

Research frontiers

Acellular dermal matrix (ADM) is a bioabsorbable xenograft for tissue defect. Application of different kinds of ADM in treatment of anorectal fistula is a hotspot in the World.

Innovations and breakthroughs

Closure of the fistula tract primary opening using ADM is an effective and acceptable alternative to complex anorectal fistula. ADM is a safe, beneficial,

minimally invasive procedure for fistula, and can protect anal function. It should be considered a first line treatment for patients with complex anorectal fistula.

Applications

This method can reduce postoperative pain, shorten fistula healing time, and improve the postoperative life quality of patients.

Peer review

This is an interesting and generally well written paper with the effects of endorectal mucosal advancement flap and acellular dermis plug on complex fistula compared. The results are striking.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3287

World J Gastroenterol 2010 July 14; 16(26): 3287-3291
ISSN 1007-9327 (print)

BRIEF ARTICLE

Safety of same-day endoscopic ultrasound and endoscopic retrograde cholangiopancreatography under conscious sedation

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Received: February 18, 2010 Revised: March 25, 2010
Accepted: April 1, 2010
Published online: July 14, 2010

Abstract

AIM: To assess the tolerability and safety of same-day tandem procedures, endoscopic ultrasound (EUS) followed by endoscopic retrograde cholangiopancreatography (ERCP) under conscious sedation.

METHODS: A retrospective review was conducted at Loma Linda University Medical Center, a tertiary-care center. All 54 patients who underwent EUS followed by ERCP (group A) from 2004 to 2006 were included in the study. A second group of 56 patients who underwent EUS only (group B), and a third group of 53 patients who underwent ERCP only (group C) during the same time period were selected consecutively as control groups for comparison.

RESULTS: Conscious sedation was used in 96% of patients in group A. Mean dosages of meperidine and midazolam used in group A were significantly higher than in group B or C. Mean recovery time in group A was not statistically longer than in group B or C. There was no significant difference in the incidence of sedation-related and procedural-related complications.

CONCLUSION: Tandem EUS/ERCP procedure can be safely performed under conscious sedation with minimal adverse events. Combined procedures, however, are associated with higher dosages of sedatives, and slightly longer recovery time.

Key words: Conscious sedation; Safety; Same-day; Endoscopic ultrasound; Endoscopic retrograde cholangiopancreatography

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Che K, Muckova N, Olafsson S, Srikureja W. Safety of same-day endoscopic ultrasound and endoscopic retrograde cholangiopancreatography under conscious sedation. *World J Gastroenterol* 2010; 16(26): 3287-3291 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3287.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3287

INTRODUCTION

Endoscopic ultrasound (EUS) and/or EUS-guided fine needle aspiration (EUS-guided FNA) is increasingly utilized for the diagnosis of pancreatico-biliary diseases including malignancies and choledocholithiasis. The diagnostic accuracy in reported studies of EUS or EUS-guided FNA in the diagnosis of obstructive jaundice is from 80% to 98%[1,2]. In addition, EUS is associated with lower morbidity compared to endoscopic retrograde cholangiopancreatography (ERCP), with an overall EUS-FNA complication rate of 1%-2%[2-4]. EUS is now being performed

first for evaluation in patients with suspected pancreaticobiliary diseases, especially those with obstructive jaundice[5]. Based on EUS findings, therapeutic ERCP interventions, such as stent placement for biliary drainage or stone extraction can then be performed in a tandem fashion.

The literature is scarce on the safety of same-day tandem EUS and ERCP procedures done under conscious sedation. The tandem procedure approach is believed to reduce procedure time and be cost-effective[6]. Potential risks associated with such a strategy are unknown. There are only 2 reported cases in the medical literature of bile leakage into the peritoneum as a result of performing ERCP immediately after EUS-guided FNA[7]. In 2007, Tarantino *et al*[8] evaluated the safety of performing ERCP immediately after EUS in 25 patients done under general anesthesia using propofol. In 1999, Duchmann *et al*[9] also examined the feasibility of performing EUS and ERCP in 57 patients during the same general anesthesia session. Although widely practiced, the safety of performing tandem procedures under conscious sedation is unclear. At our institution, a tandem approach with EUS and/or EUS-guided FNA is sometimes performed for patients with suspected obstructive jaundice, followed by therapeutic ERCP if indicated.

MATERIALS AND METHODS

From October 2004 to November 2005, 54 patients underwent same-day tandem EUS followed by ERCP procedures for the indication of obstructive, or post-hepatic jaundice. This cohort was designated as group A.

From the above time period, 60 consecutive patients who underwent EUS-only and 60 consecutive patients who underwent ERCP-only for the indication of jaundice were chosen as the EUS and ERCP control groups. Of the 60 EUS-only procedures reviewed, only 56 procedures had complete data for analysis (group B). Of the 60 ERCP-only procedures reviewed, only 53 procedures had complete data for analysis (group C).

This retrospective review included: demographics, indications, completion rate of procedures, sedation medication dosages, procedure and recovery times, and adverse events. Vital signs recorded during each procedure and during post-procedure recovery were reviewed. Adverse events included hypotension (defined as systolic blood pressure < 90 mmHg requiring intravenous fluids), bradycardia (heart rate < 60 bpm, or in patients with a baseline heart rate under 60 bpm, heart rate < 45 bpm), and oxygen desaturation (pulse oximetry reading < 90%). The use of reversal agents such as flumazenil and/or naloxone due to oversedation was examined. Post-ERCP pancreatitis was defined as amylase and lipase over five times normal values with abdominal pain and/or leukocytosis persisting 24 h after ERCP. Some of the patients in this study were inpatients, whereas the majority were outpatients. For those who underwent outpatient procedures, it was not standard practice to bring them back for scheduled follow-up laboratory tests.

Statistical analysis

Statistical analysis was performed using StatView version 5.0 for Windows. Numeric variables were expressed as mean ± SD. Differences between groups were analyzed using student's *t*-test for continuous variables. Fisher's exact test was performed for categorical variables but was not done if there was zero in a cell. All differences were considered statistically significant at the *P*-value of < 0.05.

Informed consent was waived for this retrospective study and the research committee at Loma Linda University Medical Center approved the study.

RESULTS

A total of 163 patients were included in this study: tandem EUS/ERCP (group A: $n = 54$), EUS-only (group B: $n = 56$), and ERCP-only (group C: $n = 53$). Mean age, gender, indication, and procedure completion rate are shown in Table 1. There were significantly less males in group A compared to group B, but not significantly different compared to group C. All procedures were performed for the indication of obstructive jaundice. When cholangitis was presumed to be the cause of jaundice, ERCP only, as it should be, was performed. Patients with jaundice and abnormal pancreatico-biliary imaging underwent EUS as part of the work-up.

All patients who underwent EUS in group A and in group B completed the procedures. There was no significant difference in the number of patients who had EUS-FNA in groups A *vs* B (57% *vs* 68%, $P = 0.32$). In group A, ERCP was not completed in five patients compared to seven patients in control group C. Two patients (one each from group A and C) had difficult anatomy and the ampulla was not reached. Eight patients (four from both group A and C) had failed deep cannulation of the desired duct. Two patients from group C had cardiac dysrhythmias (atrial fibrillation and ventricular tachycardia) necessitating early abortion of the procedures.

The amount and type of sedation used is displayed in Table 2. Conscious sedation was used in 96% of group A patients, 100% of group B and 98% of group C patients. General anesthesia and/or propofol were used in three patients, two patients in group A and one patient in group C. The mean total dose of meperidine used in group A was significantly higher than in group B (151.1 ± 64.0 mg *vs* 104.0 ± 43.6 mg, $P < 0.0001$) or in group C (151.1 ± 64.0 mg *vs* 104.5 ± 32.5 mg, $P < 0.0001$). The mean total dose of midazolam used in group A was significantly higher than in group B (8.5 ± 3.2 mg *vs* 6.3 ± 2.4 mg, $P = 0.0001$), or in group C (8.5 ± 3.2 mg *vs* 6.9 ± 3.4 mg, $P = 0.01$).

Procedure and recovery time data are shown in Table 3. The total procedure time for group A was 93.5 ± 36.1 min (range: 30-185 min), 59.0 ± 35.0 min (range: 10-129 min) for group B, and 40.1 ± 20.4 min (range: 20-170 min) for group C. The mean procedure time for EUS in group A, when compared as a separate procedure to the EUS procedure time in group B was not statistically significant (48.0 ± 28.0 min *vs* 59.0 ± 35.0 min, $P = 0.07$). Similarly, the mean procedure time for ERCP in group A, when compared as a

Table 1 Patient demographics, indications, and procedure completion rates *n* (%)

	Tandem (group A) (*n* = 54)	EUS only (group B) (*n* = 56)	*P*-value[1]	ERCP only (group C) (*n* = 53)	*P*-value[2]
Demographics					
Age (yr) (mean ± SD) (Range)	65.2 ± 15.6 (18-93)	63.4 ± 15.7 (19-85)	0.54	54.7 ± 21.3 (14-96)	0.004
Sex, male (%)	43%	66%	0.02	59%	0.12
Indications					
Pancreatic mass	10 (19)	16 (29)	0.26	3 (6)	0.07
Cholangiocarcinoma	3 (6)	1 (2)	0.36	0 (0)	0.24
CBD stones	3 (6)	2 (4)	0.68	6 (11)	0.32
CBD stricture	1 (2)	10 (18)	0.01	1 (2)	1.00
Cholangitis	0	0	NA	8 (15)	NA
Abnormal imaging	10 (19)	10 (18)	1.00	2 (4)	0.03
Pancreatitis	4 (7)	4 (7)	1.00	1 (2)	0.36
Completion					
Completion of procedures	EUS 54 (100) ERCP 49 (91)	56 (100)	1.00	46 (87)	0.56

[1]*P*-value of group A *vs* group B; [2]*P*-value of group A *vs* group C. EUS: Endoscopic ultrasound; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; NA: Not available.

Table 2 Sedation medication used and dosages *n* (%)

	Tandem (group A) (*n* = 54)	EUS only (group B) (*n* = 56)	*P*-value[1]	ERCP only (group C) (*n* = 53)	*P*-value[2]
Sedation type					
Conscious sedation	52 (96)	56 (100)	0.24	52 (98)	0.22
GA/propofol	2 (4)	0	0.24	1 (2)	0.22
Medications used for sedation					
Meperidine	49 (91)	56 (100)	0.03	44 (83)	0.27
Midazolam	54 (96)	56 (100)	0.24	53 (98)	1.00
Diphenhydramine	16 (30)	4 (7)	0.003	28 (53)	0.02
Promethazine	5 (9)	4 (7)	0.74	0	0.06
Fentanyl	7 (13)	0	0.01	8 (15)	0.12
Total dosage of sedatives used (mean ± SD) (range)					
Meperidine (mg)	151.1 ± 64 (25-325)	104 ± 43.6 (25-250)	< 0.0001	104.5 ± 32.5 (25-175)	< 0.0001
Midazolam (mg)	8.5 ± 3.2 (0-16)	6.3 ± 2.4 (2-13)	0.0001	6.9 ± 3.4 (0-16)	0.01
Diphenhydramine (mg)	50 ± 15.8 (25-100)	50 ± 0 (50)	1.00	51.8 ± 9.45 (50-100)	0.64
Promethazine (mg)	25 ± 0 (25)	25 ± 0 (25)	1.00	0	NA
Fentanyl (mcg)	100.0 ± 55.9 (25-175)	0	NA	146.9 ± 54.2 (75-200)	0.12

[1]*P*-value of group A *vs* group B; [2]*P*-value of group A *vs* group C. GA: General anesthesia.

separate procedure to the ERCP procedure time in group C was not significantly different (45.1 ± 20.7 min *vs* 40.1 ± 20.4 min, $P = 0.20$). The mean recovery time in group A was slightly longer than in group B (105.1 ± 74.8 min *vs* 84.0 ± 51.7 min, $P = 0.06$) or in group C (105.1 ± 74.8 min *vs* 84.5 ± 42.6 min, $P = 0.07$).

The complications among the three study groups are reported in Table 4. There was no significant difference in the number of patients who had hypotension, bradycardia, or desaturation among the groups. Reversal agents (flumazenil and/or naloxone) were used in 2% of patients in group A *vs* 0% in group B ($P = 1.00$) *vs* 6% in group C ($P = 0.36$). The number of patients with post-ERCP pancreatitis when comparing group A and group C was similar. One patient in group A was hospitalized due to rectal bleeding that was unrelated to the procedures; a colonoscopy during hospital stay revealed colon cancer. Two patients were hospitalized in group C, one due to oversedation and another due to ventricular tachycardia. The patient admitted for oversedation returned home the following day with no complications. Hypokalemia was the cause of ventricular tachycardia in one patient with metastatic colon cancer who died a month later due to septic pneumonia unrelated to the ERCP procedure.

DISCUSSION

More patients with suspected pancreatico-biliary diseases are now first undergoing EUS with possible EUS-guided FNA. Some of these patients will also undergo ERCP and interventions based on EUS findings. Performing tandem procedures starting with EUS followed by ERCP is logical and is being done at many centers. However, there is little data on its safety when done under conscious sedation. There have been 2 reported cases of bile leak with ERCP following immediately after EUS-guided FNA[7]. Mergener *et al*[10] described a case of massive pneumoperitoneum in a patient who underwent ERCP immediately after EUS-

Table 3 Procedure and recovery times (mean ± SD) (range)

	Tandem (group A) (*n* = 54)	EUS only (group B) (*n* = 56)	*P*-value[1]	ERCP only (group C) (*n* = 53)	*P*-value[2]
EUS time (min)	48 ± 28 (8-110)	59 ± 35 (10-129)	0.07	NA	NA
ERCP time (min)	45.1 ± 20.7 (10-105)	NA	NA	40.1 ± 20.4 (7-90)	0.20
EUS and ERCP time (min)	93.5 ± 36.1 (30-185)	NA	NA	NA	NA
Recovery time (min)	105.1 ± 74.8 (15-350)	84.0 ± 51.7 (9-252)	0.06	84.5 ± 42.6 (20-170)	0.07

[1]*P*-value of group A *vs* group B; [2]*P*-value of group A *vs* group C.

Table 4 Procedure complications *n* (%)

	Tandem (group A) (*n* = 54)	EUS only (group B) (*n* = 56)	*P*-value[1]	ERCP only (group C) (*n* = 53)	*P*-value[2]
Hypotension	4 (7)	7 (13)	0.53	3 (6)	1.00
Bradycardia	12 (22)	14 (25)	0.82	14 (26)	0.66
Desaturation	1 (2)	0	NA	3 (6)	0.36
Use of reversal agent	2 (4)	0	NA	2 (4)	1.00
Post procedure pancreatitis	2 (4)	0	NA	4 (8)	0.68
Hospitalization	1 (2)	0	0.49	2 (4)	0.62

[1]*P*-value of group A *vs* group B; [2]*P*-value of group A *vs* group C.

FNA. Despite this, two other studies have reported the safety of tandem procedures when done under general anesthesia[8,9]. The objective of our study was to assess safety in a retrospective review of tandem cases done under conscious sedation at our center.

Our study reviewed 54 patients with tandem procedures EUS/ERCP along with 56 control patients with EUS only and 53 control patients with ERCP only. Appropriately, patients with presumed cholangitis underwent ERCP only and more patients with obstructive jaundice and abnormal pancreatico-biliary imaging underwent EUS, EUS-FNA as part of the evaluation.

Almost all (96%) of the procedures in the tandem group were done under conscious sedation with meperidine and midazolam. The procedure time for the tandem group was longer than either the EUS or ERCP alone control groups. This is intuitive because two procedures were done at the same setting in the tandem group. Given longer procedures, the amount of meperidine and midazolam was higher in the tandem group as compared to the controls. However, a similar amount of time was needed for EUS and ERCP when the procedures were compared separately to each control group.

In our setting, EUS is performed in the GI Lab at the medical center and ERCP is done at a different location in the radiology suite under fluoroscopy. The total time reported for the tandem group would be even shorter if EUS and ERCP were performed in one place, eliminating patient transfer and transport times.

Given the higher amount of sedation used, the recovery time was slightly longer (although not statistically significant) in the tandem group compared to the two control groups. Despite the longer procedure and recovery time, no significant difference in sedation-related and procedure-related complications were noted in the tandem group compared with the controls. There was no difference in hemodynamic adverse events, use of reversal agents, or rate of hospitalization post procedures. None of the patients with bradycardia required atropine, and none of the patients were intubated due to oxygen desaturation. The post-ERCP pancreatitis rate was 4% in the tandem group, which was statistically similar to the ERCP control group and is within the accepted level. In a large prospective study by Freeman *et al*[11], post-ERCP pancreatitis occurred after 6.7% procedures. No bile leaks were noted with the tandem group.

Nonetheless, there are limitations to our study. Given that the study was a retrospective review, we were unable to assess patient tolerability through direct patient questionnaire. It was not known whether patients were comfortable during the procedures, if they had any recollection of the procedures, or if they would undergo the tandem procedure again. Another limitation was the lack of uniform follow-up of patients to evaluate long-term adverse events due to the tandem procedure. In this study, the few patients who were admitted for observation and those who were already inpatients were all discharged home without complications. It appears that tandem procedures may be safely done under conscious sedation. With the size of groups studied here, no significant complications were noted in the tandem group compared to either study alone. However, it would seem reasonable that a prospective study should be carried out, where a larger number of patients were included and patient tolerance was assessed. Overall, our study is the first of its kind to try to assess issues concerning the safety and feasibility of performing tandem EUS/ERCP procedures under conscious sedation.

In conclusion, a tandem EUS/ERCP procedure can be safely performed under conscious sedation with minimal adverse events. The combined procedure, however, is associated with higher dosages of sedatives and with slightly longer recovery time.

COMMENTS

Background

Same-day endoscopic ultrasound (EUS) followed by endoscopic retrograde cholangiopancreatography (ERCP) is performed at many medical centers for pancreatico-biliary diseases. Little data exists regarding the safety of performing tandem procedures under conscious sedation.

Research frontiers

Efficiency and cost savings are important issues in our current healthcare system, however, this should not be done at the expense of patient safety.

Innovations and breakthroughs

Several studies have examined the safety of combined procedures under propofol or general anesthesia but none, to date, have evaluated the safety of combined procedures under conscious sedation.

Applications

In medical centers where propofol or general anesthesia is not readily available, same-day combined EUS and ERCP under conscious sedation can be performed safely.

Peer review

It is a good manuscript comparing the retrospective data of the procedures (EUS, ERCP and EUS + ERCP) including a good number of patients. This manuscript will be of use for the medical fraternity.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3292

World J Gastroenterol 2010 July 14; 16(26): 3292-3298
ISSN 1007-9327 (print)

BRIEF ARTICLE

Functional magnetic resonance imaging in an animal model of pancreatic cancer

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Supported by A Society of Interventional Radiology Foundation Pilot Research Grant (to Lewandowski RJ) and a Howard Hughes Medical Institute Medical Research Training Fellow (to Eifler AC)
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Received: November 25, 2009 Revised: December 24, 2009
Accepted: December 31, 2009
Published online: July 14, 2010

Abstract

AIM: To test the hypotheses that diffusion weighed (DW)- and transcatheter intraarterial perfusion (TRIP)-magnetic resonance imaging (MRI) can each be used to assess regional differences in tumor function in an animal pancreatic cancer model.

METHODS: VX2 tumors were implanted in pancreata of 6 rabbits. MRI and digital subtraction angiography (DSA) were performed 3 wk following implantation. With a 2-French catheter secured in the rabbit's gastroduodenal artery, each rabbit was transferred to an adjacent 1.5T MRI scanner. DW- and TRIP-MRI were performed to determine if necrotic tumor core could be differentiated from viable tumor periphery. For each, we compared mean differences between tumor core/periphery using a 2-tailed paired *t*-test ($\alpha = 0.05$). Imaging was correlated with histopathology.

RESULTS: Tumors were successfully grown in all rabbits, confirmed by necropsy. On DW-MRI, mean apparent diffusion coefficient (ADC) value was higher in necrotic tumor core (2.1 ± 0.3 mm^2/s) than in viable tumor periphery (1.4 ± 0.5 mm^2/s) ($P < 0.05$). On TRIP-MRI, mean perfusion values was higher in tumor periphery (110 ± 47 relative units) than in tumor core (66 ± 31 relative units) ($P < 0.001$).

CONCLUSION: Functional MRI can be used to differentiate necrotic from viable tumor cells in an animal pancreatic cancer model using ADC (DW-MRI) and perfusion (TRIP-MRI) values.

Key words: Animal model; Functional magnetic resonance imaging; Pancreatic cancer

Peer reviewers: Dr. Mark De Ridder, MD, PhD, Professor, Department of Radiotherapy, UZ Brussels, Vrije Universiteit Brussels, Laarbeeklaan 101, B-1090 Brussels, Belgium; Xiao-Peng Zhang, Professor, Department of Radiology, Peking University School of Oncology, Beijing Cancer Hospital and Institute, No. 52 Haidian District, Beijing 100142, China

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RA. Functional magnetic resonance imaging in an animal model of pancreatic cancer. *World J Gastroenterol* 2010; 16(26): 3292-3298 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3292.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3292

INTRODUCTION

Pancreatic cancer, the fourth leading cause of cancer-related mortality in the United States[1], portends a median survival of 6 mo and a 5-year survival of less than 5%[1-4]. Operative resection is indicated in less than 20% of patients and only increases median 5-year survival to 12%[5,6]. Given these grim statistics and paucity of therapeutic options, this disease represents an arena in which the development of novel therapies is greatly needed.

Since the 1980s, innovative, minimally invasive transcatheter intraarterial techniques to treat malignancies, particularly in the liver, have been developed. These techniques, including bland-, chemo-, and radio-embolization, have been shown to improve survival and/or induce imaging responses while being well tolerated by patients[7,8]. Despite some reports demonstrating preliminary success in the treatment of advanced pancreatic cancer with the transcatheter intraarterial infusion of chemotherapy directly to the pancreas[9-12], few studies have assessed local pancreas-directed therapies.

Before catheter-directed clinical therapies can be developed, it would be useful to develop functional imaging targets in a pre-clinical animal model of pancreatic cancer. Functional imaging is important because conventional anatomic magnetic resonance imaging (MRI) has a low sensitivity to detect pancreatic cancer and separate tumor from adjacent uninvolved pancreatic tissue[13,14]. In addition, even in instances where the tumor can be readily visualized with conventional MRI, therapies might have an effect on tumor function but no effect on overall tumor size. Functional targets could include the assessment of changes in water mobility (diffusion) and tumor perfusion using MRI.

The mouse is the most commonly used animal model of pancreatic cancer[15]. However, catheter-based therapies require an animal model large enough to permit catheterization of mesenteric vessels and tumor sizes large enough that can be visible using clinical MRI scanners. The VX2 model of cancer in rabbits is established in translational studies of uterine[16], liver[17,18], lung[19,20], brain[21,22], and renal neoplasms[23,24]. We have recently established this cancer model in the pancreas[25]. The purpose of this study is to test the hypotheses that (1) diffusion weighted (DW); and (2) perfusion MRI can be used to assess regional differences in tumor function in this rabbit model of pancreatic cancer. If feasible, these functional MRI parameters could potentially be used as surrogate functional endpoints when testing future novel transcatheter pancreatic therapies.

MATERIALS AND METHODS

VX2 animal model

Our Animal Care and Use Committee approved this study. Three VX2 tumors were implanted by an attending surgical oncologist and grown in the pancreata of 6 New Zealand White rabbits using the chunk implantation method[26]. At 2 wk following pancreatic VX2 implantation, MRI scans were obtained using a 1.5-T scanner (Espree; Siemens Medical Solutions, Erlangen, Germany). Rabbits were imaged in the supine position with a single-channel head coil. We obtained T2-weighted turbo spin echo images with the following parameters: 4210/86 (repetition time ms/echo time ms), 3 mm thick sections, 180 mm × 121 mm field of view, 256 × 172 matrix, 150° flip angle, 205 Hz per pixel bandwidth, and two signals acquired. Tumor growth was considered positive when tumor was identified in axial and coronal imaging planes by two independent attending interventional radiologists.

Digital subtraction angiography and catheter placement

We performed X-ray digital subtraction angiography (DSA) using a C-arm unit (Powermobil; Siemens Medical Solutions, Erlangen, Germany) 3 wk after VX2 tumor implantation. The femoral artery was accessed through a surgical cut-down and catheterized with a 22-gauge angiocatheter (Terumo Medical, Somerset, NJ, USA). A 2-F catheter (Cook, Bloomington, IN, USA) was advanced over a 0.014-inch diameter guide wire (Terumo Medical, Tokyo, Japan). We then selectively catheterized the gastroduodenal artery. We performed X-ray DSA of the celiac artery and gastroduodenal artery by using hand injections of iohexol (Omnipaque 350; Amersham Health, Princeton, NJ, USA). Once catheter placement was complete, a 2-0 silk suture in the rabbit's groin was used to hold the catheter in place while the rabbit was transferred to the adjacent MRI suite.

MRI following catheterization

MRI after catheter placement was performed using a Siemens 1.5-T Espree clinical MR scanner. To depict tumor anatomy, we first acquired two-dimensional turbo spin-echo T2-weighted images with the above described scan parameters. DW-MRI was performed with single-shot spin-echo echo planar imaging with the following scan parameters: repetition time/echo time, 4000/93 ms; slice thickness 3 mm; bandwidth, 1185 Hz/pixel; partial Fourier factor, 6/8; nonselective fat saturation; twice refocused spin-echo diffusion weighting to reduce eddy current-induced distortion with b values of 0, 50 and 500 s/mm^2. DW-MRI measures changes in the mobility of water as a means of differentiating viable and highly cellular regions from acellular or necrotic regions of tumors. The mobility of water is measured using the apparent diffusion coefficient (ADC). ADC maps, which showed water mobility measurements corresponding to separate spatial locations, were reconstructed from each series of DW images. In these ADC maps, signal intensity directly correlates with water mobility. Using T1-weighted contrast agent-enhanced images as a reference, we drew regions of interest to calculate mean tumor ADC values. Regions of interest were also drawn to compare the ADC values for the necrotic core and the viable outer ring typically present in VX2 tumors.

For tumor perfusion imaging, we used transcatheter intraarterial perfusion (TRIP)-MRI[27], an innovative first-pass perfusion technique employing direct catheter-based intraarterial injections of contrast medium. This technique can be used to detect intra-procedural changes in perfusion to targeted tumor cells and surrounding parenchyma[28]. For TRIP-MRI, we used a three-dimensional spoiled gradient echo sequence with the following parameters: repetition time/echo time, 5/1.6 ms; 15° flip angle; contiguous axial slices of 3 mm thickness; eight partitions; 200 mm × 100 mm field of view; 128 × 64 matrix; and 660 Hz/pixel bandwidth. This TRIP-MRI sequence is a real-time three-dimensional MR fluoroscopy technique[29] that rapidly and continuously images the entire tumor during transcatheter contrast medium injection. TRIP-MRI scans were obtained during hand injections of 2 mL 20% gadopentetate dimeglumine solution (Magnevist; Berlex, Wayne, NJ, USA) over 5 s *via* a catheter previously placed during DSA. Each contrast medium injection was immediately followed by a 4-mL saline solution flush injected over 5 s. For each TRIP-MRI scan, the entire pancreas area including tumor(s) was continuously sampled at 1.6-s intervals for 100 s.

Before and after TRIP-MRI, we obtained anatomic images with two-dimensional T1-weighted gradient-echo MRI. The T1-weighted scan parameters were as follows: repetition time/echo time, 193/6 ms; average of 2; flip angle of 80°; bandwidth of 475 Hz/pixel; slice thickness of 3; 256 × 160 matrix; and field of view of 180 mm × 113 mm.

With the baseline R_{10} map, a longitudinal relaxation rate R_1 map time series was derived from the signal intensity ratio between the baseline image and each TRIP-MRI series with nonlinear curve fitting. Increases in R_1 after injection are proportional to increases in contrast agent concentration. For each R_1 map time series, we calculated the first-pass area under the curve for each voxel, thereby producing spatially resolved perfusion maps for each TRIP-MRI scan. Regions of interest were drawn to measure mean tumor area under the curve. Each TRIP-MRI area under the curve (AUC) measurement served as a semi-quantitative index of tumor perfusion. Tumor regions of interest were placed in peripheral hypervascular regions to avoid the necrotic core.

Necropsy and histopathology

Once the MRI was completed, each rabbit was sacrificed with intravenous administration of 150-200 mg/kg sodium pentobarbital (Euthasol; Delmarva Laboratories, Midlothian, VA, USA). Each rabbit tumor was harvested for pathologic confirmation of VX2 tumor growth and location in pancreatic tissue. Pancreatic tumors were inspected grossly for anatomic consistency with MR images. Tumor samples, including surrounding tissue, were then removed, embedded in paraffin, and mounted on glass slides. These 4-μm-thick slices were stained with hematoxylin and eosin. An attending surgical pathologist performed histopathologic analysis using a Zeiss Axioskop Confocal (Germany) microscope and Zeiss Plan (NEOFLUAR 2.5 ×) objective lens. Imaging was performed with a Cambridge Research and Instrumentation model N-MSI-420-FL camera and Cri Nuance Multispectral Imaging System version 1.6 software.

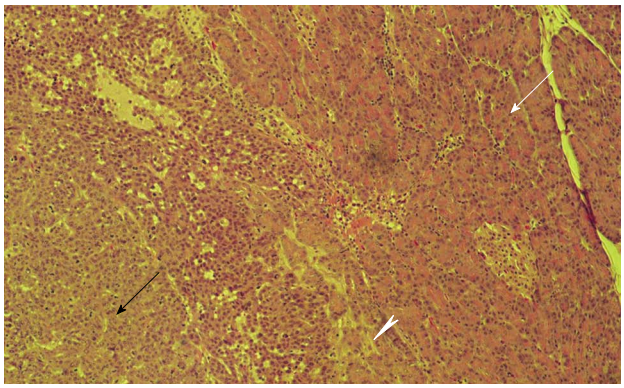

Figure 1 Histopathologic image obtained at necropsy. Border between viable VX2 tumor cells (black arrow) and healthy pancreatic tissue (white arrow) with irritated pancreatitis-like tissue at the tumor-pancreas interface (arrowhead). Note islet of Langerhans within healthy pancreatic tissue. Hematoxylin-eosin stain; original magnification × 25.

Statistical analysis

MR and X-ray DSA images were viewed in conjunction with one another. All MR images were analyzed on a computer workstation (Argus; Siemens Medical Solutions). We compared mean differences between necrotic tumor core and viable tumor periphery for DW-MRI (ADC value) and TRIP-MRI (AUC) using a 2-tailed paired *t*-test, with $\alpha = 0.05$. Mean values were reported ± 1SD. All statistical tests were performed using InStat version 3.06 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Eleven tumors were successfully grown in 6 rabbits, as confirmed by necropsy (Figure 1). Tumors in all 6 rabbits were successfully depicted using anatomic and DW-MRI. Anatomic T2-weighted images showed increased signal intensity within the tumors compared to surrounding tissues (Figure 2).

With DW-MRI, tumors showed regions of low water mobility within the viable periphery, as signified by increased signal intensity (SI) at higher *b* values (Figure 3). This finding suggests increased cellularity compared to the tumor core and surrounding tissues. The mean ADC value was higher in the necrotic tumor core (2.1 ± 0.3 mm^2/s) than in the viable tumor periphery (1.4 ± 0.5 mm^2/s) ($P < 0.05$).

TRIP-MRI was successful in 5 of 6 rabbits. One rabbit could not be catheterized for DSA due to severe vasospasm. TRIP-MRI scans showed tumor perfusion to the peripheral viable portions of the tumor, with lack of perfusion to the central necrotic portions of the tumor (Figure 4). Mean AUC was significantly higher in the periphery (110 ± 47 relative units) than in the central core (66 ± 31 relative units) ($P < 0.00002$) (Figure 5).

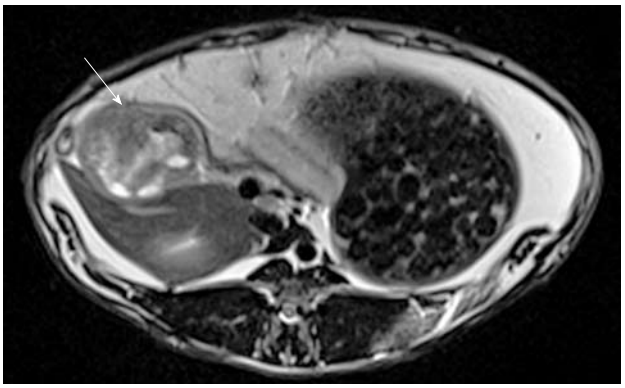

Figure 2 T2-weighted turbo spin echo magnetic resonance imaging showing pancreatic tumor (arrow) in axial view.

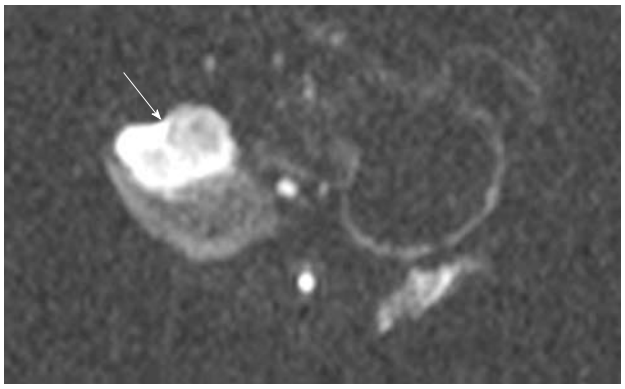

Figure 3 Single shot spin-echo planar diffusion weighted MRI (b = 500 s/mm^2) showing pancreatic tumor (arrow) in axial view.

When comparing DW- and TRIP-MRI, regions of relatively low water mobility correlated with relatively greater tissue perfusion.

DISCUSSION

The results of this study confirm the hypothesis that functional MRI can be used to differentiate necrotic from viable tumor cells in an animal model of pancreatic cancer. Regions of necrosis were delineated from viable tumor cells by larger ADC values on DW-MRI and reduced perfusion values with TRIP-MRI. These results are relevant because the differentiation between malignant and uninvolved or adequately treated pancreatic tissue is difficult with conventional anatomic MRI.

In several preliminary clinical studies enrolling 8-26 patients, DW-MRI has been shown to be able to detect pancreatic carcinoma and differentiate neoplasm from confluent pancreatitis[30-32]. Viable tumor cells are highly cellular and have intact cell membranes. This restricts the motion of water molecules and results in a decrease in the ADC value. Conversely, cellular necrosis causes increased membrane permeability, allowing free diffusion of water molecules and a marked increase in the ADC value. DW-MRI has also been shown to be able to accurately predict the degree of tumor necrosis in hepatocellular carcinoma following transcatheter liver-directed therapy[33-36]. Our current study demonstrates that DW-MRI can be used to differentiate necrotic from viable cells in this animal model of pancreatic cancer.

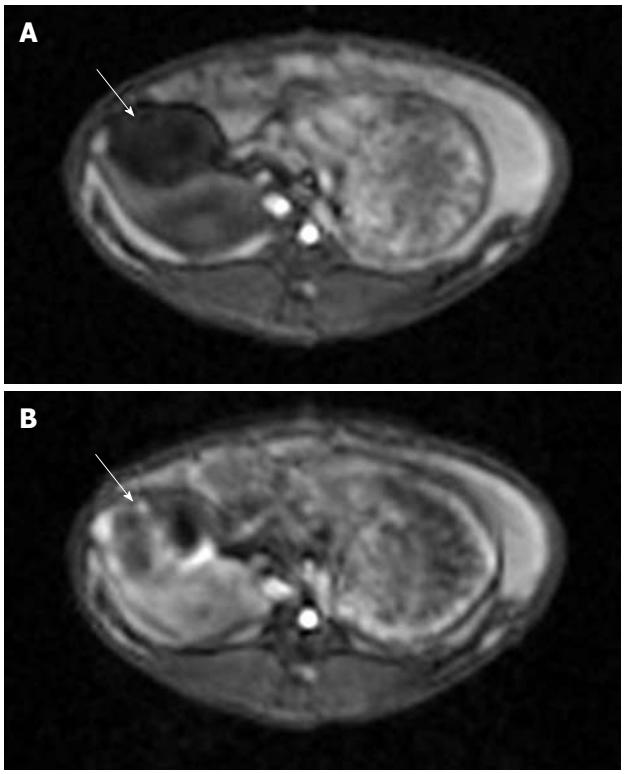

Figure 4 T1-weighted gradient-echo magnetic resonance [taken before (A) and after (B) TRIP imaging occurred]. Each image shows a VX2 tumor (solid arrows) located in the pancreas. Note the areas of increased perfusion to the viable tumor periphery in image B.

TRIP-MRI uses catheter-directed intraarterial injections of gadolinium. This technique is distinct from dynamic contrast-enhanced (DCE)-MRI, which uses intravenous injections of gadolinium. TRIP-MRI has several advantages over DCE-MRI including the capacity for dramatically lower volumes of contrast agents and the ability to perform serial injections without needing to wait > 1 h for injected contrast agent to wash-out. A pre-clinical study in the VX2 rabbit liver tumor model has validated the utility of TRIP-MRI to monitor iterative changes in liver tumor perfusion during embolization[27]. This technique has been translated clinically to measure intra-procedural changes in perfusion to targeted tumors during the treatment of patients with hepatocellular carcinoma with chemoembolization[28]. Because of its ability to enhance tissue distal to the catheter tip, TRIP-MRI can also verify that tumors will be adequately targeted by localized catheter-based techniques prior to their injection. This study demonstrates that TRIP-MRI can be used to detect differences in perfusion to necrotic and viable pancreatic tumor cells.

The development of localized therapies for unresectable pancreatic cancer represents a new avenue for research. As recently demonstrated by Olive *et al*[37], a stromal

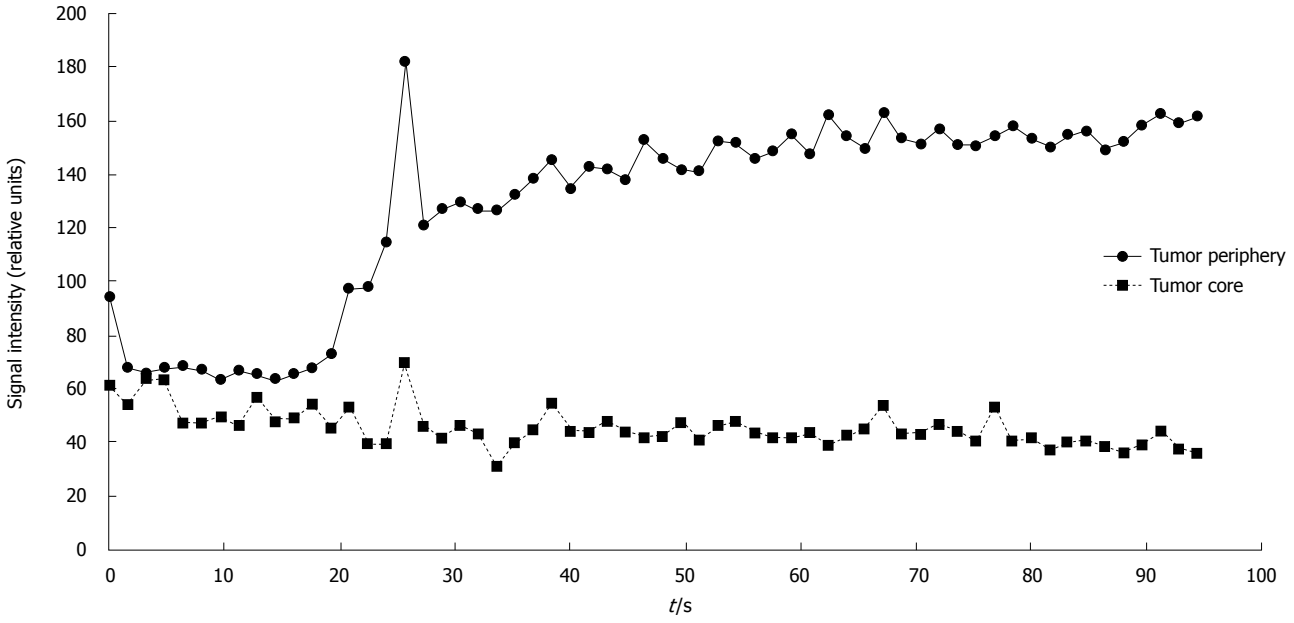

Figure 5 Signal intensity *vs* time curve for one pancreatic tumor, depicting higher signal intensity in the viable tumor periphery than in the necrotic tumor core.

desmoplastic reaction encapsulating pancreatic tumors greatly impedes systemic therapies and thus localized therapies that are able to greatly increase the local concentration of therapeutics may offer benefit in overcoming this fibrotic capsule. Preliminary studies have reported on transcatheter intraarterial infusion of chemotherapy for advanced pancreatic cancer[9-11], which may provide improved response to therapy[12]. The functional MRI parameters described in this animal model of pancreatic cancer could potentially be used as surrogate functional endpoints to test such transcatheter pancreatic therapies. The animal model and functional MRI techniques described in this paper could also be used to assess novel localized therapies intended to target some of the molecular mechanisms underlying pancreatic cancer, including a mutant *p53* tumor suppressor gene[38] or other inhibitors of apoptosis, such as survivin[39,40].

Our study had several important limitations. First, the molecular mechanisms that govern pancreatic ductal adenocarcinoma and VX2-based tumors differ. VX2 is a squamous cell carcinoma that does not have the alterations in the multiple molecular pathways seen with true pancreatic cancer[41]. Our model, however, does mimic well the position and blood supply of a human pancreatic tumor which makes it well suited to the translational development of localized therapies. Second, the smaller diameter of rabbit pancreatic arteries precluded direct catheterization of the pancreaticoduodenal, dorsal pancreatic, great pancreatic, and caudal pancreatic arteries. These arteries are also extremely difficult to selectively catheterize in patients. Additionally, TRIP-MRI verified that perfusion to viable pancreatic tumor cells could be achieved *via* catheterization of the rabbit gastroduodenal artery. Third, although functional imaging in animals is not entirely novel, previous studies have focused on rodents instead of rabbits[42], and have primarily employed high field strength magnets[43]. Using a 1.5-T scanner, we believe, allows our findings to be more readily translated into future clinical studies. Finally, we did not assess the effect of therapy in this animal model. Such assessment will be the subject of future studies.

In conclusion, functional MRI parameters can be used to differentiate necrotic from viable tumor cells in an animal model of pancreatic cancer. Regions of necrosis are delineated by larger ADC values on DW-MRI and reduced perfusion values with TRIP-MRI. In a disease that so desperately needs a more effective treatment, these functional MRI parameters could potentially be used as surrogate functional endpoints when testing future novel local transcatheter pancreatic therapies.

COMMENTS

Background

Pancreatic ductal adenocarcinoma (commonly referred to as pancreatic cancer) carries the worst prognosis of any cancer, thus novel therapies must be developed to treat this disease. Because evaluation of these new therapies may be difficult, imaging modalities to assess therapeutic efficacy based on tumor necrosis are desirable.

Research frontiers

Functional magnetic resonance imaging (MRI) modalities are quickly emerging as new imaging methods with the potential to provide more information about structures in the body than traditional anatomic MRI. In this study, the authors demonstrate that the use of two specific types of functional MRI: diffusion-weighted MRI (DW-MRI) to measure tissue diffusion; and transcatheter intraarterial perfusion MRI (TRIP-MRI) to measure perfusion, could be used to differentiate tumor cells that are dead from those that are still alive.

Innovations and breakthroughs

This study is the first to combine cutting edge imaging modalities (DW-MRI and TRIP-MRI) performed using a clinical 1.5T MRI scanner with a recently created rabbit model of pancreatic cancer which allows for intraarterial catheterization.

Applications

By characterizing this model of pancreatic cancer using functional MRI, this

study may allow for more effective development of much-needed novel therapeutics for the treatment of this disease.

Terminology

DW-MRI and TRIP-MRI are types of MRI called "functional" MRI because they provide information about tissue function in addition to anatomical information provided by traditional MRI. DW-MRI is used to acquire a value called the apparent diffusion coefficient which is a relative measure of water diffusion within a tissue. TRIP-MRI is used to acquire a measure of relative perfusion which represents the amount of blood flow to a tissue.

Peer review

The manuscript is well written and methodology is accurately described. The manuscript applied function MRI in pancreas tumor model and results showed that MR-DWI and TRIP-MRI can distinguish the necrotic tissue form living tissue of pancreas tumor model. This gives us more confidence on the potential of function MRI in the therapy response evaluation. Limitations of the study are considered by the authors themselves and substantiated in the discussion.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3299

World J Gastroenterol 2010 July 14; 16(26): 3299-3304
ISSN 1007-9327 (print)

BRIEF ARTICLE

Wireless capsule endoscopy and proximal small bowel lesions in Crohn's disease

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Supported by (in part) The Foundation "Fondazione Umberto Di Mario, Largo Marchiafava", Rome, Italy
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Received: November 6, 2009 Revised: December 11, 2009
Accepted: December 18, 2009
Published online: July 14, 2010

Abstract

AIM: To investigate the prevalence of proximal small bowel (SB) lesions detected by wireless capsule endoscopy (WCE) in Crohn's disease (CD).

METHODS: WCE was performed in 64 patients: 32 with CD of the distal ileum, and 32 controls with iron-deficiency anemia (IDA) or diarrhea. WCE was performed using the Given SB-WCE, followed by small intestine contrast ultrasonography (SICUS). Findings compatible with CD by using WCE included erosions, aphthoid or deep ulcers, and strictures/stenosis.

RESULTS: WCE detected proximal SB lesions in 16/32 (50%) patients (14 aphthoid ulcers, 2 deep ulcers, one stricture), which appeared not to be related to clinical parameters [epigastric pain, age, smoking, non-steroidal anti-inflammatory drugs (NSAIDs), IDA]. Among patients with proximal SB lesions, 6 (37%) were smokers, 3 (19%) NSAID users, 3 (19%) had epigastric pain and 4 (25%) had IDA. SICUS detected proximal SB lesions in 3/32 patients (19%) also showing lesions with WCE. No correlations were observed between proximal SB lesions assessed by WCE or by SICUS ($\chi^2 = 1.5$, $P = 0.2$).

CONCLUSION: The use of WCE allows the detection of previously unknown upper SB lesions in a high proportion of patients with a previous diagnosis of CD involving the distal ileum.

Key words: Wireless capsule endoscopy; Crohn's disease; Small bowel

Peer reviewer: Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

Petruzziello C, Onali S, Calabrese E, Zorzi F, Ascolani M, Condino G, Lolli E, Naccarato P, Pallone F, Biancone L. Wireless capsule endoscopy and proximal small bowel lesions in Crohn's disease. *World J Gastroenterol* 2010; 16(26): 3299-3304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3299.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3299

INTRODUCTION

Wireless capsule endoscopy (WCE) is a non-invasive technique for visualizing the mucosal surface of the small bowel (SB)[1-7]. However, a variable impact risk (from 0%-6.7%) has been reported[8]. WCE showed a high sensitivity and specificity for detecting lesions related to SB Crohn's disease (CD)[1-7]. A meta-analysis showed an incremental diagnostic yield of WCE *vs* small bowel follow through (SBFT) ($P < 0.001$), ileocolonoscopy ($P =$

0.02), computed tomography enteroclysis ($P = 0.001$) and push enteroscopy ($P < 0.001$)[9]. WCE has in particular been shown to be able to detect minor lesions (erosions, aphthoid ulcer), not visualized by conventional radiologic techniques, which result in high radiation exposure. WCE has therefore been proposed as an alternative non-invasive technique for assessing CD lesions.

Ultrasonography also is a non-invasive technique proposed for detecting SB lesions in CD[10]. The use of an oral contrast [small intestine contrast ultrasonography (SICUS)] significantly increases, in experienced hands, the sensitivity of ultrasonography for assessing SB lesions in CD (> 95%)[11,12].

Although several studies concordantly showed that WCE is able to visualize superficial lesions in the SB, its role in defining the extent of the lesions in CD is undefined. In particular, the frequency and clinical relevance of superficial lesions in the upper SB as detected by WCE, but not by conventional techniques, in patients with an established diagnosis of CD involving the distal ileum, is currently unknown. Disease-specificity of the small lesions as detected by WCE is also under investigation.

On the basis of these observations we therefore aimed to assess, in a prospective longitudinal study in patients with a known diagnosis of CD of the distal ileum, the prevalence of lesions in the proximal SB (jejunum, proximal ileum) compatible with CD, as assessed by WCE. The secondary end point was to evaluate the possible concordance between WCE and SICUS, in detecting SB lesions compatible with CD. Additional end points included investigation of possible correlations between proximal SB lesions in WCE and specific signs and symptoms, including anemia and/or epigastric pain in patients with CD. A comparison between characteristics of SB lesions detected by WCE in patients with CD *vs* patients undergoing WCE for other indications was also performed. The safety of WCE in CD patients with no radiological or clinical evidence of sub/obstructive symptoms was further addressed.

MATERIALS AND METHODS

Study population

In a prospective longitudinal study, WCE was performed in all consecutive CD patients referred to our Unit from May 2004 to May 2008, fulfilling the following inclusion criteria: (1) age 18-75 years; (2) regular follow-up; and (3) established diagnosis of CD involving the distal ileum, according to standard procedures.

As a control group (C), WCE was performed in all consecutive patients referred to our Unit from May 2004 to May 2008, with the following inclusion criteria: (1) age 18-75 years; and (2) clinical indication for WCE such as iron-deficiency anemia (IDA) or chronic diarrhea of unknown origin with no diagnosis by conventional procedures. No patients showed evidence of stenoses/strictures. Written informed content was provided by all patients.

Study design

CD group: Before WCE, recorded parameters included: findings at physical examination, activity (CD activity index, CDAI)[13], epigastric pain (yes/no), IDA, laboratory tests (complete blood count, hemoglobin, serum iron, ferritin, creatine phosphate, erythrocyte sedimentation rate), non-steroidal antiinflammatory drug (NSAID) use, and smoking habit. Before WCE, all 32 patients were studied by both SICUS and ileocolonoscopy (median time interval 1 mo, range 0-7 mo, and 1 mo, range 0-14 mo, respectively). After WCE, patients were clinically followed up at least 12 mo (median 24 mo, range 12-36 mo).

C group: Before WCE, recorded parameters included: findings at physical examination, gastrointestinal symptoms, laboratory tests (as above), NSAID use, and smoking habit.

SICUS

SICUS was performed after 375 mL polyethylene glycol (PEG) ingestion[11] using 3.5 and 5 MHz convex and linear-array transducers, by the same expert gastroenterologist (> 2000 examinations). Findings compatible with CD included: increased bowel wall thickness (BWT) (≥ 3 mm), SB dilation (diameter > 2.5 cm), bowel stricture (diameter < 1 cm, at the level of the maximally distended loop)[11,12]. Fistulas or abscesses were considered.

Ileocolonoscopy

All endoscopies were performed by the same gastroenterologist, according to standard procedures.

WCE

WCE was performed with the Given Pillcam SB capsule system (Given Imaging Limited, Yoqneam, Israel)[1] after 3 d of a fiber-free diet and bowel preparation [2 L PEG, (Promefarm, Milano, Italy)]. Images were reviewed by a single gastroenterologist unaware of the SICUS findings.

CD group: The following WCE findings were considered compatible with CD: aphthoid ulcers (> 3), deep ulcers, strictures or stenoses. Erosions, villous dropouts and mucosal breaks were reported, although considered not related to CD. As no standard criteria for defining upper SB lesions using WCE were available, distal SB lesions were considered lesions proximal to the ileo-cecal valve or to the ileo-colonic anastomosis. Upper SB lesions were considered the SB lesions proximal to these areas (jejunum, proximal ileum).

In a subgroup of 10 CD patients with ileo-colonic resection, WCE findings were blindly scored by 2 independent gastroenterologists. For this purpose, lesions in the peri-anastomotic area and in the upper SB were graded as follows[5]: absent (G0), erythema/loss of villi (G1), erosions/aphthoid ulcers (G2), deep ulcers (G3).

C group: Any lesion detected by WCE was reported. A comparison with the CD group considered only those lesions in the upper or distal SB compatible with CD, including: aphthoid or deep ulcers, strictures, stenoses. Planar X-ray of the abdomen was performed in all patients

Table 1 Clinical characteristics of the 32 CD patients studied with WCE

Parameter	*n* (%)
Gender	
Male	16 (50)
Female	16 (50)
Clinical activity	
Active (CDAI > 150)	5 (16)
Inactive (CDAI < 150)	27 (84)
Lesions extent before WCE	
Distal ileum only	27 (84)
Distal ileum and colon	2 (6)
Distal ileum and esophagus/stomach	3 (10)
Previous intestinal resections	
Yes	25 (78)
Smoking habits	
Smokers	14 (44)
Ex-smokers	4 (12)
Non-smokers	14 (44)
NSAID use	
Yes	3 (9)

CD: Crohn's disease; WCE: Wireless capsule endoscopy; CDAI: CD activity index; NSAID: Non-steroidal anti-inflammatory drug.

with no WCE excretion after 48-72 h. Retention was defined as WCE persistence after 14 d. Incomplete studies were defined when WCE did not reach the cecum.

Comparison between WCE and SICUS findings

Comparison between WCE and SICUS in terms of findings compatible with SB lesions related to CD was made using the following parameters: presence (yes/no), site (upper *vs* distal SB) and severity of the lesions (deep *vs* aphthoid ulcers) by using WCE and presence (yes/no) and site (upper *vs* distal SB) of increased BWT ($\geqslant$ 3 mm) using SICUS. Correlations between WCE findings compatible with upper SB lesions and clinical parameters (age, smoking habits, epigastric pain, IDA) were determined.

Statistical analysis

Results were expressed as median and range both in the text and in the tables. Differences between groups were assessed by the Student's *t*-test. The interobserver variation in terms of presence and severity of upper SB lesions detected by WCE was assessed.

RESULTS

Study population

CD group: Thirty two consecutive patients (16 male, median age 32 years, range 19-65 years) with an established diagnosis of CD of the distal ileum fulfilled the inclusion criteria. Clinical characteristics of the enrolled patients are summarized in Table 1.

Treatment included budesonide (9 mg/d) in 6 (19%) and mesalazine 2.4 g/d in 26 (81%) patients. Among the 32 patients, 4 (12%) had IDA and 7 (22%) epigastric pain.

C group: Thirty two consecutive C patients (16 male, median age 42 years, range 18-72 years) undergoing WCE fulfilled the inclusion criteria. Clinical indications for WCE included IDA in 17 (53%) and chronic diarrhea in 15 (47%) patients.

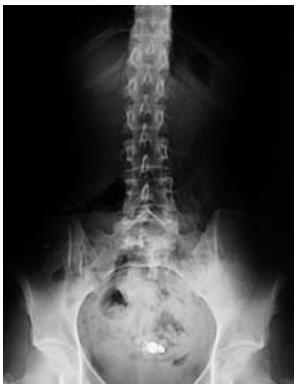

Figure 1 Plain film of the abdomen from one Crohn's disease patient showing capsule retention for 12 wk, with no associated symptoms. The patient had 2 anastomoses (ileo-ileal and ileo-colonic). Surgical removal was required, showing wireless capsule endoscopy within the "*cul de sac*" of the side-to-side ileo-ileal anastomosis not reachable by the endoscope.

Adverse events

CD group: Retention was observed in one patient (3%) with 2 anastomoses (ileo-ileal and ileo-colonic), showing no symptoms despite capsule retention for > 12 wk (Figure 1). Surgical removal of WCE was required after 2 unsuccessful therapeutic endoscopies. During surgery, WCE was detected within the "*cul de sac*" of the side-to-side ileo-ileal anastomosis not reachable by the endoscope.

C group: No adverse events were reported.

Proximal SB lesions detected by WCE

CD group: WCE detected previously unknown proximal SB lesions compatible with CD in 16/32 (50%) patients. Among these 16 patients, lesions included > 3 aphthoid ulcers in 14 (87.5%), deep ulcers in 2 (12.5%) and one ulcerated stricture identified by WCE in one patient (Figure 2A-C). All lesions compatible with CD appeared discontinuous and surrounded by macroscopically uninvolved mucosa. In all 16 patients showing lesions in the upper SB, WCE also detected lesions in the distal ileum compatible with CD.

The median age was comparable in patients showing or not (n = 16 for both) proximal SB lesions at WCE (44 years, range 20-65 years *vs* 32 years, range 19-48 years, P = NS).

Table 2 summarizes the clinical characteristics of the 32 patients grouped according to the presence or not of upper SB lesions in WCE. No statistically significant concordance was observed between upper SB lesions in WCE for both clinical parameters and risk factors considered (CD site χ^2 = 3.3, P = 0.18, epigastric pain χ^2 = 0.0, P = 1.0, smoking habits χ^2 = 1.3, P = 0.5, NSAIDs use χ^2 = 1.5, P = 0.2, IDA χ^2 = 0.0, P = 1.0).

The interobserver agreement for SB lesions visualized by WCE (score 0-4)[5] was very high when considering proximal SB lesions (κ = 0.86) and high when considering distal SB lesions (κ = 0.61). At 12 mo, none of the 32 patients showing lesions in the upper SB developed related symptoms (anemia, epigastric pain) or symptomatic SB stenosis.

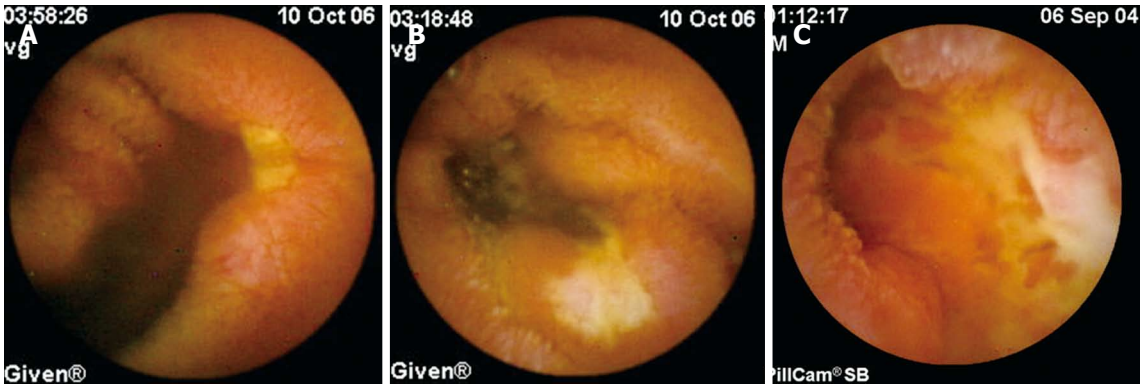

Figure 2 Upper small bowel lesions detected at wireless capsule endoscopy in 3 patients with an established diagnosis of Crohn's disease involving the distal ileum. Aphthoid ulcer (A), deep ulcer (B) and one ulcerated stricture easily identified by wireless capsule endoscopy (C). All lesions compatible with Crohn's disease detected by wireless capsule endoscopy appeared discontinuous and surrounded by macroscopically uninvolved mucosa.

Table 2 Clinical characteristics of the 32 CD patients grouped according to the presence of upper small bowel lesions at WCE

	Small bowel lesions at WCE (%)	
	Yes (n = 16)	No (n = 16)
Known CD extent before WCE		
Distal ileum	12 (75)	15 (94)
Distal ileum and colon	1 (6)	1 (6)
Distal ileum and esophagus stomach	3 (19)	0
Epigastric pain		
Yes	3 (19)	4 (25)
No	13 (81)	12 (75)
Smoking habits		
Smokers	6 (37)	8 (50)
Ex-smokers	3 (19)	1 (6)
Non-smokers	7 (44)	7 (44)
NSAID use		
Yes	3 (19)	0
No	13 (81)	16 (100)
IDA		
Yes	4 (25)	5 (31)
No	12 (75)	11 (69)

IDA: Iron-deficiency anemia.

C group: WCE detected proximal SB lesions not compatible with CD in 5/32 (16%) patients, represented by erosions in 3 (9%) and angiodysplasia in 2 (6%). In contrast to CD patients, none of the 32 C patients showed aphthoid or deep ulcers in the SB with WCE.

Proximal SB lesions detected by SICUS

SICUS detected proximal SB lesions in 3/32 (9%) CD patients, represented by increased jejunal BWT with bowel dilation, associated with stenosis in one patient.

Comparison between WCE and SICUS in detecting upper SB lesions

When considering the 16 CD patients showing upper SB lesions with WCE, only 3 (19%) also showed SICUS findings compatible with CD lesions in the same area (BWT > 3 mm). Therefore, all 3 (9%) patients showing SICUS results compatible with CD lesions in the proximal SB had the findings confirmed by WCE. Upper SB lesions in WCE in these patients included deep ulcers in 2 and aphthoid ulcers in one patient. The only stricture identified by WCE was also visualized by SICUS. However, these 2 techniques showed no significant concordance in detecting proximal SB lesions ($\chi^2 = 1.5$, $P = 0.2$).

Distal SB lesions detected by WCE

CD group: Findings compatible with CD lesions in the distal SB were detected by WCE in 30/32 (93%) patients. In the remaining 2 patients, WCE did not visualize the colon, thus not allowing the evaluation of the distal SB. Of the 30 patients with available distal SB images with WCE, lesions included erosions in 2 (7%), aphthoid ulcers in 13 (43%), deep ulcers in 11 (37%), both aphthoid and deep ulcers in 3 (10%) and one single ulcerated substenosis in one (3%) patient. In all patients showing lesions in the upper SB, WCE also showed lesions in the distal SB.

One patient (PL) showed WCE impaction at the level of the anastomosis as detected by a plain film of the abdomen showing capsule retention for 12 wk, with no associated symptoms (Figure 1). The patient had 2 anastomoses (ileo-ileal and ileo-colonic) for CD-related surgery. Surgical removal of the WCE was required, showing the capsule retained within the "*cul de sac*" of the side-to-side ileo-ileal anastomosis not reachable by the endoscope. In a second patient (CE) with IDA, WCE images stopped at the level of a bleeding ulcerated substenosis in the proximal SB, with no retention (Figure 2C). In this patient indication for surgery was also determined after WCE examination, followed by resection of the ulcerated substenosis not detected by conventional techniques, with histological findings compatible with CD.

C group: WCE detected distal SB lesions not compatible with CD in 2/32 (6%) patients, including erosions in one patient and one single angiodysplasia in the other patient. No patients showed aphthoid or deep ulcers compatible with CD.

Distal SB lesions detected by SICUS

CD group: SICUS detected distal SB lesions in 30/32

(93%) patients, including all 3 patients showing upper SB lesions by SICUS. In both patients with normal SICUS, conventional techniques detected lesions in the distal ileum.

Comparison between WCE and SICUS in detecting distal SB lesions

Findings compatible with CD in the distal SB were detected by both WCE and SICUS in 30/32 (93%) patients, as SICUS detected no lesions in 2 patients. These 2 techniques showed no significant concordance in detecting proximal SB lesions ($\chi^2 = 0.5$, $P = 0.4$).

Additional findings at WCE

CD group: WCE detected gastric and/or duodenal lesions in 7/32 (22%) patients. Gastric lesions were detected in 5/32 (15%) patients, including aphthoid ulcers in one and erosions in 4 patients. Duodenal lesions (erosions) were detected by WCE in 5/32 (15%) patients, and were also visualized in the stomach in 4 patients. Of the 7 patients showing gastric/duodenal lesions at WCE, 5 (71%) also showed upper SB lesions with WCE.

C group: Additional findings were detected in 6/32 (19%) C patients, including gastric/duodenal erosions in 5 and colonic angiodysplasia in 2 (with gastric erosions in one).

DISCUSSION

Proximal SB lesions are detected in a low proportion of CD patients (about 5%)[14-16]. However, these rates have been reported using radiologic techniques which show a low sensitivity for visualizing superficial lesions. Recently, WCE has been shown to visualize the inner SB surface, providing a high sensitivity in detecting minor lesions (i.e. erosions, aphthoid ulcers)[2-4]. Two independent studies reported that WCE visualizes lesions related to early CD recurrence in the SB[17,18]. As the frequency, natural history and clinical relevance of proximal SB lesions in CD is undefined, we investigated this issue in a prospective longitudinal study. A high frequency of WCE findings compatible with proximal SB lesions related to CD was observed. As WCE does not allow an histological characterization of the lesions, WCE findings in CD patients were compared with those observed in control patients requiring WCE for IDA or chronic diarrhea. No control patients showed aphthoid or deep ulcers, thus supporting the specificity of the upper SB lesions detected by WCE in our CD population. No correlations were observed between risk factors and proximal SB lesions, supporting the disease-specificity of our findings.

The frequency of WCE impaction was within the expected range (3%)[8]. The finding of WCE impaction within the "*cul the sac*" of a side-to-side ileo-ileal anastomosis may indicate a higher impaction risk in these patients, even in the absence of overt stenosis. In addition, a WCE examination allowed the detection of a previously unknown ulcerated substenosis in one additional patient with IDA associated with CD of the distal ileum. This observation further supports the role of WCE in identifying upper SB lesions not detected by conventional radiology.

No concordance was observed between proximal SB lesions at WCE and related signs/symptoms, even when patients were followed up for at least 12 mo. This observation provides additional evidence for a diffuse involvement of the SB in patients with CD of the distal ileum, even if not associated with overt symptoms. Present findings also suggest that no treatment changes may be required in CD patients showing upper SB lesions at WCE. The observed frequency of upper SB lesions is in agreement with previous findings in the early postoperative period (56%)[17].

When WCE and SICUS findings were compared, a small proportion of patients (3 of 16) showing upper SB lesions with WCE, also showed the same finding with SICUS. This discrepancy is in agreement with our previous studies[18,19] and may be related to the observation that WCE and SICUS provide a different view of the SB (i.e. intraluminal *vs* extraluminal). In addition, WCE allows the visualization of superficial lesions not detected by SICUS[18]. In the 3 patients showing upper SB lesions by both WCE and SICUS, lesions were represented by deep ulcers at WCE in 2, suggesting that discrepancies are mainly observed for superficial lesions. In contrast to the upper SB lesions, WCE and SICUS findings appeared comparable in the distal SB. This observation indicates that the characteristics of the SB lesions, including not only the severity (deep *vs* aphthoid ulcers) and number, but also the site (upper *vs* distal SB) may influence the sensitivity of ultrasonography. A good interobserver agreement was observed, supporting previous findings[17]. Although some patients were studied by SBFT, this technique was not included for ethical and economic reasons, in relation to both the high radiation exposure and to the known low sensitivity of SBFT in detecting superficial SB lesions. In addition, there was no clinical indication for SBFT in our population, including only 5 active patients with an established diagnosis of CD.

To our knowledge, no studies have investigated the role of WCE in comparison with SICUS in detecting the presence and clinical relevance of upper SB lesions in patients with CD involving the distal ileum, diagnosed by conventional radiological techniques. The present findings supports that WCE is a non-invasive technique which allows the visualization of superficial proximal SB lesions in a high proportion of patients with an established diagnosis of CD of the distal ileum. Despite no significant clinical relevance appearing to be associated with these findings even in the long term, the use of WCE in CD involving the distal SB may add clues in defining the extent of the lesions and its relation with clinical manifestations of the disease.

COMMENTS

Background

Wireless capsule endoscopy (WCE) is a non-invasive technique visualizing the

mucosal surface of the small bowel (SB). WCE showed a high sensitivity and specificity for detecting lesions related to SB Crohn's disease (CD). WCE has in particular been shown to be able to detect minor lesions, not visualized by conventional radiologic techniques, providing high radiation exposure. WCE has therefore been proposed as an alternative non-invasive technique for assessing CD lesions. Ultrasonography also is a non-invasive technique proposed for detecting SB lesions in CD. The use of an oral contrast (SICUS) significantly increases, in experienced hands, the sensitivity of ultrasonography for assessing SB lesions in CD (> 95%). Although several studies concordantly showed that WCE is able to visualize superficial lesions in the SB, its role in defining the extent of the lesions in CD is undefined.

Research frontiers

Proximal small bowel lesions are detected in a low proportion of CD patients (about 5%). However, these frequencies have been reported using radiologic techniques which have a low sensitivity for visualizing superficial lesions. WCE has been shown to visualize the inner SB surface, providing a high sensitivity in detecting minor lesions (i.e. erosions, aphthoid ulcers). Two independent studies reported that WCE visualizes lesions related to early CD recurrence in the SB. However, the frequency, natural history and clinical relevance of proximal SB lesions in CD is currently undefined.

Innovations and breakthroughs

The present study showed that WCE is a non-invasive technique allowing the visualization of superficial proximal small bowel lesions in a high proportion of patients with an established diagnosis of CD of the distal ileum.

Applications

Despite no significant clinical manifestations appearing to be associated with these findings even in the long term, the use of WCE in CD involving the distal SB may add clues in defining the extent of the lesions and the relationship with clinical symptoms of the disease.

Terminology

WCE is a non-invasive technique able to visualize the inner surface of the small intestine. SICUS is also a non invasive technique showing, in experienced hands, a high sensitivity and specificity in terms of assessment of small bowel lesions, including increased bowel wall thickness in ileal CD.

Peer review

This is a small (32 patients) prospective study, with a control population, that evaluates the diagnostic accuracy of WCE in CD, and compares it with different diagnostic tools. It is well presented and performed in an ethical manner.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3305

World J Gastroenterol 2010 July 14; 16(26): 3305-3309
ISSN 1007-9327 (print)

BRIEF ARTICLE

Experience with the surgical treatment of hepatic hydatidosis: Case series with follow-up

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Received: December 7, 2009 Revised: May 9, 2010
Accepted: May 16, 2010
Published online: July 14, 2010

Abstract

AIM: To describe patient characteristics and the results of surgical treatment for hepatic hydatidosis (HH) in the Hospital Hernán Henríquez Aravena from December 2001 to March 2005.

METHODS: Subjects older than 16 years with suspected HH were included based on the clinical picture and radiological studies. Variables examined included clinical and laboratory aspects, outcome, features of the parasite and surgical aspects. Descriptive statistics were used, calculating central tendency, dispersion and extreme tendency.

RESULTS: The series was comprised of 122 patients, with an average age of 44 ± 16.9 years. The most frequently used surgical technique was subtotal cystectomy in 90% of the patients, followed by hepatic resection (hepatectomy, segmentectomy and subsegmentectomy) in 5%. In 2%, a combination of subtotal cystectomy and segmentectomy was performed. In addition, 28% of the series presented complications in the postoperative period and mortality was 2%.

CONCLUSION: The most frequently used surgical technique for HH was subtotal cystectomy and the morbidity and mortality rates in this Chilean series are comparable to other national and international series.

Key words: Echinococcosis of liver; Surgery; Cases series; Mortality; Morbidity

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Losada Morales H, Burgos San Juan L, Silva Abarca J, Muñoz Castro C. Experience with the surgical treatment of hepatic hydatidosis: Case series with follow-up. *World J Gastroenterol* 2010; 16(26): 3305-3309 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3305.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3305

INTRODUCTION

Hepatic hydatidosis (HH) is a zoonosis produced by *Equinococcus granulosus* that presents a high prevalence and incidence in southern Chile, at a rate of 38 per 100 000[1-2]. There are many options for HH treatment. Based on World Health Organization guidelines and our experience, surgery is one of the most effective treatments for HH[3-5].

There is a wide range of surgical techniques for treating HH, from radical surgery with complete removal of

the cyst leaving no residual cavity (pericystectomy or regulated hepatic resection), to more conservative techniques with partial removal of the cyst and treatment of that part of the cyst remaining in the hepatic parenchyma: subtotal cystectomy plus epiploplasty, subtotal cystectomy plus capitonnage, cystostomy plus epiploplasty, among others[3-12]. Some reports show that epiploplasty decreases the incidence of deep abdominal abscess after surgical treatment[10-12].

The surgical technique preferred by our team is subtotal cystectomy, leaving the smallest amount of cyst in the hepatic parenchyma, accompanied by epiploplasty[11,12]. Performing complete dissection of the liver's ligamentous attachments we can thus provide even better exposure for resection of the cyst, only leaving the part close to vascular structures.

Our hypothesis is that morbidity and mortality rates associated with the surgical treatment of HH in the Hospital Hernán Henríquez Aravena, Temuco, Chile, are comparable to national and international figures. Our aim is to describe patient characteristics, the results of the surgical treatment and the morbidity and mortality associated with the surgical treatment of HH in the Hospital Hernán Henríquez Aravena from December 2001 to March 2005.

MATERIALS AND METHODS

Study design and population

Patients were subjects older than 16 years with suspected HH based on the clinical findings and radiological studies which were performed in Hospital Hernán Henríquez Aravena (Temuco, Chile) from December 2001 to March 2005. Our protocol did not include preoperative albendazole.

Definitions

Subtotal cystectomy: Surgical technique in which part of the adventitious membrane of the hydatid cyst is resected. In this technique, we only leave the part of the adventitia adjacent to the vascular structures. When possible, this part is covered by a vascularized omental flap (epiploplasty).

Pericystectomy: Total resection of the cyst's adventitious membrane, in which the plane between the adventitious membrane and hepatic parenchyma is dissected. This technique is used for peripheral cysts and is performed with afferent vascular control (Figure 1A).

Maneuver

The biliopancreatic surgery team prefers to treat HH *via* the abdomen no matter where the cysts are located. A subcostal laparotomy is used, either right or bilateral, depending on the location of the cysts; or a J laparotomy towards the right. The round ligament is sectioned and tied off and the liver is then moved to locate the cyst (Figure 1B). On some occasions, intraoperative echography is used when cysts are difficult to locate. Once a cyst has been located, we protect the surgical area with compresses containing either diluted povidone or 20% hypertonic saline solution as per the surgeon's choice (Figure 1C). The cyst is subsequently punctured and all the hydatid fluid and daughter vesicles are aspirated (Figure 1D and E). The germinative membrane is removed and the protection field withdrawn. Depending on the location, the subtotal cystectomy is planned, beginning the resection of the cyst wall with electrocoagulation, controlling the hemostasia, leaving only that part of the cyst which is adjacent to the vascular structures. This area is then reviewed in search of biliary communications, which are sutured with 3-0 resorbable material. Some team members perform a cholecystectomy and they inject physiological serum into the cystic duct through a nelaton probe to make small biliary communications visible (Figure 1F). Depending on the number of communications, satisfaction with the closing of the communications and the diameter of the bile duct, a choledochostomy is performed. The only absolute indication for a choledochostomy is a clinical picture compatible with cholangiohydatidosis, in which the bile duct is always drained. Nevertheless, in recent months, we have tried to drain the bile duct using endoscopic retrograde cholangiography. An epiploplasty is subsequently performed with a greater omentum pedicle flap. Drains are not positioned as a matter of course and are at the surgeon's discretion.

Study variables

Variables examined included clinical and laboratory features, clinical images and evolutionary complications, surgical and clinical evolution variables.

Variables examined included ultrasound (US) and computed tomography (CT) characteristics of the cyst, the number of cysts (continuous variable), location of the cysts (categorized into right hemi-liver, left hemiliver, bilateral or central liver), diameter of the lesion, evolutionary complications of the cyst (hepatic abscess of hydatid origin (HAH), hepatothoracic transit (HHT), rupture to the abdominal cavity and cholangiohydatidosis.

Surgical variables: The substance used to protect the surgical area is considered (povidone and/or hypertonic solution), as is the surgical technique (categorized into subtotal cystectomy, pericystectomy, segmentectomy and hepatectomy). The use of choledochostomy and/or drain (dichotomized: yes or no) is evaluated.

Clinical evolution variables: The duration of hospitalization is assessed, and the presence of postoperative complications (categorized into: residual cavity, infection of the operating site, atelectasis, pneumonia, hemoperitoneum, bilioperitoneum, evisceration and pneumothorax).

Abscess in the residual cavity is defined as any purulent collection in relation to the liver surgical bed that needs treatment (surgery or CT-guided drainage).

Postoperative follow-up was carried out by clinical assessment. For any abnormal finding US or CT was un-

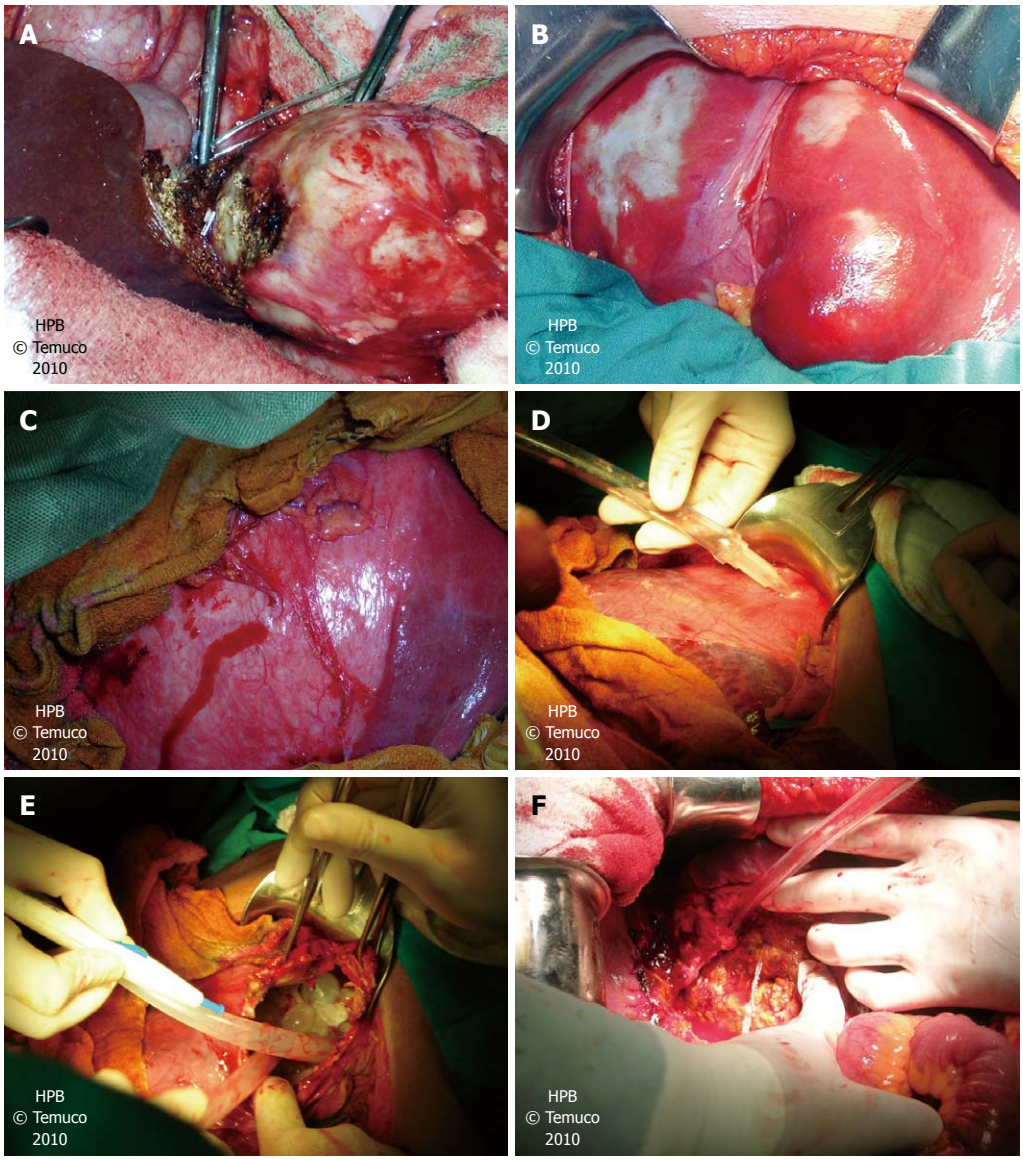

Figure 1 Surgical technique. A: Pericystectomy used for peripheral cyst; B: Subcostal laparotomy is used. Round ligament is sectioned and tied off and the liver is then moved to locate the cyst; C: Protection of the surgical area with compresses containing diluted povidone; D: Puncturing hydatid cyst with trocar; E: Aspirating daughter vesicles; F: Once the cystectomy has been performed, saline solution is injected *via* cyst duct or choledochostomy to locate the biliary communications.

dertaken. Liver recurrence was defined as any cyst image (such as hydatid cyst) with postoperative normal images.

Statistical analysis

Descriptive statistics were used, calculating measures of central tendency (mean, median) dispersion (SD) and extreme values. Database was analyzed using the program Stata® 8.0.

RESULTS

The series included 122 patients with a mean age of 44 ± 16.90 years; with a median of 44 years and range 17-85 years. The distribution by gender showed 73 (60%) male and 49 (40%) female. Forty-nine patients (40%) were admitted by the emergency service and 73 (60%) by the clinic. The mean number of cysts found by US was 1.57 ± 0.91 and the mean number found in surgery was 1.37 ± 0.63. The mean diameter of the main lesion was 10.77 ± 5.62 cm with a median of 10 cm and range 5-30 cm.

When describing the location of the largest cyst, we found that 73 (60%) were located in the right hemi-liver and 49 (40%) in the left hemi-liver. At the time of this study only 49 patients (40%) had undergone CT. Ninety-eight lesions (80%) were hypodense.

The most frequently used surgical technique was subtotal cystectomy in 110 (90%) cases (Table 1). In those patients who underwent a subtotal cystectomy, 90 (82%) also received epiploplasty.

There were evolutionary complications of HH in 20 patients (16%). Intraoperatively, evolutionary complications were found in 49 patients (40%), with the most frequent being cholangiohydatidosis in 38 (31%), followed by HHT in 24 (20%), HAH in 18 (15%) and rupture in 12 (10%).

The surgical area was protected with povidone in 107 patients (88%) and hypertonic solution in 15 (12%).

Table 1 Surgical techniques for HH in this patient series

Technique	*n* (%)
Subtotal cystectomy	110 (90)
Liver resection	6 (5)
Subtotal cystectomy + liver segmentectomy	2 (2)
Others	4 (3)
Total	122 (100)

HH: Hepatic hydatidosis.

The bile duct was explored *via* choledochostomy in 39 patients (32%), with the most common indication being suspected cholangiohydatidosis. Cholangiohydatidosis was treated by bile duct exploration and antibiotic treatment. A drain was left in the cystectomy site at the end of the surgery in 81 patients (66%). Twenty-eight per cent of the series (34 patients) presented postoperative complications. The most frequent was atelectasis and pneumonia in 28 patients (23%), surgical site infection in 6 (5%), hemorrhage in 6 (5%). Three patients presented abscess in the residual cavity. Two were solved surgically and 1 patient was treated by CT-guided drainage.

The clinical, evolutionary and laboratory variables according to the presence of complications are described in Table 2.

The mortality of the series was 3 patients (2%). Six patients (5%) received albendazole as a pharmacological therapy subsequent to the surgery, despite our protocol. Twenty-three patients (19%) were operated on for pulmonary hydatidosis at some point in the postoperative evolution, with the most frequent surgery being cystectomy in 111 patients (91%), followed by pulmonary lobectomy (9%).

With an average follow-up of 22.4 ± 8 mo, 13 patients (11%) presented recurrence. In 10 patients recurrence was in relation to a surgical site.

When the biopsies of the patients operated for suspected HH were reviewed, we found that HH was confirmed in 120 patients (98%). In only 3 patients did the biopsy show different results: in 2 patients the biopsy revealed a simple hepatic cyst with signs of inflammation and in 1 patient a primitive neuroectodermic tumor was discovered.

The mean hospital stay was 16.33 ± 8 d with a median of 13 d and range 3-62 d.

Table 2 Clinical, evolutionary and laboratory variables according to postoperative complications (mean ± SD)

Variables	Patients with postoperative complications (*n* = 25)	Patients without postoperative complications (*n* = 97)
Age (yr)	48.2 ± 15	43.63 ± 17
Sex (% male)	60	33
Evolutionary complications of the cyst (before surgery) (%) (HAH, HHT, rupture to the abdominal cavity and cholangiohydatidosis)	20	9
Protection of the surgical area (% povidone)	24	21
Number of cysts	1.56 ± 0.9	1.32 ± 0.5
Leukocyte count (cells/mm^3)	10245 ± 3955	9095 ± 4605
Total bilirubin (mg/dL)	2.03 ± 2.4	1.43 ± 3.7
Alkaline phosphatase (U/L)	616 ± 576	397 ± 440
AST (U/L)	85.8 ± 39	55.8 ± 91
ALT (U/L)	83.47 ± 94	73 ± 96

HAH: Hepatic abscess of hydatid origin; HHT: Hepatothoracic transit; AST: Aspartate transaminase; ALT: Alanine transaminase.

DISCUSSION

All the patients in our series had abdominal pain as the main symptom upon admittance. Our center does not run routine immunological exams for *Equinococcus* because several previous reports from our center question their diagnostic value[13]. Our diagnostic suspicion is based on epidemiological history, the study of images and the clinical presentation of the patient.

The mean age of the series (44 years) and the predominance of males are remarkable aspects in that these vary from what was recently reported by a Greek series where female gender was higher and patients were younger[14].

The average diameter of the main lesion (10.77 ± 5.62 cm) is less than that reported in a previous cohort conducted in our center (14.5 ± 6 cm). Predominant location in the right hemi-liver tallies with national and international series[11-15].

Among the evolutionary complications of HH, the most frequent was cholangiohydatidosis (31%), followed by hepatothoracic transit (20%). This contrasts with a previous cohort studied in our center, where the most frequent evolutionary complication was HAH (51.5%) followed by cholangiohydatidosis (10.6%)[11].

We have changed the preference of surgical area protection to diluted povidone, due to some cases of hypernatremia which occurred with use of hypertonic saline solution.

With respect to the surgical technique, our team prefers subtotal cystectomy with epiploplasty. We are emphatic about resecting most of the content of the cyst, leaving only the surface in contact with the vascular structures, which we would call an "almost total cystectomy". To this end, it is necessary to have a complete mobilization of the liver and to be familiar with the vascular structures of the hepatic segments.

Our service does not perform routine choledochostomies in HH surgery. This is demonstrated in the 32% of patients in the series who underwent this procedure, where the majority had suspected cholangiohydatidosis.

Unlike other centers[14], postoperative treatment with albendazole is not routine in our service; this is based on previous studies made in our center that showed a low concentration in the interior of the cyst and the non-existence of an association between the intracystic concentration of albendazole and the viability of the scolices[16].

The recurrence of abdominal hydatidosis (11%) is comparable to a recently published series carried out in Turkey with similar surgical approach[15].

The morbidity (28%) is comparable to that reported

by 2 recent international series and 1 cohort conducted in our center[11,12,14] and the mortality (2%) is comparable to that reported recently in a Greek series[14].

A greater prevalence of evolutionary complications and a greater alteration in hepatic test results can be seen in those patients who presented postoperative complications. This tendency must be corroborated in a cohort study designed for this purpose.

The median duration of the hospital stay (16.33 d) is comparable to that published by a previous Chilean series (17 d)[17].

In conclusion, in our Chilean series, subtotal cystectomy was the most common procedure undertaken for HH. The rates of morbidity and mortality are comparable to those reported by other national and international series.

COMMENTS

Background

Hepatic hydatidosis (HH) is a zoonosis produced by *Equinococcus granulosus* that presents a high prevalence and incidence in southern Chile. Based on World Health Organization guidelines and our experience, surgery is one of the most effective treatments for HH.

Research frontiers

Patient characteristics and the results of surgical treatment for HH in the Hospital Hernán Henríquez Aravena from December 2001 to March 2005 were described.

Applications

The authors are very experienced with the treatment of HH and the number of patients presented would allow for a meaningful analysis, which could help to improve the quality of care for these patients.

Peer review

The authors describe their surgical technique used in HH treating and compare morbidity and mortality with the available literature.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3310

World J Gastroenterol 2010 July 14; 16(26): 3310-3317
ISSN 1007-9327 (print)

BRIEF ARTICLE

Comparison of different nutritional assessments in detecting malnutrition among gastric cancer patients

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Received: December 2, 2009 Revised: January 27, 2010
Accepted: February 3, 2010
Published online: July 14, 2010

Abstract

AIM: To evaluate the prevalence of preoperative and postoperative malnutrition and the relationships between objective and subjective nutritional assessment of gastric cancer patients.

METHODS: From October 2005 to July 2006, we studied 80 patients with no evidence of recurrent disease and no loss to follow-up after curative surgery for gastric cancer. In this group, 9 patients underwent total gastrectomy and 71 patients subtotal gastrectomy. At admission, 6 and 12 mo after surgery, the patients were assessed on the subjective global assessment (SGA), nutritional risk screening (NRS-2002), nutritional risk index (NRI) and by anthropometric measurements and laboratory data. Differences between the independent groups were assessed with the Student's *t* test and one-way analysis of variance. Spearman's rank correlation coefficients were calculated to evaluate the association between the scores and variables.

RESULTS: The prevalence of malnutrition at admission was 31% by SGA and 43% by NRS-2002. At admission, the anthropometric data were lower in the malnourished groups defined by the SGA and NRS-2002 assessments, but did not differ between the groups using the NRI assessment. Body weight (BW), body mass index (BMI), triceps skin fold and midarm circumference were significantly reduced, but the total lymphocyte count, albumin, protein, cholesterol and serum iron levels did not decrease during the postoperative period. Six months after surgery, there was a good correlation between the nutritional assessment tools (SGA and NRS-2002) and the other nutritional measurement tools (BW, BMI, and anthropometric measurements). However, 12 mo after surgery, most patients who were assessed as malnourished by SGA and NRS-2002 had returned to their preoperative status, although their BW, BMI, and anthropometric measurements still indicated a malnourished status.

CONCLUSION: A combination of objective and subjective assessments is needed for the early detection of the nutritional status in case of gastric cancer patients after gastrectomy.

Key words: Gastrectomy; Malnutrition; Nutritional assessment; Nutritional risk screening; Postoperative follow up; Subjective global assessment

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Ryu SW, Kim IH. Comparison of different nutritional assessments in detecting malnutrition among gastric cancer patients. *World J Gastroenterol* 2010; 16(26): 3310-3317 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3310.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3310

INTRODUCTION

We should pay special attention to the alarming report that 30%-50% of patients in general hospitals have some degree of malnutrition[1-5]. Malnutrition is defined as a state of deficiency in energy, protein or other specific nutrients, producing an appreciable change in body function[6]. Patients who have had gastrointestinal problems and who have undergone gastrointestinal surgery constitute an important risk group for malnutrition[7,8]. Malnutrition is an important factor, influencing both their morbidity and recovery after gastrectomy surgery including radical lymphadenectomy[9,10]. The early detection of nutritional risk would allow early intervention, which may prevent later complications.

The assessment of a patient's initial nutritional status and its evaluation during the disease and/or treatment plays an important role in tailoring nutritional support[11]. The goals of a formal nutrition assessment are: to identify patients who are malnourished or are at risk of malnutrition; to collect the information necessary to create a nutrition care plan; and to monitor the adequacy of nutritional therapy[12]. Studies have consistently revealed the inadequacy of any single method or tool in assessing a patient's nutritional status. The absence of a single gold-standard objective measure has led investigators to develop various nutritional indices that can be used to stratify patients at increased risk of poor outcomes[13]. As a result, combinations of diverse measurements have been developed into subjective scoring systems designed to increase the sensitivity and specificity of nutritional status determinations[14]. Traditionally, scoring systems have been based on objective measurements of nutritional status, such as oral energy intake, body weight, weight loss over time, loss of subcutaneous fat, muscle wasting, serum protein levels, and immune competence. These prognostic indices include the nutritional risk index (NRI)[15], which is based on mathematical equations, and the subjective global assessment (SGA)[16] and nutritional risk screening (NRS-2002), which are based on clinical and subjective assessments[17].

This study was performed to evaluate the prevalence of preoperative and postoperative malnutrition in patients with gastric cancer who underwent radical gastrectomy, and the relationships between the objective variables (anthropometric and laboratory measurements) and the subjective scoring systems in the assessment of nutritional status during the postoperative follow-up period.

MATERIALS AND METHODS

Patients

Between October 2005 and July 2006, 80 patients were studied following curative surgery for gastric cancer. Among this group, 9 patients underwent total gastrectomy and 71 patients underwent subtotal gastrectomy. We assessed the nutritional status and laboratory parameters of the patients on admission and at 6 and 12 mo after surgery. Patients with evidence of recurrent disease or who were lost to follow-up were excluded.

This study was approved by the research ethics committee of the institution, and informed consent was obtained from all patients.

Nutritional measurements

The patients were assessed on the following items: SGA, NRS-2002, NRI, anthropometric measurements and laboratory data. The nutritional assessments were performed by a trained nurse specializing in nutrition and a dietitian.

SGA questionnaire

The SGA is a screening tool to determine the nutritional status of patients and was developed by Detsky *et al*[16]. The SGA is a clinical technique with subjective elements and assesses nutritional status based on features of the patient's history and physical examination. During the SGA, two trained investigators used a standardized questionnaire concerning the patients' height and weight (current, before illness, and weight range during the previous 6 mo) and took a nutritional history (appetite, intake, gastrointestinal symptoms). In addition, the dietitian evaluated their physical appearances (subjective assessment of fat loss, muscle wasting, edema and ascites) and noted any existing medical conditions (e.g. encephalopathy, infection, renal insufficiency). Based on this evaluation, the patients were classified as being well nourished (SGA A), moderately malnourished (SGA B), or severely malnourished (SGA C). The SGA examiner was not aware of the laboratory test results at the time of the assessment.

Nutritional risk screening

The NRS-2002 was introduced by the European Society of Parenteral and Enteral Nutrition as the preferred method for screening and assessing hospital patients[17]. Its stated purpose was "Identification of those hospitalized patients, who are malnourished or at risk for malnourishment and who would gain benefit from the improvement of their nutritional situation." The NRS-2002 consists of a nutritional score and a severity of disease score and an age adjustment for patients aged > 70 years (+1). Nutritional score: weight loss > 5% in 3 mo or food intake below 50%-75% in the preceding week = 1; weight loss > 5% in 2 mo or BMI 18.5-20.5 kg/m^2 and impaired general condition or food intake 25%-60% in the preceding week = 2; weight loss > 5% in 1 mo or > 15% in 3 mo or BMI < 18.5 kg/m^2 and impaired general condition or food intake 0%-25% in the preceding week = 3. Severity of disease score: hip fracture, chronic patients with acute complications = 1; major abdominal surgery, stroke, severe pneumonia, hematological malignancies = 2; head injury, bone marrow transplantation, intensive care patients with APACHE > 10 = 3. The NRS-2002 score is the total of the nutritional score, severity of disease score and age adjustment. Patients are classified as no risk = 0, low risk = 0-1, medium risk = 3-4 and high risk = > 5.

Nutritional risk indicator

The NRI was developed by the Veteran's Affairs Total

Parenteral Nutrition group[15] in 1991 for use in the evaluation of the efficacy of perioperative total parenteral nutrition in patients undergoing thoracic or abdominal surgery. The NRI is a simple equation that uses serum albumin and recent weight loss: NRI = [1.519 × serum albumin (g/L)] + 0.417 × (present weight/usual weight × 100). An NRI score higher than 100 indicates that the patient is not malnourished, a score of 97.5 to 100 indicates mild malnourishment, a score of 83.5 to 97.5 indicates moderate malnourishment, and a score lower than 83.5 indicates severe malnourishment.

Anthropometric measurements

Body weight (BW; nearest 0.1 kg) and height (nearest cm) were measured while the patient was standing without shoes and in light clothes. Body mass index (BMI) was derived as weight (kg) divided by height (m) squared (kg/m^2). The triceps skinfold thickness (TSF), to the nearest mm, was measured at the midpoint between the acromion and olecranon processes on the nondominant side with a Holtain caliper (Holtain Ltd., Crymych, UK). The midarm circumference (MAC) was measured to the nearest 0.1 cm with a tape at the same point as the TSF. All anthropometric measurements were made at least three times by the same investigator, and the reported values are the means of the repeated measurements.

Serological measurements

Blood samples were taken from the cubital vein and tests included the measurement of serum protein, albumin, and cholesterol, and total lymphocyte counts (TLC). Laboratory data were collected using standard laboratory methods.

Statistical analysis

The data were analyzed with the statistical software "Statistical Package for Social Science (SPSS)" version 12.0 for Windows (SPSS, Inc., Chicago, IL, USA). Differences between the independent groups were assessed with Student's *t* test and one-way analysis of variance. Spearman's rank correlation coefficients were calculated to evaluate the association between the scores and variables. Data are presented as mean ± SD. Differences were considered to be statistically significant at $P < 0.05$. Agreement between two assessment methods was analyzed with the κ statistic. The value of κ varies from 0 to 1; a value of 0.4 or less indicates that chance alone can account for the observed agreement, and a value of 1 indicates perfect concordance.

RESULTS

Eighty patients who were treated with gastrectomy for gastric carcinoma were enrolled. The patients' characteristics are summarized in Table 1.

Preoperative nutritional status

We assessed the nutritional status and laboratory parameters of patients within 24 h of their hospital admission. The prevalence of malnutrition at admission was 31% when determined with the SGA (moderately and severely malnourished) or 43% when determined with the NRS-2002 (medium and high risk). The frequency of any degree of malnutrition at admission was 31% according to the NRI (mild, moderate, and severe malnutrition). There was no difference in age or TLC between malnourished and well-nourished groups defined according to the three assessments, but the percentage weight loss differed between the groups (Table 2). The anthropometric data were lower in the malnourished groups based on SGA and NRS-2002 assessments, but did not differ between the groups defined with the NRI assessment. Albumin, protein, and total cholesterol levels differed between the malnourished and well-nourished groups based on the NRI assessment, but there was no significant difference between the groups defined with the SGA and NRS-2002 techniques (Table 2).

Table 1 Demographic characteristics of gastric cancer patients (mean ± SD) *n* (%)

	Subtotal gastrectomy (*n* = 71)	Total gastrectomy (*n* = 9)	*P* value
Age (yr)	58.5 ± 11.9	56.5 ± 13.2	0.641
Sex			
Male	37 (52.1)	6 (66.7)	0.409
Female	34 (47.9)	3 (33.3)	
Cancer Stage			0.003
I	57 (81.4)	5 (55.6)	
II	8 (11.4)	0 (0)	
III	2 (2.9)	3 (33.3)	
IV	3 (4.3)	1 (11.1)	
Complications			0.219
Major	2 (2.8)	1 (11.1)	
Minor	24 (33.8)	1 (11.1)	
Hospital stay (d)	12.8 ± 8.4	16.4 ± 6.8	0.223

P values were determined with the use of the Pearson χ^2 test and independent *t* test.

Malnutrition scores correlated significantly with the percentage weight loss according to the SGA and NRS-2002 groupings. BMI and anthropometric data correlated inversely in the SGA and NRS-2002 groupings, but did not correlate in the NRI grouping, which correlated inversely with the nutrition factors albumin, protein, and total cholesterol (Table 3). Concordance between the SGA and NRS-2002 assessments was observed in 68 of the 80 (85%) patients, but was not observed between the SGA and NRI assessments in 50 of the 80 (63%) patients (Table 4). Sensitivity was 80% with the NRS-2002 and 73% with the NRI. Specificity was 96% and 40% with the NRS-2002 and NRI, respectively. Agreement was higher between the SGA and NRS-2002 (κ = 0.685, $P = 0.000$) than between the SGA and NRI (κ = 0.127, $P = 0.255$) (Table 4).

Postoperative nutritional status

At 6 and 12 mo after surgery, BW, BMI, TSF and MAC were significantly reduced, whereas the TLC, and albumin, protein, cholesterol and serum iron levels did not decrease

Table 2 Patient characteristics and anthropometric and laboratory data according to nutritional status (mean ± SD)

	SGA			NRS-2002			NRI		
	Well-nourished (*n* = 55)	Malnourished (*n* = 25)	*P* value	Well-nourished (*n* = 45)	Malnourished (*n* = 35)	*P* value	Well-nourished (*n* = 55)	Malnourished (*n* = 25)	*P* value
Age (yr)	57.58 ± 11.5	60.00 ± 13.2	0.410	56.53 ± 9.9	60.65 ± 14.1	0.130	58.07 ± 12.3	58.92 ± 11.6	0.769
Weight (kg)	62.04 ± 9.1	59.30 ± 8.3	0.207	63.64 ± 8.4	58.03 ± 8.6	0.005	61.80 ± 9.0	59.83 ± 8.7	0.360
Weight loss (%)	1.03 ± 1.5	7.31 ± 4.5	0.000	0.89 ± 1.4	6.42 ± 4.6	0.000	2.71 ± 3.1	5.32 ± 5.9	0.047
TSF (mm)	17.88 ± 7.5	13.72 ± 6.5	0.020	18.25 ± 7.8	14.44 ± 6.4	0.020	16.99 ± 8.0	15.69 ± 6.2	0.435
MAC (cm)	27.95 ± 2.5	26.70 ± 2.3	0.040	28.37 ± 2.4	26.51 ± 2.2	0.001	27.81 ± 2.6	26.99 ± 2.1	0.155
BMI (kg/m^2)	24.26 ± 2.7	22.47 ± 2.6	0.008	24.69 ± 2.4	22.42 ± 2.7	0.000	23.88 ± 2.9	23.29 ± 2.6	0.366
Albumin (g/dL)	3.86 ± 0.3	3.85 ± 0.3	0.924	3.87 ± 0.3	3.84 ± 0.3	0.618	4.02 ± 0.2	3.50 ± 0.1	0.000
Total protein (g/dL)	6.89 ± 0.6	6.75 ± 0.6	0.391	6.88 ± 0.6	6.80 ± 0.6	0.568	7.13 ± 0.5	6.23 ± 0.4	0.000
Total cholesterol (mg/dL)	169.65 ± 42.3	167.8 ± 42.5	0.863	166.04 ± 42.2	173.02 ± 42.2	0.466	176.4 ± 39.6	152.8 ± 43.6	0.026
TLC (× 10^3/mm^3)	1856.6 ± 503	1905.9 ± 569	0.698	1874.7 ± 536	1868.6 ± 511	0.959	1903.9 ± 511	1801.8 ± 549	0.698

All *P* values were determined with the use of independent *t* test. SGA: Subjective global assessment; NRS-2002: Nutritional risk screening; NRI: Nutrition risk index; TSF: Triceps skinfold; MAC: Midarm circumference; BMI: Body mass index; TLC: Total lymphocyte count.

Table 3 Correlation coefficients and *P* values for patient data and nutritional assessment techniques

	SGA[1]		NRS-2002[2]		NRI[3]	
	r	*P* value	*r*	*P* value	*r*	*P* value
Age (yr)	0.118	0.297	0.246	0.028	0.035	0.758
Weight (kg)	-0.132	0.243	-0.314	0.005	-0.091	0.425
Weight loss (%)	0.754	0.000	0.690	0.000	0.199	0.166
TSF (mm)	-0.272	0.015	-0.234	0.037	-0.048	0.669
MAC (cm)	-0.228	0.042	-0.378	0.001	-0.170	0.132
BMI (kg/m^2)	-0.279	0.012	-0.393	0.000	-0.109	0.335
Albumin (g/dL)	0.004	0.971	-0.043	0.703	-0.783	0.000
Total protein (g/dL)	-0.086	0.448	-0.062	0.583	-0.636	0.000
Total cholesterol (mg/dL)	-0.006	0.955	0.088	0.436	-0.285	0.010
TLC (× 10^3/mm^3)	0.038	0.738	-0.008	0.943	-0.116	0.305

[1]SGA rating: 0, not malnourished; 1, moderate malnutrition; 2, severe malnutrition; [2]NRS-2002 rating: 0, no risk; 1, medium risk; 2, high risk; [3]NRI rating: 0, not malnourished; 1, mild malnutrition; 2, moderate malnutrition; 3, severe malnutrition; All *P* values were determined with the use of Pearson's correlation coefficient.

Table 4 Statistical comparison of nutritional assessments and screening tool values at hospital admission: NRS-2002 and NRI *vs* SGA

	NRS-2002			NRI		
	Low	Medium/ high	Total	Low	Medium/ high	Total
SGA well-nourished	44	11	55	40	15	55
SGA malnourished	1	24	25	15	10	25
Total	45	35	80	55	25	80
Sensitivity	80.0% (44/55)			72.7% (40/55)		
Specificity	96.0% (24/25)			40.0% (10/25)		
	κ = 0.685, *P* = 0.000			κ = 0.127, *P* = 0.255		

κ statistic: percent of agreement.

Table 5 Patient anthropometric and laboratory data after curative surgery for gastric cancer

	Preoperative day 1	Postoperative 6 mo	Postoperative 12 mo	*P* value
Weight (kg)	61.4 ± 8.8	55.4 ± 8.2	55.2 ± 8.6	0.000
TSF (mm)	17.0 ± 7.5	12.7 ± 6.5	12.1 ± 6.2	0.000
MAC (cm)	27.7 ± 2.5	25.1 ± 4.9	25.5 ± 2.4	0.000
BMI (kg/m^2)	23.9 ± 2.7	21.4 ± 2.4	21.5 ± 2.4	0.000
Albumin (g/dL)	3.88 ± 0.3	4.28 ± 0.2	4.30 ± 0.2	0.000
Total protein (g/dL)	6.87 ± 0.6	7.18 ± 0.5	7.25 ± 0.5	0.000
Total cholesterol (mg/dL)	170.9 ± 37.4	168.2 ± 38.1	173.3 ± 31.4	0.679
TLC (× 10^3/mm^3)	1863 ± 493	1799 ± 633	1752 ± 578	0.328
Serum iron (μg/dL)	72.9 ± 37.6	110.4 ± 52.8	118.2 ± 57.8	0.000
Serum ferritin (μg/L)	77.7 ± 75.3	74.9 ± 81.3	60.2 ± 64.4	0.358
Vitamin B12 (pg/mL)	686.5 ± 249	666.8 ± 281	709.1 ± 330	0.731

P values were determined with the use of ANOVA or the Kruskal-Wallis nonparametric test.

during the postoperative period (Table 5). The nutritional status of the patients who had undergone subtotal gastrectomy stabilized 6 mo after surgery, but the total gastrectomy patients showed a significantly reduced nutritional status in terms of BW, BMI and anthropometric measurements 12 mo after surgery (Figure 1).

Relationship between the nutritional assessment tools and nutritional status after gastrectomy

At 6 mo after surgery, a good correlation was observed between the results of the nutritional assessment tools (SGA, and NRS-2002) and those of the other nutritional measurement tools (BW, BMI, and anthropometric measurements).

According to the SGA and NRS-2002, the proportion of malnourished patients was 80% and 83%, respectively, 6 mo after surgery. At 12 mo after surgery, most patients who had been assessed as malnourished by SGA and NRS-2002 had returned to their preoperative status (Figure 2), although the other nutritional measurement tools (BW, BMI, and anthropometric measurements) still showed a malnourished status.

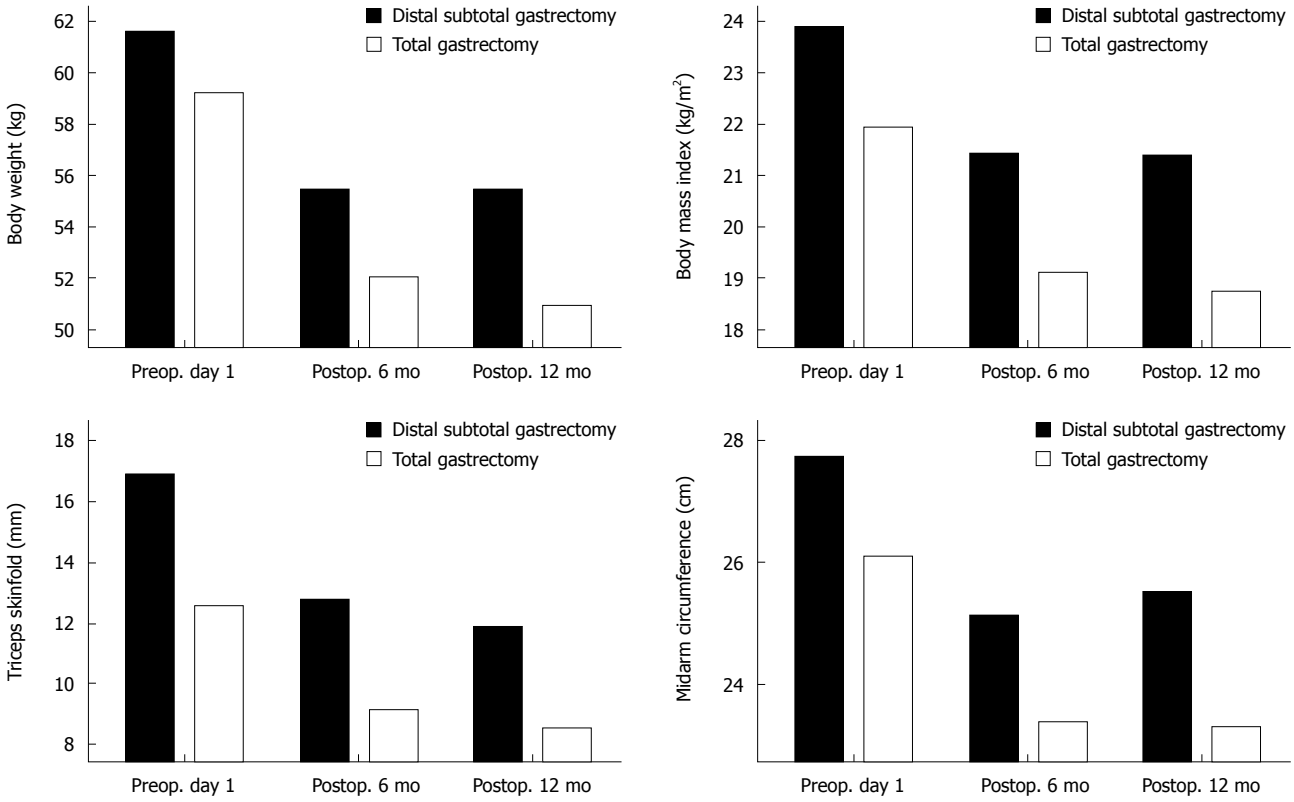

Figure 1 Postoperative nutritional status. Comparison of postoperative changes in objective nutritional parameters of patients undergoing distal subtotal gastrectomy and total gastrectomy from preoperative day 1 until 12 mo after surgery.

DISCUSSION

Nutritional depletion is a common problem in critically ill patients with cancer and is associated with a poor outcome. It is specifically reversible with nutritional support. Several studies have shown that patients with malignant gastrointestinal disease have a higher prevalence of weight loss before surgery, and during the first postoperative months, an additional weight loss of approximately 10% has been reported[7,18-21]. Plausible reasons for the development of malnutrition are a reduced food intake because of poor appetite, postprandial symptoms, and malabsorption[22,23]. Hospitalization, surgery, and chemo/radiotherapy can also cause malnutrition. In this study, the overall prevalence of malnutrition in patients with gastric cancer at admission was 31% according to the SGA and 43% according to the NRS-2002. Based on the objective assessment techniques, BW loss, BMI and anthropometric data were lower in the malnourished groups.

The purpose of nutritional screening is to identify those patients who are at nutritional risk and therefore at higher risk of complications. Malnutrition in hospitalized patients is a critical issue and has been associated with a significant increase in morbidity and mortality[9,10,24]. The detection of malnourished patients is possible if the importance of the issue is understood and the patient's nutritional status is evaluated on admission to hospital. Multiple clinical parameters are available to assess the nutritional status of critically ill patients, but no standard recommendation can be made at this time. Each method has its own advantages and disadvantages[25].

A traditional nutritional assessment often includes dietary and medical evaluations to identify significant weight loss over time, significantly low or high BW or BMI, reduction in MAC, SFT, serum protein levels, or immune competence, and functional measurements of muscle strength may be incorporated into the overall final assessment[26]. Individually, these measurements often have limited value in accurately determining a patient's nutritional risk. Studies have consistently revealed the inadequacy of any single assessment method or tool in evaluating a patient's nutritional status. An effective nutritional screening tool will generally combine both objective and subjective factors.

In this analysis of the preoperative and postoperative anthropometric p arameters of patients with gastric cancer, an interesting observation was that, although the mean BMI was within the normal range, malnutrition scores correlated significantly with the percentage weight loss according to the SGA and NRS-2002. This means that BMI alone is not sufficient to determine the real malnutrition rate. Aydin *et al*[27] reported that a patient can be malnourished even when the BMI is normal and that the SGA can detect malnutrition before the BMI drops below 20 kg/m^2. For this reason, it is very important to use several methods in combination to evaluate a patient's nutritional status.

Albumin is commonly considered a good marker of

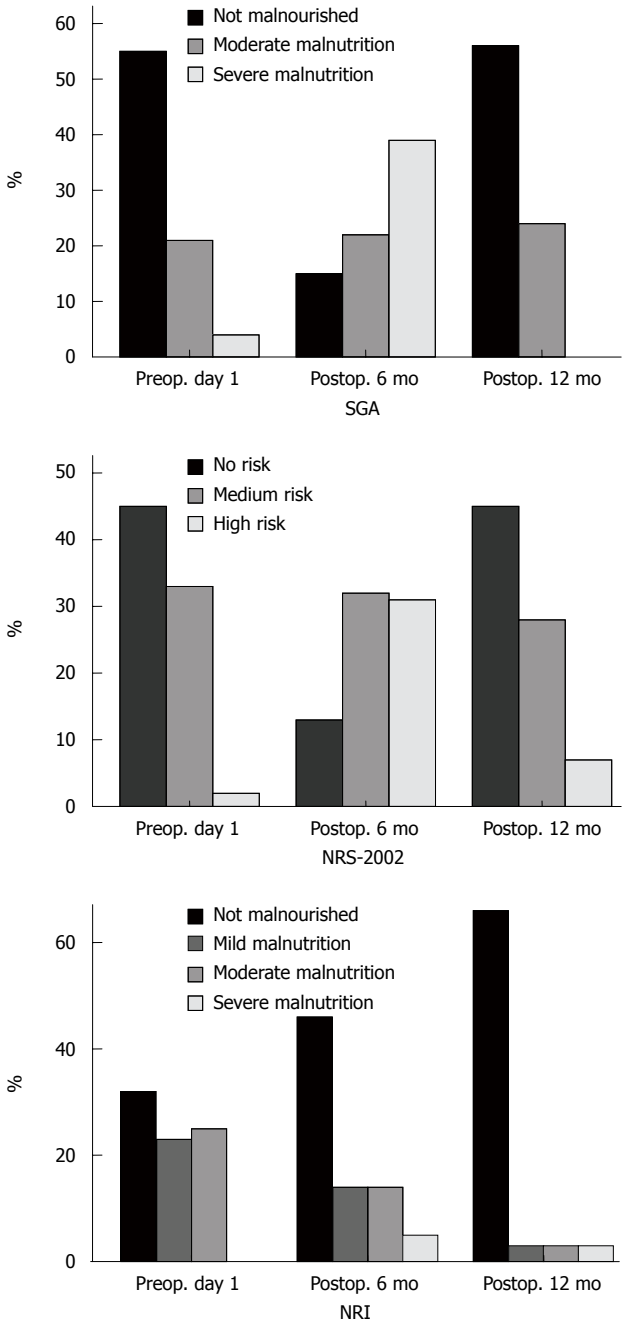

Figure 2 Malnutrition rate. Frequency of malnutrition during the postoperative period assessed with various subjective scoring techniques. SGA: Subjective global assessment; NRS-2002: Nutritional risk screening; NRI: Nutritional risk index.

nutritional status and visceral protein stores. The NRI is derived with an equation from the serum albumin concentration and the ratio of the actual to the usual weight. In this study, serum protein and albumin levels correlated statistically with the malnourished and well-nourished groups based on NRI assessment, but there was no statistical correlation with the groups defined by the SGA or NRS-2002. From this result, it can be inferred that albumin and serum protein parameters are not as sensitive as anthropometric measurements in the evaluation of nutritional status. Some studies have demonstrated that low serum albumin concentrations correlate with longer hospital stays, medical complications, and increased mortality[28,29], whereas other studies have reported that low serum protein levels do not always indicate malnutrition and malnutrition does not always accompany low serum protein levels[27,30,31]. Usually, many serum proteins and albumin are affected by the inflammatory response, liver disease, cancer, or idiopathically[32]. For these reasons, hypoalbuminemia has been reported to be a predictor of risk in a broad sense, rather than a parameter that indicates malnutrition[33]. Therefore, there are arguments for discounting hypoalbuminemia as a marker of malnutrition because patient populations differ. In this study, we evaluated only preoperative patients with gastric cancer who had no other serious medical problems. There was no good correlation between the NRI and objective assessments. The concordance and agreement were higher between the SGA and NRS-2002 than between the SGA and NRI. In this respect, the NRI may not be specific for the diagnosis of malnutrition in preoperative cancer patients.

Weight loss is a common problem after gastrectomy. The main mechanisms implicated include impaired food intake and malabsorption[34]. Patients who undergo gastrectomy consume fewer calories during the first 3-6 mo after surgery, after which their intake improves[20]. In this study, mean BW, BMI, TSF, and MAC were significantly reduced from the time of hospital discharge until 6 mo after surgery. Conversely, serum albumin levels, total protein, cholesterol, and TLC were similar between the groups before and 6 mo after surgery.

According to the subjective assessment of nutritional status 6 mo after surgery, 80% and 83% of the patients were malnourished according to the SGA and NRS-2002, respectively, compared with 31% and 43% of patients who were malnourished on preoperative day 1, respectively. In the group of patients who underwent subtotal gastrectomy, the patients' anthropometric parameters did not change between 6 and 12 mo after surgery. At 12 mo after surgery, their nutritional status was assessed as similar to its preoperative value according to the SGA, NRS-2002 and NRI, but their objective nutritional parameters were still low, especially mean BW, BMI, TSF, and MAC. Subjective assessment is a validated method of nutritional assessment when based on a medical history (weight change, dietary intake change, gastrointestinal symptoms, changes in functional capacity) and physical examination (loss of subcutaneous fat, muscle wasting). Therefore, according to the SGA and NRS-2002, the proportion of malnourished patients was high at 6 mo after surgery, but weight loss was not significant between 6 and 12 mo after surgery. Most of the patients who were assessed as malnourished had returned to their preoperative status, although the other nutritional measurement tools (BW, BMI, and anthropometric measurements) still indicated a malnourished status.

Patients with malignant gastrointestinal disease have a high prevalence of malnutrition. In cancer, reduced food intake and an increased energy gap result in the deterioration of nutritional status. It is very important to detect malnourished patients during the preoperative period and

postoperative follow-up. Not only objective nutritional parameters but also subjective assessments have some limitations in the accurate measurement of nutritional status. Therefore, measuring the nutritional status of patients who have undergone gastrectomy requires a combination of objective variables (anthropometric and laboratory measurements) and a subjective scoring system during the postoperative follow-up period.

COMMENTS

Background

Nutritional depletion is a common problem in critically ill patients with cancer and is associated with a poor outcome. The assessment of nutritional status and its evaluation plays an important role in tailoring nutritional support. Multiple clinical parameters are available to assess the nutritional status of gastric cancer patients, but no standard recommendation can be made at this time. This study would suggest that a specific tailored nutritional assessment is needed for the accurate measurement of nutritional status in patients.

Research frontiers

A traditional nutritional assessment often includes dietary and medical evaluations to identify significant weight loss over time, significantly low or high body weight, skinfold thickness, serum nutritional factor levels and functional measurements of muscle strength. Individually, these measurements often have limited value in accurately determining a patient's nutritional risk. As a result, combinations of diverse measurements have been developed into subjective scoring systems [subjective global assessment (SGA) and nutritional risk screening (NRS-2002)] designed to increase the sensitivity and specificity of nutritional status determinations. Scoring systems have been based on objective measurements of nutritional status, such as oral energy intake, body weight, weight loss over time, loss of subcutaneous fat, muscle wasting, serum protein levels, and immune competence.

Innovations and breakthroughs

When the authors analyzed the nutritional status in gastric cancer patients after gastrectomy surgery, body weight (BW), body mass index (BMI) and fat thickness were significantly reduced, but the total lymphocyte count, albumin, protein, cholesterol and serum iron levels did not decrease during the postoperative period. From this result, it can be inferred that albumin and serum protein parameters are not as sensitive as anthropometric measurements in the evaluation of nutritional status. Six months after surgery, there was a good correlation between the scoring nutritional assessment tools and the other general nutritional measurement tools (BW, BMI, and anthropometric measurements). However, 12 mo after surgery, most patients who were assessed as malnourished by the scoring nutritional assessment tool had returned to their preoperative normal nutritional status, although their BW, BMI, and anthropometric measurements still indicated a malnourished status.

Applications

The authors studied the prevalence of preoperative and postoperative malnutrition in patients with gastric cancer who underwent radical gastrectomy. This is the first study to report on the relationship between nutritional assessment tools and the nutritional status of gastric cancer patients after gastrectomy. From this study, not only objective nutritional parameters but also subjective scoring assessments have some limitations in the accurate measurement of nutritional status. Therefore, measuring the nutritional status of patients who have undergone gastrectomy requires a combination of objective variables (anthropometric and laboratory measurements) and a subjective scoring system during the postoperative follow-up period.

Peer review

This is a nicely written paper and well executed small study. There is enough presented to alert clinicians to both the problem of malnutrition in the sample studies and the problem of nutritional assessment tools used. The combination of assessment tools should allow for improvement in the identification of at risk patients.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3318

World J Gastroenterol 2010 July 14; 16(26): 3318-3324
ISSN 1007-9327 (print)

BRIEF ARTICLE

Oncological outcome of unresectable lung metastases without extrapulmonary metastases in colorectal cancer

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Received: March 23, 2010 Revised: April 22, 2010
Accepted: April 29, 2010
Published online: July 14, 2010

Abstract

AIM: To explore the oncological outcomes of unresectable lung metastases without extrapulmonary metastases in colorectal cancer.

METHODS: Patients with unresectable isolated lung metastases from colorectal cancer were prospectively collected in a single institution during a 5-year period. All patients received either the fluorouracil/leucovorin plus oxaliplatin, fluorouracil/leucovorin plus irinotecan or capecitabine plus oxaliplatin regimen as first-line treatment. The resectability after preoperative chemotherapy was evaluated. Patients' outcome and predictive factors for overall survival were also investigated by univariate and multivariate analysis.

RESULTS: A total of 70 patients were included in the study. After standardized first-line chemotherapy, only 4 patients (5.7%) were converted to resectable disease. The median overall survival time in all patients was 19 mo (95% CI: 12.6-25.4), with a 2-year overall survival rate of 38.8%. No survival difference was found among different first-line chemotherapeutic regimens. Prognostic analysis demonstrated that only the first response assessment for first-line treatment was the independent factor for predicting overall survival. The median survival time in partial response, stable disease and progressive disease patients were 27 mo, 16 mo and 8 mo (P = 0.00001).

CONCLUSION: Pulmonary metastasectomy can only be performed in a small part of unresectable lung metastases patients after chemotherapy. Patients' first response assessment is an important prognostic factor.

Key words: Colorectal cancer; Lung; Metastases; Chemotherapy

Peer reviewer: Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, Singapore 228510, Singapore

Li WH, Peng JJ, Xiang JQ, Chen W, Cai SJ, Zhang W. Oncological outcome of unresectable lung metastases without extrapulmonary metastases in colorectal cancer. *World J Gastroenterol* 2010; 16(26): 3318-3324 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3318.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3318

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the USA, and its frequency is increasing in China in recent years. In Shanghai, colorectal cancer has

become the third most prevalent malignancy[1]. Approximately 50%-60% of patients diagnosed with colorectal cancer will develop metastases[2,3]. The liver and lungs are the most common sites of metastases for colorectal cancers. Nearly 50% of patients with colorectal cancer will develop liver metastases, while only approximately 10%-15% of patients will develop lung metastases ultimately[4,5]. Commonly, metastases from colorectal cancer occur first in the liver and then later appear systemically, because of venous drainage *via* the portal system. However, lung metastases without extrapulmonary metastasis, also called isolated lung metastases, have been reported in several studies[4,6,7]. Through the bypass route of venous or lymphatic drainage, isolated lung metastases were only found in 2%-8% of all colorectal cancers[4,6].

Since the majority of metastases from colorectal cancer are considered initially unresectable disease, neoadjuvant chemotherapy has been applied in potentially resectable liver metastases for years. Studies demonstrated that 10%-15% of initially unresectable liver metastases could be converted to resectable disease after preoperative chemotherapy. However, the experience in unresectable isolated lung metastasis was rare. Current opinions consider that the recommendation in liver metastases may be reasonable to apply to the treatment of lung metastasis[8], but the evidence is weak and the result of this application is unknown, especially for initially unresectable isolated lung metastases.

In this current study, we prospectively collected data from all patients with unresectable isolated lung metastases from colorectal cancer at the first recurrence in a single institution during a 5-year period. The resectability after chemotherapy, outcomes of the patients, results with different chemotherapeutic regimens, and predictive factors for overall survival were all studied in the current cohort.

MATERIALS AND METHODS

Between May 2003 and January 2008, a prospective study was performed to collect data from all the colorectal cancer patients who developed unresectable lung metastasis without extrapulmonary lesions as the first recurrence from the outpatient and inpatient services of Fudan University Shanghai Cancer Center. All the unresectable patients had multiple lung metastastic lesions in two or more than two lobes and had no possibility of radical resection. According to the institutional protocol, all patients who were diagnosed with recurrence received intensive oncological assessment, including chest computed tomography (CT) scans, abdominopelvic CT or magnetic resonance imaging scans and serum carcinoembryonic antigen (CEA). Positron emission tomography-CT scans were performed in some patients, and digital rectal examinations were performed in all patients whose primary tumor was located in rectum.

For viable patients, core biopsy or fine needle biopsy was performed to confirm the diagnosis. For patients whose cytological or histological diagnosis was unavailable, the diagnoses of lung metastases were discussed by the colorectal cancer multidisciplinary treatment (MDT) team, which included a specialized radiologist. When there was doubt about the case, close follow-up without chemotherapy and dynamic change in CT scans were observed to confirm the diagnosis. The resectability of lung metastases was discussed by the colorectal MDT team and a specialized thoracic surgeon according to image study.

Patients with extrapulmonary metastasis or local recurrence were excluded from the current study. Patients, who refused to receive chemotherapy (3 cases) or sign the informed consent form (2 cases), were also excluded from the study. Besides concurrent chemotherapy, 4 patients received weekly administration of cetuximab 8, 8, 11 and 16 times, respectively, in the first line setting; they were excluded from the following outcome analysis because of the small number of cases. After screening, a total of 70 colorectal cancer patients who developed synchronous or metachronous unresectable isolated lung metastasis were included in the study.

Chemotherapeutic regimens and response assessment

According to the medical oncologists' preference, either the fluorouracil/leucovorin plus oxaliplatin (FOLFOX), fluorouracil/leucovorin plus irinotecan (FOLFIRI) or capecitabine plus oxaliplatin (XELOX) regimen was used as first-line treatment for patients who never received chemotherapy or who had received adjuvant chemotherapy over 12 mo ago. For patients who received adjuvant chemotherapy within 12 mo, a different regimen from their original first-line treatment was used. Second-line or third-line treatment regimens were determined by considering the first-line regimen, adjuvant regimen and patients' consent. The "stop-and-go" strategy using a modified FOLFOX6 regimen with maintenance capecitabine during the interval period was applied to 5 patients in our cohort, and they were considered to have received first-line treatment of FOLFOX in the following analysis.

Physical examination, chest CT scans, abdominopelvic ultrasound or CT scans, and serum CEA were used according to the institutional routine assessment for metastatic colorectal cancers. During chemotherapy, response assessment was performed after the first 3 (XELOX regimen) or 4 cycles (FOLFOX or FOLFIRI regimen) and then repeated every 2 to 3 mo. In patients who stopped treatment, similar oncological assessments were asked to be performed every 3 to 6 mo. The response to treatment was defined based on Response Assessment Criteria in Solid Tumors (RECIST)[9].

Statistical analysis

All surviving patients were followed up between January and March 2009 for the purpose of this project. Progression free survival (PFS) was calculated from the start of first-line treatment to the time when disease progression was determined by response assessment. In patients with metachronous metastasis, the time from resection of the primary lesion to occurrence of metastases in the lung was defined as time to metastasis in the current study. The

prognostic value of the first response assessment, which was performed after the first 3 (XELOX regimen) or 4 cycles (FOLFOX or FOLFIRI regimen) of chemotherapy, was analyzed as an independent factor.

The distribution of PFS and overall survival (OS) was calculated by the Kaplan-Meier method, and a log-rank test was used to compare the differences among curves in univariate analyses. Cox regression was used in multivariate analyses, and hazard ratios were calculated including 95% confidence interval (CI). $P < 0.05$ was considered statistically significant.

RESULTS

Basic clinical characteristics

Of the 70 patients who had unresectable lung metastases, 19 patients (27.1%) had synchronous lung metastases with primary colorectal cancers, while the other 51 patients (72.9%) had metachronous metastatic disease. All the patients with synchronous metastases in our series underwent resection of primary colorectal cancer first, and chemotherapy started within 2 mo of the surgery. All 70 patients received first-line treatment with a regimen of FOLFOX, FOLFIRI or XELOX. The detailed clinicopathological features are summarized in Table 1. In 51 patients who had metachronous lung metastasis, the median time to metastasis was 17 mo (range: 3-67 mo). The cumulative proportions of time to metastasis within 1, 2 and 3 years were 29.4%, 70.6% and 84.3%, respectively.

Table 1 The clinicopathological characteristics and survival time of all the unresectable pulmonary metastases patients

Characteristics	Number (%)	Median OS (mo)	95% CI	*P* value
Gender				
Male	21 (30)	22	9.0-35.0	0.487
Female	49 (70)	19	10.8-27.1	
Age (yr)				
≤ 60	42 (60)	24	19.5-28.5	0.093
> 60	28 (40)	13	7.8-13.2	
Location of primary lesion				
Colon	23 (32.9)	22	6.8-37.2	0.957
Rectum	47 (67.1)	19	10.7-27.2	
Stage of primary lesion				
Ⅰ	4 (5.7)	11	5.1-16.9	0.766
Ⅱ	11 (15.7)	27	14.1-39.9	
Ⅲ	36 (51.4)	18	8.6-27.4	
Ⅳ	19 (27.1)	22	11.7-32.3	
Unilateral or bilateral				
Unilateral	3 (4.3)	Not reached	Not reached	0.769
Bilateral	67 (95.7)	19	12.6-25.4	
Synchronous or metachronous				
Synchronous	19 (27.1)	22	11.5-32.5	0.693
Metachronous	51 (72.9)	18	11.0-24.9	
Regimen of first-line treatment				
FOLFOX	32 (45.7)	17	9.4-24.6	0.556
XELOX	12 (17.1)	24	18.5-29.5	
FOLFIRI	26 (37.1)	17	8.0-26.0	
First response assessment				
PR	25 (35.7)	27	23.4-30.6	0.00001
SD	36 (51.4)	16	8.3-23.7	
PD	9 (12.9)	8	5.2-10.8	

FOLFOX: Fluorouracil/leucovorin plus oxaliplatin; XELOX: Capecitabine plus oxaliplatin; FOLFIRI: Fluorouracil/leucovorin plus irinotecan; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Treatment outcomes

With a median follow-up time of 17 mo (range: 5-44 mo), 81.4% of patients (57 cases) had disease progression, and 26 patients were still alive (37.1%). The median PFS for first-line treatment was 8 mo (95% CI: 6.4-9.6 mo, range: 1-32 mo, Figure 1A). The median PFS for FOLFOX, XELOX and FOLFIRI regimens in the first-line setting were 10, 8 and 8 mo, respectively ($P = 0.693$). The median overall survival time was 19 mo (95% CI: 12.6-25.4 mo, range: 5-44 mo), with a 2 years OS rate of 38.8% (Figure 1B). At the patients' first response assessment, none of the patients had complete response; partial response (PR), stable disease (SD) and progressive disease (PD) were observed in 35.7%, 51.4% and 12.9% of patients, respectively. Four patients (5.7%) in our cohort underwent pulmonary resection of metastases for curative intent after first-line chemotherapy. We analyzed the 4 patients whose pulmonary metastatic lesions converted to resection in more detail: all 4 patients had multiple lesions in bilateral lungs. Two patients received preoperative chemotherapy with a regimen of modified FOLFOX6, while the other two received FOLFIRI. All 4 patients had a PR after first-line chemotherapy. The decision to perform metastatectomy in the 4 patients was made on the basis of the disappearance of lesions in one side of lung and stillness of residual disease in the other side of lung during ongoing chemotherapy. One patient underwent thoracoscopic surgery and the other three underwent open surgery. Wedge resections were performed and postoperative chemotherapy was applied using the same regimen before resection in all 4 patients. However, 1 patient developed disseminated liver and pelvic metastases later and died of cancer 7 mo after surgery; 1 patient had lung metastasis recurrence in the place where lesions disappeared after first-line chemotherapy. The other two patients were still free of recurrence until the last follow-up.

Clinical characteristics, including patients' gender, age, location of primary lesion, stage of primary lesion, metachronous or synchronous lung metastasis, unilateral or bilateral lung metastases, first-line chemotherapeutic regimens and first response assessment, were analyzed in univariate analyses to find potential predictive factors for patients' OS. The time to metastasis (continuous variable) in 51 patients with metachronous lung metastases was also studied using univariate Cox regression. Univariate analysis revealed that only the first response assessment was related to overall survival. Using Cox regression, multivariate analyses also demonstrated that first response assessment was the only independent factor for predicting OS. The risks of death within 2 years in SD and PD patients at their first response assessment were 3.5 times (95% CI: 1.4-8.7, $P = 0.007$) and 18.2 times (95% CI: 6.1-54.5, $P = 2 \times 10^{-8}$) higher than that of PR patients at the first response assessment. The median survival time in PR, SD and PD patients was 27 mo, 16 mo and 8 mo ($P = 0.00001$, Figure 1C).

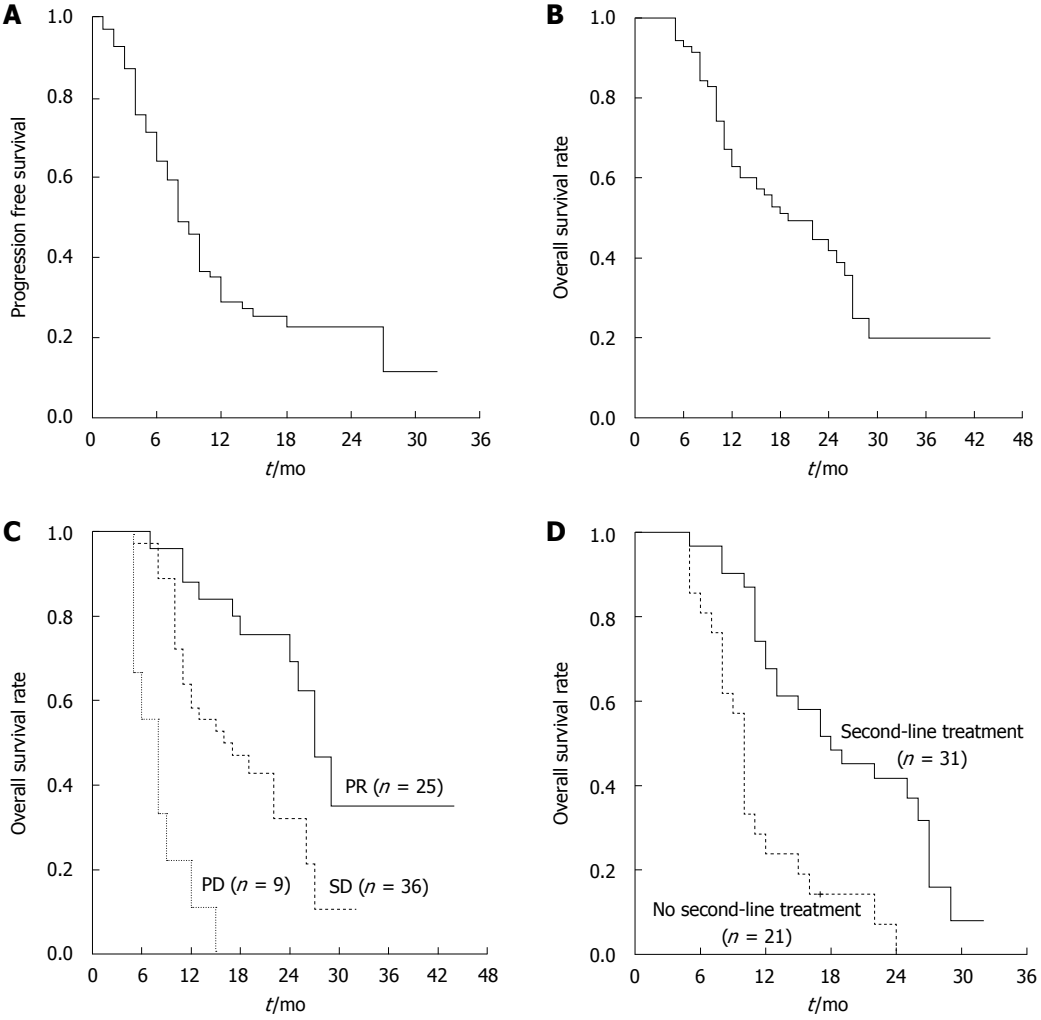

Figure 1 The median progression free survival and overall survival time. A: Progression free survival time of first-line chemotherapy; B: Overall survival rate for colorectal cancer patients with unresectable pulmonary metastases; C: Kaplan and Meier plots for median survival time according to chemotherapy efficacy; D: Kaplan and Meier plots for median survival time according to whether the second-line treatment was received. PR: Partial response; SD: Stable disease; PD: Progressive disease.

Second-line treatment

None of the patients without disease progression received second-line treatment. A total of 52 patients (74.3%) had disease progression during first-line chemotherapy. Thirty-one patients (59.2% of all first-line PD patients) underwent second-line chemotherapy. Unlike the first-line regimens, the chemotherapeutic regimens in the second-line were varied. Three patients only had a single agent regimen with 5-FU/leucovorin or capecitabine; 8 patients received FOLFOX or XELOX regimen; 16 patients received FOLFIRI or XELIRI regimen; 4 patients received a combination of chemotherapy and cetuximab. In 52 first-line PD patients, the median survival time was 18 mo for those receiving any of the above second-line regimens, compared with 10 mo for patients without any further chemotherapy ($P = 0.0004$, Figure 1D) .

DISCUSSION

In the current study, we focused on a typical group of patients with isolated lung metastasis. Lung metastases in all of our patients were the first and the only site of metastasis after diagnosing the primary tumor in the colon or rectum. Isolated lung metastasis is considered uncommon in colorectal cancers, as the major venous drainage is through the portal vein system and metastases in lungs usually occur along with concomitant systemic metastases. Pihl *et al*[4] reported 8.6% of 1578 patients with colorectal cancer who had undergone curative resection developed lung metastases, and that 11.5% of lung metastases were found in patients with rectal cancer, compared with 3.5% in colon cancer. Tan *et al*[6] reported 56 cases (7.4%) of isolated lung metastasis in 754 patients with colorectal cancer, and 13.3% of lung metastases were found in patients with primary rectal cancer *vs* 6.3% with primary colon cancer ($P = 0.011$). In our series, we also observed more isolated lung metastases in patients with primary rectal cancer. This result may partly be attributed to the bypass route of spreading into systemic circulation *via* the hemorrhoidal veins. However, the precise mechanism for this phenomenon of skip metastasis bypassing the first venous or lymphatic draining route is unclear. Differences in tumor biology among various patients, tumor types, or even within a given tumor, may also be related to this result[10,11].

Most of the lung metastases from colorectal cancer were unresectable disease because of synchronous metastases in other organs or multiple unilateral or bilateral lesions. Although resection of synchronous liver and lung metastases was performed in selected patients in several studies, a favorable outcome was only observed in a study with a small number of patients with limited metastases in liver and lung[12-14]. Different from the situation in the liver, lung metastases are more prone to disseminating to the lungs bilaterally, making the resection rate lower than that of liver metastasis[15,16]. The use of CT scans in diagnosing multiple lesions and thoracoscopic resection in the management of metastatic disease were also questioned[17-19]. Most of the patients selected for pulmonary metastasectomy presented with a single lesion in lung, and patients with a single metastasis were proven to have a better outcome compared with patients with multiple metastases[20-22]. The effectiveness of pulmonary resection in patients with multiple lesions is still unclear, and no consensus has been obtained for the indication of resection concerning the number of metastases[8,22-25]. Multiple lesions in bilateral lungs were usually considered unresectable disease. In our study, after discussing the resectability of isolated lung metastases, all cases with unresectable metastases were treated with first line chemotherapy. We reported the possibility of converting to resection in patients with multiple bilateral lesions by preoperative chemotherapy for the first time. Four cases (5.7%) were converted to resectable disease after first line treatment. To our knowledge, this is the first study reporting the resectability of initially unresectable lung metastases after preoperative chemotherapy. The proportion of resection seems lower than that of inoperable liver metastases[26], suggesting a different strategy might be used when considering preoperative chemotherapy for initially unresectable lung metastases. However, there are still other reasons which may be responsible for the low rate of conversion to resection, including the undetermined surgical indication, doubtful nodules in other areas, multiple lobular lesions after chemotherapy, and patients' consent.

In our study, the median PFS for first-line treatment and OS was similar to other studies in stage Ⅳ colorectal cancer, which combined all metastatic disease together[27,28]. Different chemotherapeutic regimens in the first-line setting, including FOLFOX, FOLFIRI and XELOX, resulted in a similar PFS and response rate. The National Comprehensive Cancer Network (NCCN) guidelines recommended that the practice in liver metastases can be applied to lung metastases[29]. However, few data were focused on this group of patients with unresectable isolated lung metastases. Our data confirmed the similar oncological results in the management of isolated lung metastases. Although the second-line chemotherapy regimens varied in progressed patients, we confirmed the benefit of continuing chemotherapy for patients with isolated lung metastases who progressed after first-line treatment. However, which regimen would be better and whether a monoclonal antibody should be used were unknown according to our study.

During first-line chemotherapy, the first response assessment is usually performed after the second month of treatment in most clinical trials of metastatic colorectal cancer. In our cohort, multivariate analysis demonstrated that the first response assessment was the independent factor predicting patients' overall survival. The risk of death within 2 years was found to be 3.5 times higher in SD patients than that in PR patients. This result raised doubts about whether we should continue first-line treatment in SD patients. In our study, the response assessment was performed according to RECIST guidelines. Patients assessed to have SD included patients whose targeted lesions showed less than 20% increase to less than 30% decrease in the sum of the longest diameter. Changing of chemotherapeutic regimens might be reasonable in selected SD patients, such as patients with increased lesions. For patients with metachronous lung metastases, the time to relapse was considered a risk factor predicting survival in several studies[30], but most other studies did not find the time to relapse a significant risk factor[31-33]. In 906 patients who relapsed after curative treatment of colorectal cancer, Kobayashi *et al*[16] found that the time to relapse was only related to the survival of liver metastases and local recurrence, but no survival difference was found in patients with lung metastases.

Novel treatment approaches have been introduced into the local treatment of unresectable lung metastases. Radiofrequency ablation was studied for patients with unresectable lung metastases in several studies. Marginal survival benefit and improvement in quality of life were observed in selected cases[34-36], and it is only used as an adjunct to resection or as an alternative for unresectable lesions. Transpulmonary chemoembolization is another new technique used for the local treatment of inoperable lung metastases. Preliminary results found this technique was a well-tolerated procedure for palliative treatment, but the benefit of survival was still unknown[37-39].

In conclusion, isolated lung metastases were relatively rare in metastatic colorectal cancer. The outcomes of chemotherapy in unresectable lung metastases patients were similar to the results of liver metastases, and first response assessment was an important risk factor to predict survival. Pulmonary metastasectomy can only be performed in a small group of patients with initially unresectable lung metastases after preoperative chemotherapy. Further studies are needed to validate our results and evaluate the rate of resectability after chemotherapy.

COMMENTS

Background

Approximately 50%-60% of colorectal cancer patients will develop metastases. Nearly half of patients with colorectal cancer will develop liver metastases, while only approximately 10%-15% of patients will develop lung metastases ultimately. Studies demonstrated that 10%-15% of initially unresectable liver metastases could be converted to resectable disease after preoperative chemotherapy. However, there was little experience in unresectable isolated lung metastasis.

Research frontiers

Current opinions consider that it may be reasonable to apply the recommendations for treatment of liver metastases to the treatment of lung metastasis, but

the evidence is weak and the result of this application is unknown, especially for initially unresectable isolated lung metastases.

Innovations and breakthroughs

This study prospectively collected data from all patients with unresectable isolated lung metastases from colorectal cancer as the first recurrence in a single institution during a 5-year period. The outcomes of chemotherapy were similar to the results of liver metastases, and first response assessment was an important risk factor to predict survival. Furthermore, only 5.7% of patients were converted to resectable disease after preoperative chemotherapy, which was obviously lower than the conversion rate in liver metastasis. It is the first study reporting the resectability of initially unresectable lung metastases after preoperative chemotherapy.

Applications

The results of this study suggested that a different strategy might be used when considering the preoperative chemotherapy for initially unresectable lung metastases.

Peer review

This is an interesting and novel paper looking at isolated lung metastases in colorectal cancer.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3325

World J Gastroenterol 2010 July 14; 16(26): 3325-3329
ISSN 1007-9327 (print)

BRIEF ARTICLE

Relationship between let-7a and gastric mucosa cancerization and its significance

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Received: January 12, 2010 Revised: April 30, 2010
Accepted: May 7, 2010
Published online: July 14, 2010

Abstract

AIM: To investigate let-7a expression and analyze the correlation between let-7a and progression of gastric mucosa cancerization.

METHODS: The tissue microarray was constructed previously in 52 cases of human gastric carcinoma, 17 cases of chronic atrophic gastritis (atypical hyperplasia) and 11 cases of normal gastric tissue, and tissue microarrays combined with *in situ* hybridization were used to detect the expression of let-7a.

RESULTS: The positive rates of let-7a in normal gastric tissue, chronic atrophic gastritis and gastric carcinoma were 90.9%, 88.2% and 86.5%, respectively, without significant differences among the groups ($P > 0.05$). However, an intense signal of let-7a was observed in gastric epithelial cells, whereas a less intense signal was found in gastric atypical hyperplasia epithelial cells and a weak signal in gastric carcinoma epithelial cells. The expression of let-7a decreased along with the progression of gastric mucosa cancerization ($P < 0.05$). In the group of gastric carcinoma, the expression of let-7a was even significantly lower in gastric carcinomas with lymph node metastasis than in those without metastasis ($P < 0.05$).

CONCLUSION: Gastric carcinoma has relatively lower expression of let-7a. Reduced let-7a may be a fundamental factor in the formation and lymph node metastasis of gastric carcinoma.

Key words: Tissue microarray; *In situ* hybridization; MicroRNA; Gastric carcinoma; Precancerous lesions

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Zhu YM, Zhong ZX, Liu ZM. Relationship between let-7a and gastric mucosa cancerization and its significance. *World J Gastroenterol* 2010; 16(26): 3325-3329 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3325.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3325

INTRODUCTION

MicroRNAs (miRNAs) are a recently discovered class of short noncoding RNA genes that act post-transcriptionally as negative regulators of gene expression[1,2]. A large body of research shows that animal miRNAs play a fundamental role in many biological processes, including cell growth and apoptosis, hematopoietic lineage differentiation, insulin secretion, brain morphogenesis, and muscle

cell proliferation and differentiation[3]. About 1000 miRNA genes are thought to be encoded in the human genome[4,5]. Although it is still difficult to identify accurately individual miRNA/target interactions[6], computational predictions of miRNA target genes indicate that as many as one-third of all human protein-encoding genes may be regulated by miRNAs[7]. This suggests that miRNAs could be involved in a wide variety of human diseases, including cancer. As a result of in-depth researches over the last 5 years, miRNA-mediated regulation of tumorigenesis is emerging as a new paradigm in the field of cancer biology[8,9]. It has also been reported that these miRNA signatures may be a more robust tool than expression patterns of protein-encoding genes for distinguishing normal from tumor tissues[10,11]. One particular miRNA, let-7, was discovered in the early 1990s in humans and mice. It has been reported that let-7 as a novel candidate of tumor suppressor gene is involved in several cancers such as lung carcinoma and colon carcinoma.

Gastric carcinoma is one of the most frequent cancers and one of the most common causes of cancer-related mortality in China, with an incidence of 0.4 million new cases and 0.3 million deaths annually. Gastric carcinoma is also an inadequately understood, and often fatal disease when not detected at early stages. Atypical hyperplasia is generally accepted as a precancerous lesion. Cancerization from normal gastric mucosa, chronic atrophic gastritis to gastric carcinoma has been well acknowledged. In this study, the expression of let-7a in normal gastric tissue, chronic atrophic gastritis (atypical hyperplasia) and gastric carcinoma was detected by *in situ* hybridization (ISH) using a locked nucleic acid (LNA) probe and gastric mucosa cancerizing tissue microarrays; the relation between expression level of let-7a and the progression of gastric mucosa cancerization was discussed.

MATERIALS AND METHODS

Tissue microarray and probe

The tissue bank at Shanxi Chaoying Biotechnology Company maintains a series of formalin-fixed, paraffin-embedded (FFPE) tissue blocks of patients with gastric carcinoma, chronic atrophic gastritis (atypical hyperplasia) and normal gastric tissue. Information on tumor histologic grade, patient's age and sex was collected and anonymized in a secure database. We generated a tissue microarray (TMA) from archived FFPE blocks of 80 cases, including 11 cases of normal gastric tissue, 17 cases of chronic atrophic gastritis with atypical hyperplasia (incomplete intestinal metaplasia) and 52 cases of gastric carcinoma (intestinal type). In the gastric carcinoma group, there were 10 cases with lymph node metastasis and 42 cases without lymph node metastasis. This study complied with the rules and regulations of the Ethics Committee of the hospital. Informed consent was obtained from all subjects. All FFPE blocks were reviewed and tissue cores were selected by a board-certified pathologist. The 5'Digoxin-labeled, LNA-modified oligonucleotide probe (sequence 5'-3': aactatacaacctactacctca) complementary to the entire mature sequence of let-7a, was synthesized by Exiqon DNA technologies.

Detection of miRNAs by ISH in FFPE sections

The experiment was conducted at a laboratory of Shanxi Chaoying Biotechnology Company in November 2009. Sections (5 μm) of archived paraffin-embedded specimens were deparaffinized in xylenes and then rehydrated through an ethanol dilution series (from 100% to 25%). Slides were submerged in diethyl pyrocarbonate-treated water and subjected to proteinase K digestion (5-10 μg/mL) and 0.2% glycine treatment, refixed in 4% paraformaldehyde, and treated with acetylation solution [66 mmol/L HCl, 0.66% (v/v) acetic anhydride, and 1.5% (v/v) triethanolamine]; slides were rinsed thrice with 1 × PBS between the treatment. Slides were prehybridized in hybridization solution (50% formamide, 5 × SSC, 500 Ag/mL yeast tRNA, and 1 × Denhardt's solution) at 50℃ for 30 min. Then 5-10 pmol of Digoxin-labeled, LNA-modified probe, was added to 150 μL hybridization solution and hybridized for 18 h at a temperature of 42℃ below the calculated Tm of the LNA probe. Subsequently, the samples were incubated at 37℃ for 90 min with 50 μL biotin-labeled anti-digoxin antibody, rinsed with 0.5 × SSC four times for 5 min, incubated at 37℃ for 30 min with 50 μL SABC solution, and rinsed with 0.5 × SSC solution three times for 5 min. After added 50 μL biotin-labeled peroxidase and incubated at 37℃ for 30 min, the samples were rinsed five times with 0.5 × SSC for 5 min, then stained with Nitro-Blue-Tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate. Finally, the sections were restained with eosin before covering. For a negative control, the hybridization solution was replaced with pre-hybridization solution. Likewise, β-actin was used as the probe of positive control.

Determination of results

Signals were visually quantified by a pathologist according to the reference of Frantz *et al*[12]. A total of 100 × 10 cells were counted randomly. Then, tissue sections were blindly examined by a second individual for an agreement with the initial quantifications. If there was no positive cell, the sample was scored as 0 point. If the percentage of positive cells was less than or equal to 30%, the sample was scored as 1 point; less than or equal to 70% as 2 points; and more than 70% as 3 points. The staining intensity was recorded as negative, light, moderate, or strong, and scored as 0, 1, 2 or 3 points, respectively. The two values were multiplied, and a sum of 0 point represented negative expression, and 1-9 points represented positive expression. Points 1-2 were marked ±, 3-4 marked +, 6 marked ++, and 9 marked +++.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 Package. The χ^2 and correlation tests were used in this study. A difference was considered significant when *P* value was less than 0.05.

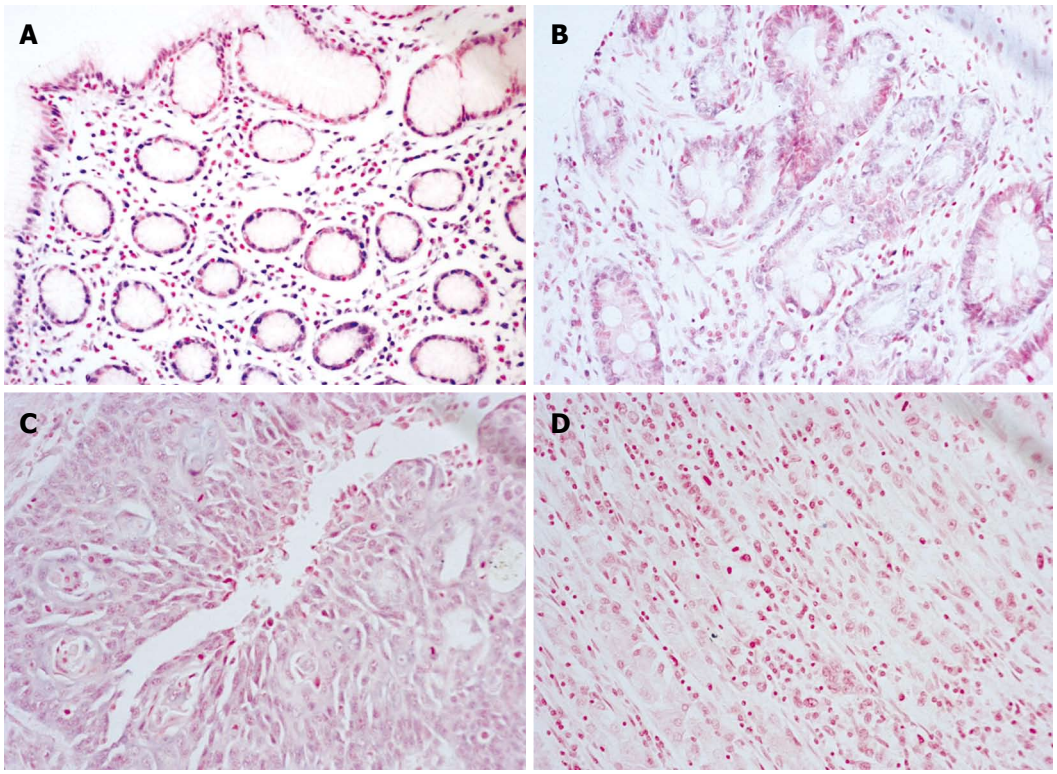

Figure 1 Expression let-7a in stomach cancer progression tissue array (*in situ* hybridization, Nitro-Blue-Tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate staining, original magnification × 200). A: Positive expression (++) of let-7a in gastric tissue; B: Positive expression (+) of let-7a in chronic atrophic gastritis; C: Positive expression (±) of let-7a in gastric carcinoma; D: Negative expression of let-7a in gastric carcinoma.

Table 1 Expression of let-7a in gastric mucosa cancerization tissue array

	Cases (*n*)	Results					Positive rate (%)
		-	±	+	++	+++	
Normal gastric mucosa	11	1	0	2	8	0	90.9
Chronic atrophic gastritis	17	2	2	7	5	1	88.2
Gastric carcinoma	52	7	18	18	7	2	86.5

Table 2 Expression of let-7a in gastric carcinomas

Lymph node metastasis	Cases (*n*)	Results					*P* value
		-	±	+	++	+++	0.043
Negative	42	4	11	18	7	2	
Positive	10	3	6	1	0	0	

RESULTS

Expression of let-7a was determined on 5-μm sections obtained from each of the TMA blocks. Generally, let-7a appeared as blue-purple particles in the cytoplasm or nucleus after staining, and predominantly expressed in normal gastric mucosa epithelia and its expression was down-regulated in atypical hyperplasia epithelia, especially in malignant cells. The let-7a probe revealed an intense cytoplasmic signal in gastric epithelial cells, whereas a less intense signal was observed in gastric atypical hyperplasia epithelial cells and a weak signal in gastric carcinoma epithelial cells (Figure 1). Let-7a was also detected in endothelial cells lining blood vessels and in fibroblasts (data not shown). The classification of let-7a expression grade in each group is shown in Table 1. There was no significant difference in the positive rates of let-7a among gastric carcinoma, chronic atrophic gastritis and normal gastric tissues ($P > 0.05$). However, by analysis of the signal levels of let-7a in each group, the levels of let-7a expression were reversely related to the progression of gastric mucosa cancerization ($P = 0.008$). The expression of let-7a decreased along with the progression of gastric mucosa cancerization. In the gastric carcinoma group, the expression of let-7a in the 10 cases with lymph node metastasis was even lower than in the 42 cases without lymph node metastasis ($P = 0.043$) as shown in Table 2. In this study, β-actin showed an intense cytoplasmic signal in each of the TMA blocks in positive control while no blue-purple staining was found in blocks of negative control.

DISCUSSION

The let-7 family consists of 12 genes encoding for nine distinct miRNAs (let-7a to let-7i). Historically, let-7 was found to bind to the 3' UTR of lin-41 and hbl-1 (lin-57)

to inhibit their translation in nematodes[13], and control the developmental transition from the L4 stage into the adult stage[14-16]. An evolutionarily conserved regulation of the RAS family of growth control proteins by let-7 from nematodes to human cancer cells has also been demonstrated. Recently, let-7 implication in cancerogenesis has been extended to the repression of high mobility group A2, thus preventing oncogenic transformation in many tumors[17]. Johnson *et al*[18] showed that let-7 miRNA expression was lower in lung tumors than in normal lung tissues. The detection of mature miRNAs by ISH is technically challenging because of their small size. LNAs are a class of bicyclic high-affinity RNA analogues, in which the furanose ring in the sugar-phosphate backbone is chemically locked in a conformation mimicking the North-type (C3'-endo) conformation of RNA[19,20]. This results in an unprecedented hybridization affinity of LNA toward complementary single-stranded RNA molecules. A limitation to identifying and validating potential biomarkers makes it difficult to conduct retrospective studies with archived tumor specimens due in part to protein and RNA degradation[21]. Hence, the very small size of miRNAs offers a unique advantage because short RNA molecules are substantially less susceptible than mRNAs to enzymatic and mechanical degradation. In this study, we used a method for detection of miRNAs in gastric carcinoma specimens, LNA-ISH, which offers a spatial resolution of miRNA expression unsurpassed by other available techniques. Zhang *at al*[22] established an accurate and rapid stem-loop reverse transcriptional real-time PCR (RT-PCR) method to quantify human let-7a miRNA in gastric cancer. It was found that the expression level of let-7a miRNA in gastric tumor tissues was significantly lower than in normal tissues of 14 samples from 32 patients. There might be a discordance between ISH and RT-PCR results, which could be explained by the overrepresentation of epithelial cells in tumor lesions, resulting in a high let-7 signal in gross tissue biopsies. However, let-7a expression is diminished within individual cancer cells, which can be determined only by the ISH approach. We noticed that long fixation periods in 10% formalin (more than 12-18 h) can affect negatively the intensity of the miRNA *in situ* signal. There is no significant difference of the positive rates of let-7a expression among gastric carcinoma, chronic atrophic gastritis and normal gastric tissue, which may be partly due to the limitation of normal gastric tissue cases in the TMA. Decrease of let-7a expression is already apparent in hyperplasia (possibly preneoplastic). Its further decrease in gastric carcinoma is reminiscent of reduction of let-7a related to the gastric mucosa cancerization progression. Hence, this suggests that reduction of let-7a is an early event during intestinal-type gastric carcinoma formation. The most essential behavior of the malignant tumor is metastasis. Lymph nodes metastasis is an important index for clinical stages of gastric carcinoma before surgery and it is also an important prognosis factor for gastric carcinoma patients. Our study showed that the expression of let-7a was even lower in gastric carcinoma with lymph nodes metastasis than in those without lymph nodes metastasis. Decreased expression or absence of let-7a may determine initially the occurrence, development, metastasis and prognosis of gastric carcinoma. Using combined LNA-ISH and RT-PCR, quantitative detection of let-7a may have a potential clinical application as a novel biomarker for early diagnosis and prognosis of gastric carcinoma. Infecting breast tumor-initiating cells with let-7-lentivirus reduced proliferation, mammosphere formation, and the proportion of undifferentiated cells *in vitro* and tumor formation and metastasis in NOD/SCID mice[23]. Thus, the reason why let-7a in human gastric carcinoma has generated great interest is that it not only has a potency in diagnosis, but also can shed light on the molecular mechanisms of the tumorigenic process and has a high potential as novel targets for therapeutic intervention using synthetic oligonucleotide technologies. Further studies are being carried out on the relationship between gastric carcinoma and let-7a in our laboratory.

COMMENTS

Background

Gastric carcinoma is one of the malignancies with highest incidence and mortality rates and it is of great significance to investigate the mechanisms behind the occurrence and development of gastric carcinoma. Down-regulated expression let-7a has been shown to play an important role in the tumorigenesis and cancer progression. Therefore, the exploration into the expression of let-7a in gastric carcinoma and precancerous lesions may shed a new light on early detection and therapy of gastric carcinoma.

Research frontiers

Let-7a has been shown to exert tumor suppressor gene effects in various human neoplasms. In this study, the author investigated the expression of let-7a in normal gastric mucosa, chronic atrophic gastritis and gastric carcinoma to explore its correlation with gastric carcinogenesis and metastasis. They found that reduced expression of let-7a may take part in early carcinogenesis and metastasis of gastric carcinoma.

Innovations and breakthroughs

Let-7a is down-regulated in a wide range of human cancers such as gastric, colorectal and breast carcinomas, and may play a key role in cancer pathogenesis. Down-regulated let-7a increases cancer cell proliferation, migration and invasion. This is the first study to report that expression of let-7a has a reverse correlation with gastric cancerization.

Applications

The expression level of let-7a was significantly lower in gastric carcinoma than in chronic atrophic gastritis and normal gastric mucosa. In the malignant tumor group, the expression level of let-7a was even significantly lower in gastric carcinoma with lymph node metastasis than in those without metastasis. Detection of let-7a expression might be helpful in early detection and prognosis judgment of gastric carcinoma patients. In addition, let-7a may serve as a potential therapeutic tool for gastric carcinoma.

Peer review

In this descriptive study, let-7a miRNA expression is analyzed in human gastric mucosa, chronic atrophic gastritis, and gastric cancer with *in situ* hybridization technique on tissue microarray. The study confirms the results by Zhang *et al* demonstrating a decrease of let-7a in gastric carcinogenesis.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3330

World J Gastroenterol 2010 July 14; 16(26): 3330-3334
ISSN 1007-9327 (print)

BRIEF ARTICLE

Paclitaxel ameliorates fibrosis in hepatic stellate cells *via* inhibition of TGF-β/Smad activity

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Author contributions: Zhou J did most of the experiments and data acquisition; Wang QW, Miao XY and Xu XD were involved in initial experiments in this paper; Zhou J and Zhong DW participated actively in the design of experiments, interpretation of data, and preparation of the manuscript.

Supported by Grants from the Creative Research Group Fund of the National Foundation Committee of Natural Science of China, No. 30871169/C140405

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Received: January 29, 2010 Revised: March 9, 2010
Accepted: March 16, 2010
Published online: July 14, 2010

Abstract

AIM: To investigated if paclitaxel can attenuate hepatic fibrosis in rat hepatic stellate cells (RHSCs).

METHODS: RHSCs were cultured *in vitro* and randomly assigned to four groups: normal control group (treated only with Dulbecco's Modified Eagle's Medium), Taxol group (200 nmol/L paclitaxel was added to the cell culture), transforming growth factor (TGF)-β group (5 ng/mL recombinant human TGF-β1 was added to the cell culture), and TGF-β + Taxol group. TGF-β signaling cascade and status of various extracellular matrix proteins were evaluated by real time reverse transcriptase polymerase chain reaction and Western blotting.

RESULTS: The paclitaxel treatment markedly suppressed Smad2/3 phosphorylation. This was associated with attenuated expression of collagen Ⅰ and Ⅲ and fibronectin in RHSCs.

CONCLUSION: These data indicate that 200 nmol/L paclitaxel ameliorates hepatic fibrosis *via* modulating TGF-β signaling, and that paclitaxel may have some therapeutic value in humans with hepatic fibrosis.

Key words: Transforming growth factor-β; Hepatic fibrosis; Paclitaxel; Smad; Microtubules

Peer reviewer: Dariusz M Lebensztejn, Associate Professor, IIIrd Department of Pediatrics, Medical University of Bialystok, 17 Waszyngtona Str., Bialystok, 15-247, Poland

Zhou J, Zhong DW, Wang QW, Miao XY, Xu XD. Paclitaxel ameliorates fibrosis in hepatic stellate cells *via* inhibition of TGF-β/Smad activity. *World J Gastroenterol* 2010; 16(26): 3330-3334 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3330.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3330

INTRODUCTION

Hepatic fibrosis is the key stage in the pathological process from hepatic injury to cirrhosis or even to tumor. Transforming growth factor (TGF)-β1 is widely acknowledged as a key factor in acceleration of the hepatic fibrosis process. TGF-β1 mainly activates hepatic stellate cells (HSCs) through the TGF-β1/Smad signaling pathway, thus causing hepatic fibrosis[1-5]. TGF-β evokes diverse cellular responses by binding to and activating specific cell-surface receptors that have intrinsic serine/threonine kinase activity. The activated TGF-β receptors stimulate the phosphorylation of receptor-regulated Smad2 and Smad3 proteins (R-Smads), which in turn form complexes with Smad4. This complex translocates from the cytoplasm into the nucleus, where the Smads regulate the transcription of target genes. Inhibitory Smad7 acts in an opposing manner to the R-Smads, and downregulates TGF-β signaling[6].

Some studies have previously demonstrated that endogenous Smad-2, 3 and 4 bind to microtubules in several cell lines, and the binding provides a negative regulatory mechanism to modulate TGF-β activity. Disruption of the microtubule network by chemical agents, such as nocodazole and colchicine, leads to ligand-independent Smad nuclear accumulation and transcription of TGF-β-responsive genes, which in turn increases TGF-β-induced Smad activity[7].

The aim of this study was to assess if microtubule stabilization with low-dose paclitaxel (Taxol) could inhibit TGF-β/Smad signaling and ameliorate hepatic fibrosis in HSCs.

MATERIALS AND METHODS

Cell culture and grouping

HSCs (supplied by the Institute of Liver Diseases, the Second Xiangya Hospital, Hunan, China) were seeded in 24-hole plastic culture plates with a density of 1×10^6/mL. The cell viability was > 95% and purity was > 90%. After cell culture for 2 wk, nearly all HSCs were activated. These cells were first cultured for 3 d, followed by a cell cycle of synchronous culture for 48 h, then the HSCs were randomly divided into four groups: normal control group (treated only with Dulbecco's Modified Eagle's Medium), normal + Taxol group [200 nmol/L paclitaxel (Taxol; Sigma, St. Louis, MO, USA) was added to the cell culture], TGF-β group [5 ng/mL recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) was added to the cell culture], and TGF-β + Taxol group. The examinations were carried out after 48 h of culture. All the examinations were repeated three times for accuracy and consistency.

Real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated using the High Pure RNA Isolation Kit according to the manufacturer's instructions (Roche, Switzerland). Contaminated DNA was removed by treating the samples with RNase-free DNase I (Promega, Madison, WI, USA). Real-time polymerase chain reaction (PCR) was performed using Bio-Rad (Hercules, CA, USA) iQ SYBR Green supermix with Opticon (MJ Research Inc., Waltham, MA, USA) by following the vendor's instructions. One hundred micrograms of total RNA was reverse-transcribed and subjected to PCR as follows: 94℃ for 2 min followed by 40 cycles of: 94℃ for 15 s, 58℃ for 30 s and 72℃ for 30 s, and a final extension at 72℃ for 10 min. The primers used were as follows. Rat Smad2, forward: 5'-TCACAGCCATCATGAGCTCAAGG-3', reverse: 5'-TGTGACGCATGGAAGGTCTCTC-3'; Smad3, forward: 5'-AGCACACAATAACTTGGACC-3', reverse: 5'-TAAGACACACTGGAACAGCGGATG-3'; collagen Ⅰ, forward: 5'-GAGCGGAGAGTACTGGATCG-3', reverse: 5'-TACTCGAACGGGAATCCATC-3'; collagen Ⅲ, forward: 5'-GTGCGGTTTGTGAAGCACCG-3', reverse: 5'-GTTCTTCTCATGCACACTT-3'; fibronectin, forward: 5'-TGACTCGCTTTGACTTCACCAC-3', reverse: 5'-TCTCCTTCCTCGCTCAGTTCGT-3'. All samples were subjected to reverse transcriptase (RT)-PCR along with the housekeeping gene GAPDH with the following primer sequences: forward: 5'-TGCTGAGTATGTCGTGGAGTCTA-3', reverse: 5'-AGTGGGAGTTGCTGTTGAAATC-3' as an internal standard. Reaction specificity was confirmed by gel electrophoresis of products after real-time PCR and melting curve analysis. Ratios for Smad2/GAPDH, Smad3/GAPDH, collagen Ⅲ/GAPDH, fibronectin/GAPDH and collagen Ⅰ/GAPDH mRNA were calculated for each sample and expressed as the mean ± SD.

Western blotting

Total protein was extracted from cells and analyzed with bicinchoninic acid protein concentration assay kit (Beijing Biosea Biotechnology Co. Ltd., China). Samples (20 μg) were fractionated by SDS-PAGE. After transfer onto nitrocellulose membrane (Amersham International, Bucks, UK), the blots were probed with a mouse monoclonal antibody to p-Smad3 (Cell Signaling Technology, Beverly, MA, USA 1:1000 dilution), a goat polyclonal antibody to p-Smad2 (Upstate Biotechnology, Billerica, MA, USA 1:1000 dilution) and rabbit polyclonal antibodies to collagen Ⅰ (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:2000 dilution), collagen Ⅲ (Abcam, Cambridge, MA, USA 1:2000 dilution), and fibronectin (Santa Cruz Biotechnology, 1:1000 dilution). The second antibodies were peroxidase-conjugated goat anti-mouse IgG (1:20 000 dilution)and the swine anti-rabbit IgG or rabbit anti-goat IgG , which be diluited in PBS that contained 1% normal goat serum or 1% fetal calf serum. β-actin was used as an internal control.

Statistical analysis

Data were calculated as the mean ± SD and the groups were compared using one-way ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Paclitaxel inhibits extracellular matrix expression in rat HSCs

The suppressive effects of paclitaxel on mRNA and protein expression of fibronectin and collagen Ⅰ and Ⅲ were assessed by real-time RT-PCR and Western blotting. Figure 1A shows RT-PCR analysis that indicated that paclitaxel had no effect on basal mRNA expression for fibronectin and collagen Ⅰ and Ⅲ in rat HSCs. The expression of integrin-linked kinase, α-smooth muscle actin and collagen Ⅰ was reduced in cells treated with paclitaxel and TGF-β at 48 h ($P < 0.01$). Western blotting analyses revealed similar trends in mRNA expression ($P < 0.05$, $n = 6$) (Figure 2A).

Blockade of Smad2/3 activation is a key mechanism by which paclitaxel prevents hepatic fibrosis in rat HSCs

The effects of paclitaxel on expression of Smad2 and Smad3 mRNA were assessed by real-time RT-PCR. The analysis showed that paclitaxel had no effect on Smad2

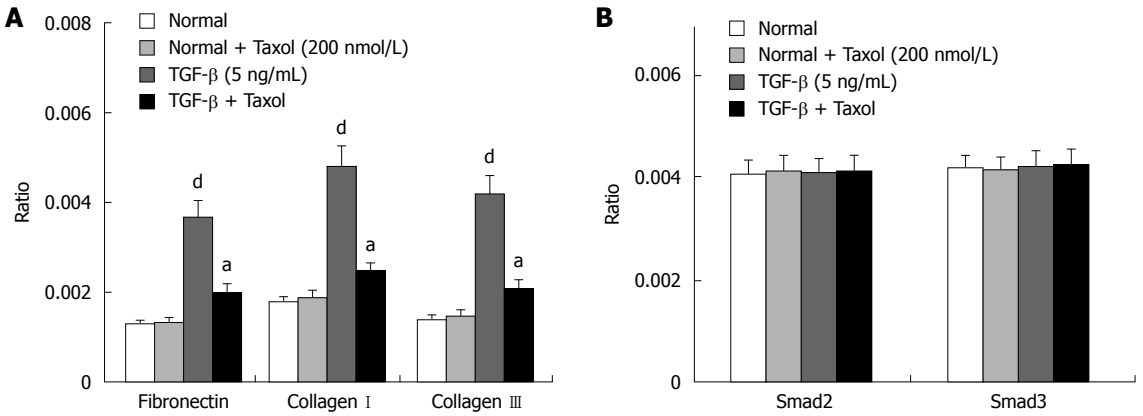

Figure 1 The mRNA levels changes *in vitro* after treatment. A: Real-time PCR depicting fibronectin and collagen Ⅰ and Ⅲ mRNA expression *in vitro* following paclitaxel treatment of hepatic stellate cells (HSCs). Fibronectin and collagen Ⅰ and Ⅲ expression was significantly higher in the TGF-β group at 48 h compared with the normal or Taxol groups ($P < 0.05$, $n = 6$). Treatment with paclitaxel resulted in a decrease in fibronection and collagen Ⅰ and Ⅲ expression ($P < 0.05$, $n = 6$). $^{a}P < 0.05$ *vs* TGF-β group; $^{d}P < 0.01$ *vs* normal or Taxol group ($n = 6$); B: Real-time PCR showing Smad2 (left) and Smad3 (right) mRNA expression *in vitro* following treatment with paclitaxel in HSCs. Smad2 and Smad3 mRNA expression was more or less similar in the normal and Taxol groups at 48 h ($P < 0.05$, $n = 6$). Densitometric analyses were performed from six independent experiments. Each bar represents the mean ± SD for six animals. PCR: Polymerase chain reaction; TGF-β: Transforming growth factor β.

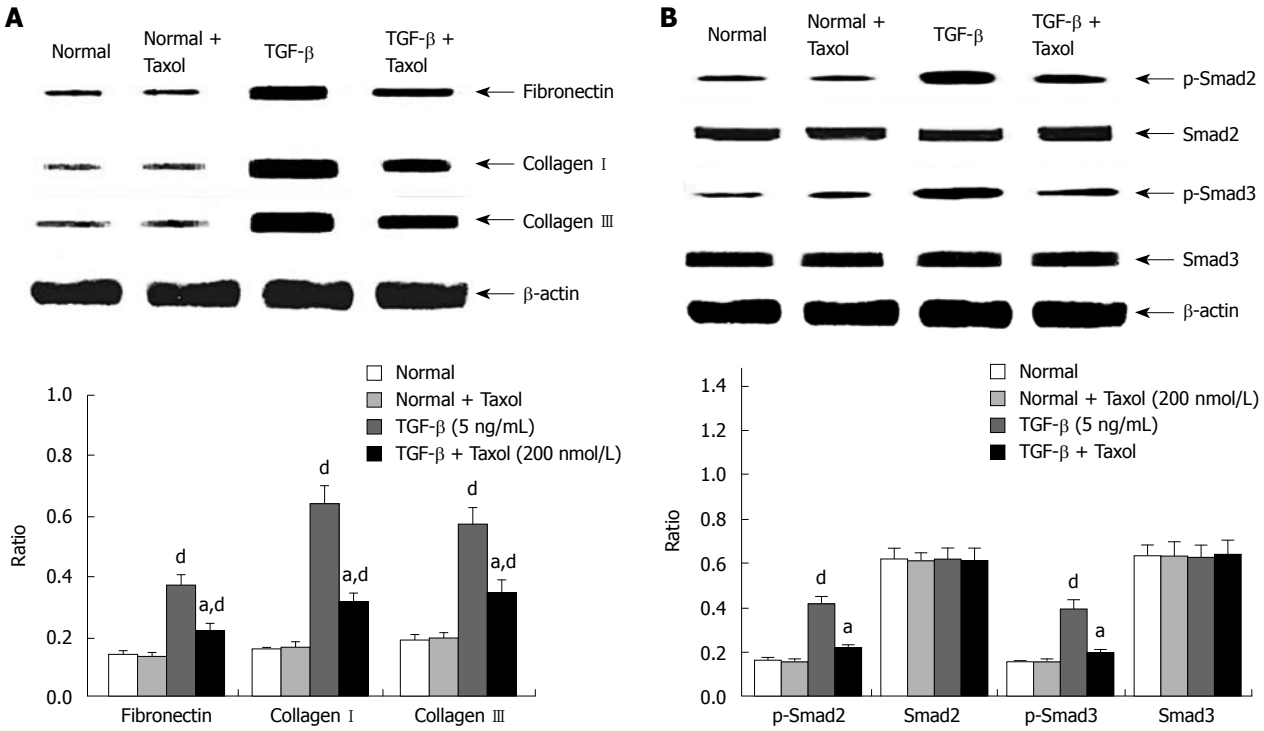

Figure 2 The protein levels changes *in vitro* after treatment. A: Western blotting analyses depicting fibronectin and collagen Ⅰ and Ⅲ mRNA expression *in vitro* following paclitaxel treatment in HSCs. Whole hepatic cell extracts were immunoblotted with the indicated antibodies. Fibronectin and collagen Ⅰ and Ⅲ expression was significantly higher in the TGF-β group at 48 h compared with the normal or Taxol group ($P < 0.05$, $n = 6$). Treatment with paclitaxel resulted in a decrease in fibronectin and collagen Ⅰ and Ⅲ expression ($P < 0.05$, $n = 6$); B: Western blotting analysis showing Smad2/3 protein expression *in vitro* following treatment with paclitaxel in HSCs. Phosphorylated Smad2 and Smad3 but not total Smad2 or Smad3 expression was significantly higher in the TGF-β group at 48 h compared with the normal or Taxol group ($P < 0.05$, $n = 6$). Treatment with paclitaxel resulted in a decrease in phosphorylation of Smad2 and Smad3 ($P < 0.05$, $n = 6$). Densitometric analyses were performed from six independent experiments. Each bar represents the mean ± SD for six animals. $^{a}P < 0.05$ *vs* TGF-β group; $^{d}P < 0.01$ *vs* normal or Taxol group ($n = 6$).

and Smad3 mRNA expression in the normal, Taxol and TGF-β + Taxol groups at 48 h (Figure 1B). Western blotting analysis showed that phosphorylated Smad2/3 but not total Smad2/3 expression was markedly reduced in HSCs treated with paclitaxel and TGF-β at 48 h ($P < 0.01$). In contrast, paclitaxel had no effect on expression and phosphorylation of Smad2/3 in the Taxol group (Figure 2B).

DISCUSSION

In the present study, we observed that fibrosis in HSCs was substantially decreased by paclitaxel, a microtubule-stabilizing agent, thus suggesting that low-dose paclitaxel has therapeutic benefits in the amelioration of hepatic fibrosis.

The TGF-β/Smad signaling pathway plays an impor-

tant role in hepatic fibrosis. In chronic hepatic injury, after HSCs are transformed into myofibroblasts, Smad2 and Smad3 are continuously phosphorylated and the inhibitory expression of Smad7 is at a low level. As a result, TGF-β1 signal transduction cannot be effectively inhibited. This might be one of the mechanisms for progression from chronic hepatic injury to hepatic fibrosis[8,9]. The worse the hepatic fibrosis and the higher the expression of TGF-β1, the higher is the Smad3 protein expression. Therefore, whatever is transferred by Smad3 might be the signal that can induce hepatic fibrosis[10-12]. Currently, TGF-β1 is the most effective fibrosis-promoting known, and it can promote activation of HSCs, and increase synthesis of extracellular matrix (ECM)[4,5]. Furthermore, it has been previously shown in several different cell lines that microtubules serve as a negative regulator for TGF-β/Smad signaling by forming a complex with endogenous Smad2, 3 and 4, thus sequestering the R-Smads away from the TGF-β receptor[7]. Therefore, it is conceivable that stabilization of microtubules by low-dose paclitaxel can dampen the exacerbated TGF-β signaling, as reported in TGF-β-induced inhibition of myogenesis in C2C12 myoblasts[13]. Similarly, in an earlier study, Liu *et al*[14] also have found that paclitaxel can significantly suppress TGF-β/Smad activity in SCID mice. In the present study, we provided evidence that low-dose paclitaxel suppressed phosphorylation of Smad2 and Smad3, two homologous Smad proteins that transduce signals from TGF-β and activin, in rat HSCs. These data support the notion that TGF-β/Smad signaling is regulated by the dynamic stability/instability of microtubules that are sensitive to low-dose microtubule-stabilizing agents, like paclitaxel.

Paclitaxel is an anticancer agent[15], which by stabilizing polymerized microtubules and enhancing microtubule assembly, arrests the cell cycle in the G0/G1 and G2/M phases, thus leading to cell death[16,17]. Prolonged treatment with paclitaxel has been associated with scleroderma-like changes or pulmonary fibrosis, albeit in only a small fraction of patients. It is noteworthy that inhibition of tumor cell proliferation can be achieved by much higher doses of paclitaxel. The inhibition of TGF-β/Smad signaling, however, can be attained with very low doses of paclitaxel. However, some of the recent studies have indicated that low-dose paclitaxel has minimal, if any, detectable effects on cell proliferation and other cellular activities, including fibrosis. Intriguingly, low-dose paclitaxel has been shown to inhibit collagen-induced arthritis and fibrosis associated with systemic sclerosis in SCID mice[14,18,19]. Type I collagen, the major ECM component of fibrotic tissue, is a heterotrimer composed of two α1 chains and one α2 chain. Increased production of type I collagen is a common hallmark of fibrotic diseases in various organs including the liver. Once stimulated by fibrogenic stimuli, HSCs are the only cells that respond by expressing increased amounts of all three different isoforms of TGF-β. As activated HSCs are the principal cells to produce type I collagen in fibrotic liver, they contribute to the development of liver fibrosis through autocrine and paracrine loops of TGF-β-stimulated collagen production[4]. In the present study, low-dose paclitaxel treatment effectively reduced expression of type I and Ⅲ collagen and fibronectin in rat HSCs.

In conclusion, we demonstrated that low-dose paclitaxel significantly suppressed the exacerbated TGF-β/Smad signaling and decreased interstitial fibrosis in rat HSCs. It is hoped that the current results will give an impetus to future investigations to explore the therapeutic potential of paclitaxel in the amelioration of hepatic fibrosis.

COMMENTS

Background

Hepatic fibrosis is the key stage in the pathological progress from hepatic injury to cirrhosis. Transforming growth factor (TGF)-β is widely acknowledged as a key factor in accelerating hepatic fibrosis. Smad proteins have been identified to play an important role in regulating the expression of extracellular matrix (ECM) proteins *via* the TGF-β signaling pathway. Aberrant TGF-β/Smad signaling can be modulated by stabilization of microtubules with paclitaxel.

Innovations and breakthroughs

In this study, the authors' research group for the first time reported that 200 nmol/L paclitaxel ameliorated fibrosis in rat hepatic stellate cells (HSCs) *via* inhibition of TGF-β/Smad activity.

Applications

In future experiments, the authors will use the hepatic fibrosis model to observe the treatment effect of paclitaxel *in vivo*.

Terminology

Paclitaxel is an anticancer agent. It is noteworthy that the inhibition of tumor cell proliferation can be achieved by much higher doses of paclitaxel. The inhibition of TGF-β/Smad signaling, however, can be attained with very low doses of paclitaxel.

Peer review

The findings that low-dose paclitaxel significantly suppressed the exacerbated TGF-β/Smad signaling and decreased interstitial fibrosis in rat HSCs are interesting and important.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3335

World J Gastroenterol 2010 July 14; 16(26): 3335-3338
ISSN 1007-9327 (print)

CASE REPORT

Vanishing bile duct syndrome in human immunodeficiency virus: Nevirapine hepatotoxicity revisited

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Received: January 30, 2010 Revised: April 27, 2010
Accepted: May 4, 2010
Published online: July 14, 2010

Abstract

Vanishing bile duct syndrome (VBDS) refers to a group of disorders characterized by prolonged cholestasis as a result of destruction and disappearance of intrahepatic bile ducts. Multiple etiologies have been indentified including infections, neoplastic disorders, autoimmune conditions and drugs. The natural history of this condition is variable and may involve resolution of cholestasis or progression with irreversible damage. VBDS is extremely rare in human immunodeficiency virus (HIV)-infected patients and anti-retroviral therapy has never been implicated as a cause. We encountered a young pregnant female with HIV and VBDS secondary to anti-retroviral therapy. Here, we report her clinical course and outcome.

Key words: Vanishing bile duct syndrome; Ductopenia; Acquired immune deficiency syndrome; Cholangiopathy; Cholestasis; Drug-induced liver injury

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Kochar R, Nevah MI, Lukens FJ, Fallon MB, Machicao VI. Vanishing bile duct syndrome in human immunodeficiency virus: Nevirapine hepatotoxicity revisited. *World J Gastroenterol* 2010; 16(26): 3335-3338 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3335.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3335

INTRODUCTION

The destruction and disappearance of intrahepatic bile ducts leading to "ductopenia" and cholestasis is termed "vanishing bile duct syndrome (VBDS)". The exact mechanism underlying this syndrome is unknown; however, common causes include autoimmune disorders such as primary biliary cirrhosis (PBC), neoplasms, infections and drug toxicities[1-7]. Several patients with VBDS respond to treatment of the underlying condition and/or removal of the offending agent. However, other patients progress to biliary cirrhosis and end stage liver disease. VBDS is rare in the human immunodeficiency virus (HIV)-infected population and to date, only one case has been reported in the literature[8]. In addition, none of the anti-retroviral medications have been implicated as a cause of ductopenia. We report the case of a young female with HIV and VBDS secondary to anti-retroviral therapy.

CASE REPORT

In August, 2009, a 28-year-old African American female in the 3rd trimester of pregnancy (G1P0, 31 wk) presented to the emergency room with jaundice for 3 d. She also complained of pruritus, light-colored stools and dark urine. She denied any abdominal pain, fever,

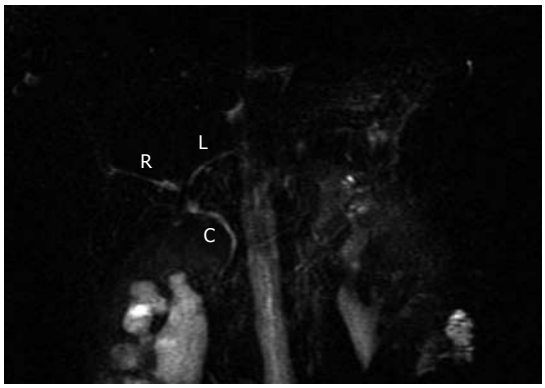

Figure 1 Magnetic resonance cholangiopancreatography. Patent biliary system without evidence of strictures or obstruction with normal caliber common bile duct (C), right hepatic duct (R), and left hepatic duct (L).

chills, vomiting or diarrhea. The patient had a history of heavy alcohol use (approximately 5 drinks/d) for 3 years discontinued at the beginning of her pregnancy. She reported no history of intravenous drug use or unprotected sexual exposure. She had no history of taking herbal supplements or over-the-counter medications. Her past medical history was significant for HIV infection diagnosed in 2000. She had not been on highly active antiretroviral therapy (HAART) since 2003 but recently started triple drug therapy to minimize risk of vertical transmission, with zidovudine, lamivudine and nevirapine 4 wk prior to presentation. She had no history of any opportunistic infections and was not taking any prophylactic medications. Her only other medications included folic acid and multivitamins. Her CD4 count and viral load prior to initiating therapy were 234 cells/μL (normal range: 410 to 1590 cells/μL) and 35 853 copies/mL [reverse transcriptase polymerase chain reaction (RT-PCR) lower limit of detection: 48 copies/mL], respectively.

Physical examination revealed icterus and gravid uterus consistent with duration of pregnancy. There were no stigmata of chronic liver disease. Abdomen was nontender and without hepatosplenomegaly or ascites. Fetal exam was normal. Her liver tests revealed total bilirubin of 10.5 mg/dL (normal range: 0.2-1.3 mg/dL), direct bilirubin 6.7 mg/dL, aspartate aminotransferase 103 U/L (normal range: 0-37 U/L), alanine aminotransferase 179 U/L (normal range: 0-40 U/L), and alkaline phosphatase 496 U/L (normal range: 39-117 U/L). Hematologic and electrolyte values were within normal range, and serum ethanol and urine drug screen were negative. Serologic testing was negative for viral and autoimmune hepatitis including hepatitis E IgM, Epstein Barr virus PCR and cytomegalovirus PCR (CMV-PCR), antinuclear antibody, antismooth muscle antibody, and antimitochondrial antibody. Her CD4 cell count and HIV-1 RNA viral load were measured as 224 cells/μL and 304 copies/mL, respectively. Fungal, mycobacterial, CMV, aerobic and anaerobic blood cultures as well as tests for *Cryptosporidium parvum*, *Microsporidium* and *Toxoplasma* were negative.

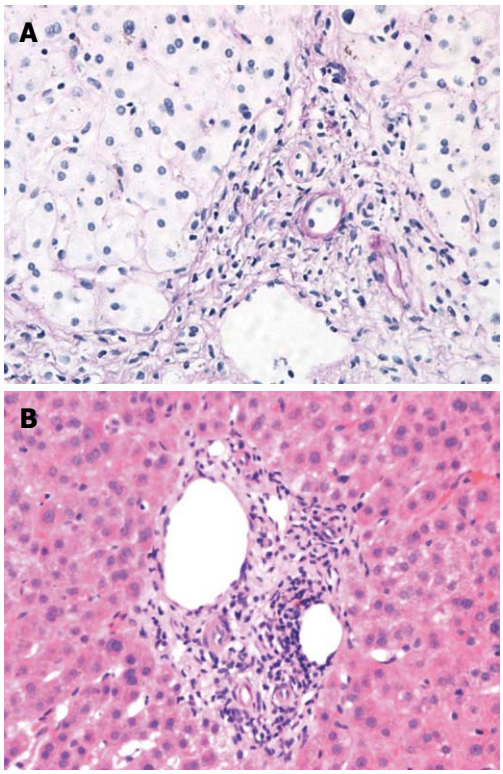

Figure 2 Liver biopsy showing portal tract with normal appearing hepatic vein and hepatic artery, and absence of bile ducts. A: PAS-D stain, × 200 magnification; B: Hematoxylin and eosin stain, × 200 magnification.

Right upper quadrant ultrasound revealed normal size liver with normal echogenicity and no focal lesions, cholelithiasis or biliary tract obstruction. Magnetic resonance cholangiopancreatography (MRCP) was performed. There was no cholangiographic evidence of extra- or intra-hepatic biliary duct dilatation (Figure 1), sclerosing cholangitis or discrete liver mass. A liver biopsy was performed which revealed marked ductopenia with cholestasis (Figure 2). Cytokeratin immunostain confirmed ductopenia and showed one irregular bile duct and scattered ductular proliferation among 11 visualized portal tracts (Figure 3). There was mild lymphocytic portal inflammation and CMV, AFB and GMS stains were negative. A diagnosis of VBDS was made.

HAART medications were withdrawn and the patient was started on ursodeoxycholic acid at 15 mg/kg per day to treat cholestasis and provide symptomatic relief. Over the next few weeks, the patient continued to have severe pruritus and worsening jaundice. Her liver tests deteriorated further with peak levels as shown in Figure 4. A second liver biopsy was performed 8 wk later which showed worsening cholestasis and persistent ductopenia. Owing to the progressive nature of her disease course, liver transplant evaluation was initiated for the patient.

DISCUSSION

VBDS refers to a group of disorders resulting in cholestasis as a consequence of progressive destruction and

Table 1 Drugs reported to cause chronic cholestasis and ductopenia

Antibiotics	Psychiatric drugs	Antiepileptic drugs	Oral hypoglycemics	Hormonal agents	Others
Ampicillin	Amitriptyline	Phenytoin	Carbutamide	Estradiol	Ibuprofen
Amoxicillin/ clavulanic acid	Chlorpromazine	Carbamazepine	Tolbutamide	Methyltestosterone	Phenylbutazone
Clindamycin	Haloperidol	Barbiturates	Glibenclamide	Norandrostenolone	Cyproheptadine
Trimethoprim/ sulfamethoxazole	Imipramine	Diazepam			Cimetidine
Flucloxacillin	Prochlorperazine				Cromolyn sodium
Tetracyclines	Trifluperazine				Chlorothiazide
Thiabendazole					
Erythromycin					
Troleandomycin					

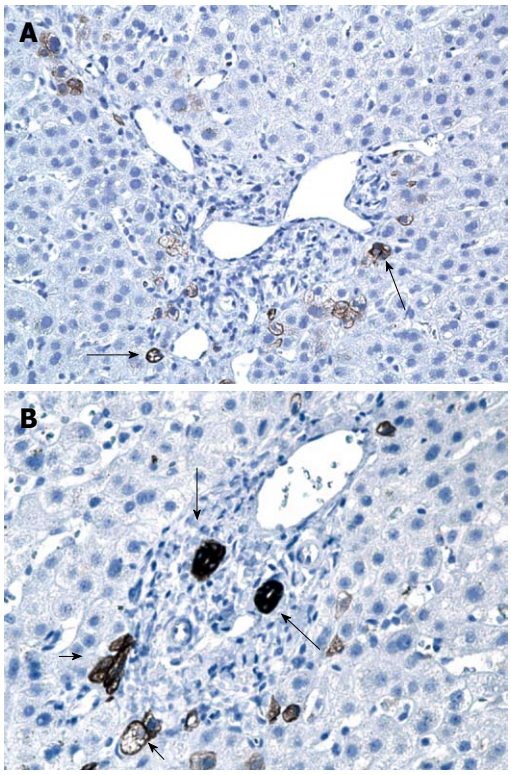

Figure 3 Liver biopsy specimen with cytokeratin staining for biliary elements. A: No bile ducts are seen, minimal ductular proliferation is apparent at the edges of the portal tract (long arrows); B: Solitary portal tract with presence of irregular bile ducts (long arrows) and ductular proliferation (short arrows).

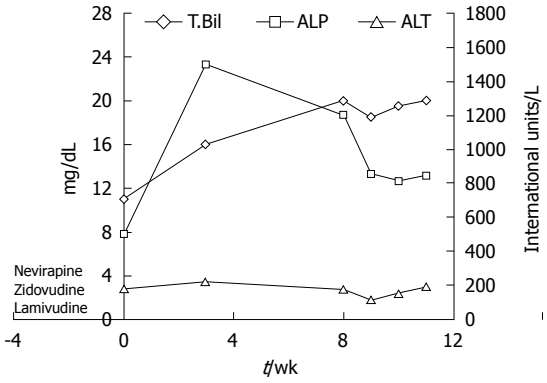

Figure 4 Trend of liver enzymes and bilirubin during the patient's clinical course.

disappearance of intrahepatic bile ducts. This condition was first described in 1988 in a case series of 3 patients by Ludwig *et al*[1] as "idiopathic adulthood ductopenia". Ductopenia is defined as the absence of interlobular bile ducts in greater than 50% of small portal tracts in a liver biopsy specimen.

Several causes of VBDS have been identified with varying mechanisms of duct injury and loss. PBC is one of the most common causes of ductopenia and is likely secondary to T-cell mediated bile duct injury[1,2]. Small duct primary sclerosing cholangitis, acute and chronic hepatic cellular rejection and sarcoidosis are other causes of VBDS with an underlying immune mechanism[3,4]. Infections and neoplastic disorders commonly implicated include CMV, reovirus 3, syphilis, cryptosporidium, Hodgkin's disease and histiocytosis X[5-7]. In addition, several drugs have been reported to cause VBDS, the most frequent ones include antibiotics and psychiatric medications (Table 1)[7].

Intrahepatic cholestasis of pregnancy was considered upon presentation as a possibility in our patient. However, this is a physiologic response to hormonal fluctuation during pregnancy and usually resolves spontaneously after delivery without persistent hyperbilirubinemia and ductopenia as seen in our patient[9]. There is no known association between pregnancy and VBDS, and the few existing case reports have been ascribed to medications such as chlorpromazine[10,11]. VBDS is even rarer in patients with HIV infection and has not been well described. Only a single case has been reported in the literature, in a patient with advanced AIDS (CD4 cell count: 7 cells/μL) and cholestasis with ductopenia presumably due to CMV infection[8]. A second case with HIV infection, low CD4 count (108 cells/μL) and idiopathic ductopenia has been reported, but does not fit the definition of VBDS since bile ducts were absent only in 6 of 15 portal tracts[12]. Cholestasis in patients with HIV infection is commonly due to sclerosing cholangitis and AIDS cholangiopathy which involves obstruction of the biliary tract due to infection-related strictures. Other causes include drug toxicity from HAART medications

and antimicrobials, neoplasms (Kaposi sarcoma and lymphoma) and opportunistic infections[13]. Our patient did not have biliary obstruction on ultrasound or MRCP, and had significantly higher levels of serum bilirubin than is commonly seen in AIDS cholangiopathy. Liver biopsy was negative for malignancy and did not show any features of sclerosing cholangitis or PBC. No strictures were seen on MRCP and tests for specific opportunistic pathogens were negative, ruling out AIDS cholangiopathy and infection.

The most likely cause of cholestasis in our patient was drug-induced liver injury. Medications used by our patient prior to onset of jaundice included zidovudine, lamivudine, nevirapine, multivitamins and folic acid. Zidovudine or lamivudine toxicity is associated with lactic acidosis, elevated lactate dehydrogenase, amylase and lipase along with abnormal liver tests[14]. None of these findings were seen in our patient. In addition, these two drugs were less likely to be the culprits since their toxic effects on the liver usually manifest after 6 mo of use. Nevirapine, a non-nucleoside reverse transcriptase inhibitor, is being increasingly used in pregnant patients owing to its favorable side effect profile and lower teratogenicity compared to protease inhibitors[14]. However, hepatotoxicity is the major adverse effect of nevirapine (5%) and most often manifests as a hypersensitivity reaction with fever, rash and elevated liver tests within the first few weeks of therapy. Late onset hepatotoxicity after several weeks of nevirapine use has also been described in several cases and may be an idiosyncratic reaction to the drug[14]. Although several case reports have demonstrated a cholestatic pattern of nevirapine toxicity, VBDS has never been reported[15,16]. Our patient did not have fever and rash, but had evidence of cholestatic hepatitis and a temporal association between nevirapine use and development of biochemical abnormalities. Therefore, nevirapine toxicity was felt to be the most likely cause of cholestasis and VBDS in our patient.

In summary, VBDS can result from multiple etiologies including autoimmune disorders, infections, neoplastic disorders, genetic abnormalities and medications. The diagnosis is suggested by abnormal liver tests and paucity of interlobular bile ducts on liver biopsy. To the best of our knowledge, this is the first reported case of nevirapine-induced cholestatic hepatitis in a patient with HIV leading to severe ductopenia and VBDS. VBDS should be considered in all HIV patients with chronic cholestasis, especially those with a history of nevirapine use.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3339

World J Gastroenterol 2010 July 14; 16(26): 3339-3342
ISSN 1007-9327 (print)

CASE REPORT

Isolated ileal perforation due to cytomegalovirus reactivation during management of terbinafine hypersensitivity

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Received: December 30, 2009 Revised: March 12, 2010
Accepted: March 19, 2010
Published online: July 14, 2010

Abstract

We report a case of 71-year-old man who developed a hypersensitivity syndrome associated with terbinafine. He was placed on terbinafine (250 mg/d) for the treatment of tinea pedis due to diabetes mellitus. Following the treatment with terbinafine, he developed drug-induced hypersensitivity syndrome (DIHS). Systemic corticosteroid led to transient improvement of his clinical manifestations. Three months after disease onset, he presented with panperitonitis due to ileal perforation, and underwent an emergency operation. The affected ileum was resected and ileostomy was performed in the terminal ileum. Cytomegalovirus (CMV)-specific IgG antibodies were significantly increased, high-titer CMV antigenemia was detected, and pathological examination of the resected ileum confirmed CMV infection. Based on these observations, we strongly recommend that physicians monitor reactivation of the family of herpesvirus other than herpesvirus 6, to manage DIHS properly.

Key words: Terbinafine; Drug hypersensitivity; Human herpesvirus 6; Cytomegalovirus; Intestinal perforation; Ileum

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Sano S, Ueno H, Yamagami K, Yakushiji Y, Isaka Y, Kawasaki I, Takemura M, Inoue T, Hosoi M. Isolated ileal perforation due to cytomegalovirus reactivation during management of terbinafine hypersensitivity. *World J Gastroenterol* 2010; 16(26): 3339-3342 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3339.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3339

INTRODUCTION

Terbinafine has become established as a drug of choice to treat onychomycosis and other dermatomycoses. Although previously reported cases of drug-induced hypersensitivity syndrome (DIHS) are notably uncommon (anticonvulsants, salazosulfapyridine, allopurinol, minocycline, and mexiletine), the number of reported cases of DIHS induced by terbinafine has been increasing.

It is true human herpesvirus 6 (HHV-6) reactivation has been the most frequently described infection associated with this syndrome, but recent articles have suggested that certain herpesviruses other than HHV-6 might be reactivated in a sequential manner in the development of DIHS (multiple and sequential herpesvirus reactivation)[1-7].

Although rare, cytomegalovirus (CMV) is known to cause ileal perforation, not only in immunocompromised

patients such as those with the acquired immunodeficiency syndrome (AIDS) but also immunocompetent patients[8-11]. To the best of our knowledge, especially in the English-language literature, isolated cases of ileal perforation due to CMV infection have never been reported in patients with DHIS.

Here, we report a case of isolated ileal perforation due to CMV in a case of DIHS due to terbinafine, in whom reactivation of HHV-6, Epstein-Barr virus (EBV) and CMV was detected.

CASE REPORT

A 71-year-old Japanese man with diabetes mellitus with onychomycosis was treated with oral terbinafine. Other medical history was not remarkable: any anticonvulsants, mexiletine, and antibiotics such as minocycline and salazosulfapyridine, were not prescribed. One week after taking terbinafine, he developed a high fever, erythema covering the whole body, and superficial lymph node swelling. Laboratory data at that time showed leukocytosis (16 670/mm^3) with 30.2% eosinophils. Atypical lymphocytes were not present. Liver enzymes were slightly elevated: 47 IU/L alanine aminotransferase and 78 IU/L aspartate aminotransferase. DIHS was suspected and terbinafine administration was discontinued soon after the onset of illness. As a result of sustained fever and erythroderma for 1 mo, 30 mg/d systemic corticosteroid was started. After 7 d, his symptoms improved temporarily. However, tapering the corticosteroid dose to 25 mg/d led to recrudescence of his fever and generalized rash (on day 50). Other results of systemic corticosteroids were not remarkable. Retrospectively, seroconversion of anti-HHV-6 antibody was detected; the titer increased from < 10 (on day 14) to 40 (on day 90). Judging from his drug history, characteristic generalized erythematous rash and laboratory data, the diagnosis of DIHS was confirmed. He was again administrated 30 mg/d prednisolone from day 50. Then, the patient was referred to our hospital (Osaka City General Hospital).

Examination on admission revealed erythematous rash over his entire body, with partially exfoliated lesions and superficial lymphadenopathy. Laboratory data included a normal count of white blood cells without eosinophilia and normal liver enzymes. Antinuclear and antineutrophil cytoplasmic antibodies were negative. The patient was negative for human immunodeficiency virus (HIV). Histological examination of the skin was not performed. Seventy days after the onset of DIHS, the patient complained of abdominal discomfort, and occult blood tests were positive on two occasions. On day 80, colonoscopic examination was performed and no abnormality around the whole colon and terminal ileum was revealed. At that time, CMV antigenemia was strongly suggested by the direct detection of viral antigen in the peripheral blood: CMV antigenemia-1, 56 and antigenemia-2, 89. CMV IgG titer increased to 128 and IgM titer was negative (< 0.8). Intravenous administration of 500 mg/d ganciclovir was started. However, fever with abdominal pain was present on day 82, and physical examination showed muscular defense in the entire abdomen. Abdominal computed tomography (CT) revealed free air, ascites, and swelling of the ileal loop (Figure 1). He was diagnosed as having panperitonitis due to ileal perforation, and underwent an emergency operation. The affected ileum was resected and ileostomy was performed. Pathological examination of the resected ileum revealed acute inflammation with vasculitis and CMV inclusions in the macrophages and endothelial cells, and evidence of CMV on immunostaining (Figure 2). There was no evidence of cancer cells or any other pathogens. Administration of 500 mg/d ganciclovir was continued, and CMV antigenemia was not detected on day 89. Unfortunately, the patient died from pneumonia.

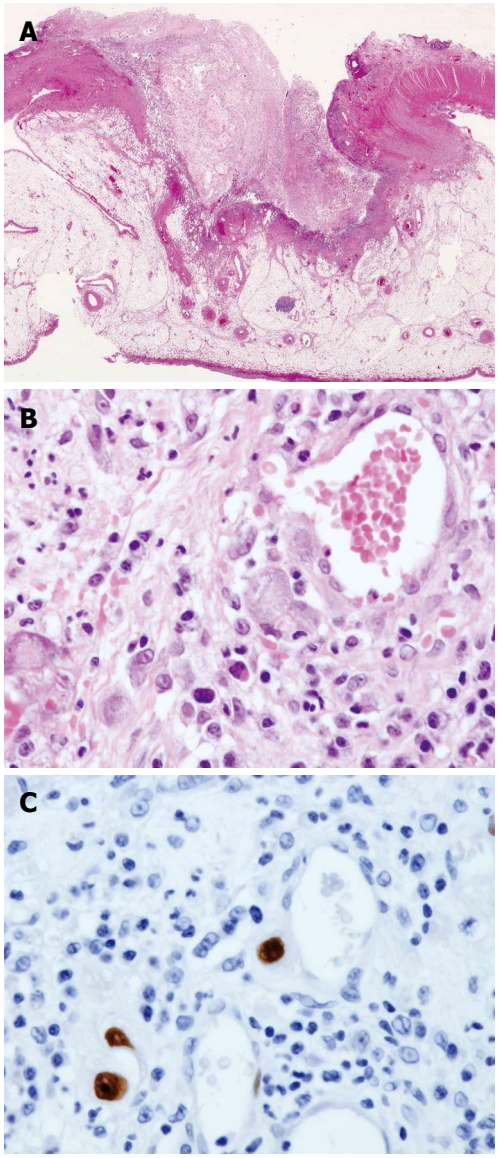

Figure 1 Histological examination of perforated ileum. A: Perforated ileal lesion (hematoxylin and eosin stain) × 40; B: × 400; C: Cytomegalovirus infection was evident in the same lesion, by immunohistochemical staining with anti-cytomegalovirus antibody (× 200).

DISCUSSION

Terbinafine has become established as a drug of choice

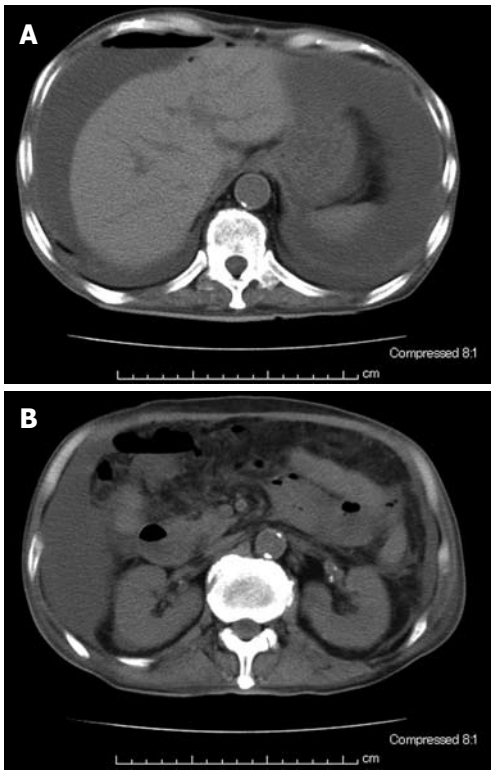

Figure 2 Abdominal computed tomography. A: Free-air, ascites; B: Swelling of the ileal loop.

to treat onychomycosis and other dermatomycoses. Although previously reported cases of DIHS are notably uncommon (anticonvulsants, salazosulfapyridine, allopurinol, minocycline, and mexiletine), the number of reported cases of DIHS induced by terbinafine has been increasing. It was thought that the present case met the criteria for DIHS because the patient had clear exposure to a given drug (in this case, terbinafine), high fever (> 39℃); erythroderma followed by exfoliative dermatitis; diffuse lymphadenopathy; and hypereosinophilia (absolute eosinophil count, 5032/mm^3). The results of a lymphocyte-stimulating test for terbinafine performed in our hospital were negative, and it was thought that treatment with prednisolone may have affected the results.

Since Aihara *et al*[2] in 2001, it has been recognized that CMV is associated with hypersensitivity syndrome. Recent studies have suggested that reactivation of members of the herpesvirus family other than HHV-6 could be associated with DIHS. Moreover, multiple herpesvirus reactivations and sequential reactivation have been reported in the case of DIHS. The list of viruses that have been documented to be reactivated include HHV-6, HHV-7, EBV and CMV[2-7]. In the present case, reactivation of HHV-6, EBV and CMV was confirmed on the basis of serological tests on paired serum samples for HHV-6 and EBV, and polymerase chain reaction for CMV. EBV reactivation was asymptomatic, which might have been due to administration of corticosteroids.

The clinical course of this patient can be divided in three phases: (1) hypersensitivity reaction with eosinophilia; (2) relapse of fever and rash due to HHV-6 reactivation; and (3) ileal perforation due to CMV reactivation. Retrospectively, the worsening of exanthema and poor control of second fever in spite of treatment with prednisolone reflect virus reactivation. We speculate that the virus responsible for this phase was HHV-6, although we could not confirm the absence of CMV infection in this period. The following abdominal pain and ileal perforation was due to CMV reactivation, as confirmed by pathological examination of the resected ileum.

Table 1 Previously reported causes of isolated ileal perforation due to CMV

AIDS
Non-Hodgkin's lymphoma with chemotherapy
Systemic lupus erythematosus
Liver transplantation
Ulcerative colitis
Recurrent gastric cancer
Blood transfusion associated with trauma
Immunocompetent elderly/neonatal
DIHS (present case)

CMV: Cytomegalovirus; AIDS: Acquired immunodeficiency syndrome; DIHS: Drug-induced hypersensitivity syndrome.

Our patient was unique in that CMV reactivation resulted in ileal perforation during the course of DIHS. Although rare, CMV is known to cause ileal perforation, especially in immunocompromised patients, such as those with AIDS. To the best of our knowledge, isolated cases of ileal perforation due to CMV infection have never been reported in a patient with DIHS (Table 1)[8-11]. Although it remains possible that systemic corticosteroid administration precipitated CMV reactivation, there is also a case report that demonstrated that a patient without any corticosteroids showed reactivation of HHV-6, HHV-7 and CMV[3]. Based on these observations, it is important for physicians to be aware of the possible reactivation of CMV and other herpesviruses in every patient with DIHS, to avoid awkward small intestinal ulcer and perforation.

In summary, we have reported a case of isolated ileal perforation due to CMV reactivation during the management of terbinafine hypersensitivity. Although the prognosis in bowel perforation related to CMV infection is extremely poor, emergency surgery saved our patient's life. However, earlier diagnosis and prompt administration of antiviral drugs could have avoided surgical treatment. Although CMV could affect multiple organ functions and might be the direct cause of death, effective medication such as ganciclovir is available. Therefore, it is strongly recommended that physicians monitor reactivation of herpesviruses other than HHV-6, to manage DIHS properly.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i26.3343

World J Gastroenterol 2010 July 14; 16(26): 3343-3346
ISSN 1007-9327 (print)

CASE REPORT

Abdominal separation in an adult male patient with acute abdominal pain

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Received: February 2, 2010 Revised: March 18, 2010
Accepted: March 25, 2010
Published online: July 14, 2010

Abstract

We report a male patient with prolonged post-prandial abdominal distension and a sudden onset of epigastric pain initially diagnosed as acute abdomen. The patient had no history of surgery. Physical examination revealed peritonitis and abdominal computed tomography scan showed upper abdominal mesentery intorsion. The patient then underwent surgical intervention. It was found that the descending mesocolon dorsal root was connected to the ascending colon and formed a membrane encapsulating the small intestine. The membrane also formed an orifice in the ileal pars caeca, from which a 30 cm herniated ileum formed a "C"-shaped loop which was strangulated by the orifice. An abdominal separation was diagnosed after surgery. We liberated the membranous peritoneum which incarcerated the intestinal canal from the root of ileocecal junction to Treitz ligament, and reduced the small intestinal malrotation. The patient had an uneventful recovery after operation with his abdominal distention disappeared during the follow-up. Abdominal separation is a rare situation, which may be related with embryo development. Surgery is a choice of treatment for it.

Key words: Abdominal separation; Abdominal pain; Internal hernia; Malrotation; Acute abdomen

Peer reviewer: Yasuhiro Fujino, MD, PhD, Director, Department of Surgery, Hyogo Cancer Center, 13-70 Kitaoji-cho, Akashi, 673-8558, Japan

Liu BL, Chen Y, Liu SQ, Zhang XB, Cui DX, Dai XW. Abdominal separation in an adult male patient with acute abdominal pain. *World J Gastroenterol* 2010; 16(26): 3343-3346 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i26/3343.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i26.3343

INTRODUCTION

Acute abdominal pain, a common chief complaint encountered in emergency department, accounts for about 5% of all emergency department visits[1] and can be caused by a variety of conditions[2]. Many factors can result in acute abdominal pain, such as appendicitis, bowel obstruction, and cholecystitis[3]. This report describes a rare case of abdominal separation in an adult male patient with strangulated internal hernia of the terminal ileum complicating malrotation of intestine, which has not been reported in the literature.

CASE REPORT

A 43-year-old man visited the emergency department of our hospital on December 31, 2007 for a sudden onset of persistent epigastric pain for 10 h. He had a history

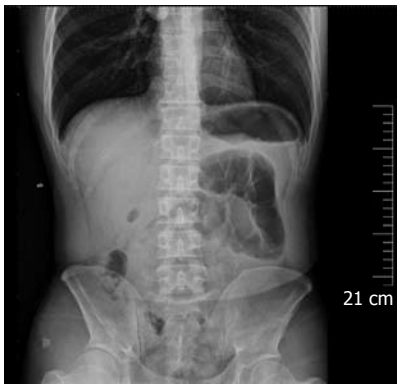

Figure 1 Plain abdominal X-ray. Mildly dilated pneumatic intestinal canal loops but no typical air-fluid level shadow in the left upper quadrant of abdomen.

of prolonged episodes of postprandial abdominal distension. The pain was associated with nausea and vomiting without any clear inducing factors, and did not radiate to the back and shoulders. The symptoms included anorexia, constipation, obstipation and poor sleep. Furthermore, he had no history of abdominal surgery or trauma. On admission, his temperature was 36.5℃, blood pressure was 127/72 mmHg, respiratory rate and pulse rate were 17 breaths/min and 70 beats/min, respectively. Physical examination showed tenderness, rebound tenderness and slight muscular rigidity in the right lower quadrant of abdomen. Laboratory findings included 86.8% of neutrophils and 116.0 U/L of hemoglobin. Plain abdominal X-ray revealed mildly dilated pneumatic intestinal canal loops but no typical air-fluid level shadow in the left upper quadrant of abdomen (Figure 1). The patient was initially diagnosed as localized peritonitis which was differentially diagnosed from acute appendicitis and intestinal mesentery intorsion.

The patient had no significant improvement after initial treatment with fluid replacement, antibiotics and gastrointestinal decompression. He developed peritonitis with the pain exacerbated. Computed tomography (CT) scan revealed suspicious upper abdominal mesentery intorsion (Figure 2). Exploratory laparoscopy demonstrated appendix in the retroileal position, descending mesocolon dorsal root that was connected to the ascending colon encapsulating the small intestine, an orifice in the ileal pars caeca from which a 30 cm herniated ileum formed a "C"-shaped loop at the root of ileocecal junction strangulating at the orifice, and a purple incarcerated intestinal canal.

We liberated the incarcerated intestinal canal by opening the membrane at the mesentery root from the ileocecal junction to Treitz ligament and reduced the mesoileal malrotation. The strangulated intestine was about 50 cm from the ileocecal junction. After liberation, the intestinal canal was recovered with its color changed from purple to pink, and vascular pulsation was obvious and peristalsis was resumed.

The patient had an uneventful recovery and was discharged on January 8, 2008. The patient was diagnosed as localized peritonitis, small intestinal malrotation, internal herniation, strangulation of small bowel and abdominal separation after operation, and followed up for 18 mo during which no complications and post prandial abdominal distention occurred.

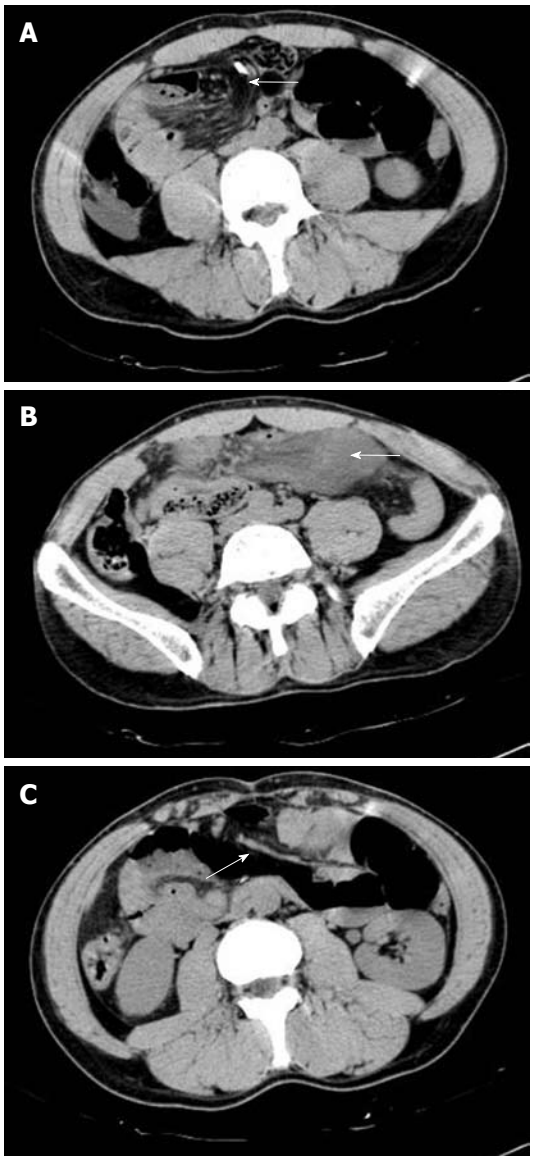

Figure 2 Computed tomography scan. Suspicious upper abdominal mesentery intorsion (A, arrow), right lower quadrant intestinal loop (B, arrow), middle abdominal line separation (C, arrow).

DISCUSSION

Since a large number of patients visiting emergency department complain of acute abdominal pain and most of them have typical symptoms and signs, appropriate evaluation can be accomplished with the aid of relevant auxiliary investigations[3]. The complaints are usually relieved after symptomatic treatment. In some rare cases, its initial diagnosis may be difficult as in our case. The incidence of internal hernia is low, accounting for less than 1% of all acute abdomen cases[4-6]. However, the overall mortality may be higher than 50% if it is not ap-

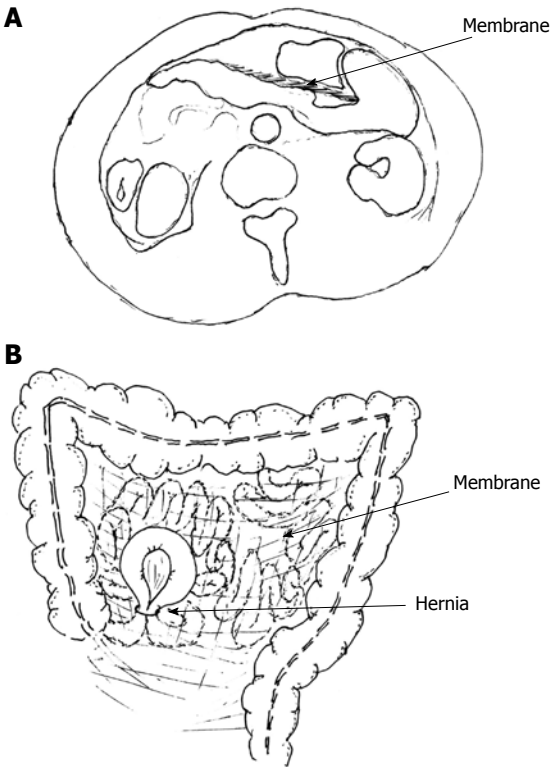

Figure 3 Schema showing a membrane separating the peritoneal cavity into two compartments (A) and descending mesocolon dorsal root connecting with the ascending colon (B).

propriately treated, especially when strangulation occurs. A fast and correct diagnosis is therefore necessary.

Our patient suffered from abdominal pain which exacerbated although it was treated with fluid resuscitation and antibiotics. Nasogastric tube decompression did not produce desirable effect and vomiting continued. Since the condition was nonspecific, its differential diagnosis was difficult from acute abdomen caused by factors such as mechanical intestinal obstruction. Plain abdominal X-ray has been recommended for patients with highly suspected obstruction[7-9] according to The Royal College of Radiologists Guidelines[10]. A single plain abdominal X-ray is not adequate to make a definitive judgment of suspected obstruction[9], and CT scan is the reasonable next step for further investigation of abdomen[2,11]. Abdominal CT scan has a good inter-observer agreement in unselected patients with acute abdominal pain at emergency department and plays an important role in diagnosis of acute intestinal obstruction and in planning of surgical treatment. Although internal hernias are uncommon, they should be included in the differential diagnosis from intestinal obstruction, especially in the absence of abdominal surgery or trauma. Recognition of the characteristic findings on CT scan may assist in identification of internal hernias in most cases of small bowel obstruction. Since the general condition of this patient deteriorated very quickly and CT revealed a mesentery intorsion, surgery was performed. The function of strangulated bowel was recovered after its loop was released and the mesenteric malrotation was reduced.

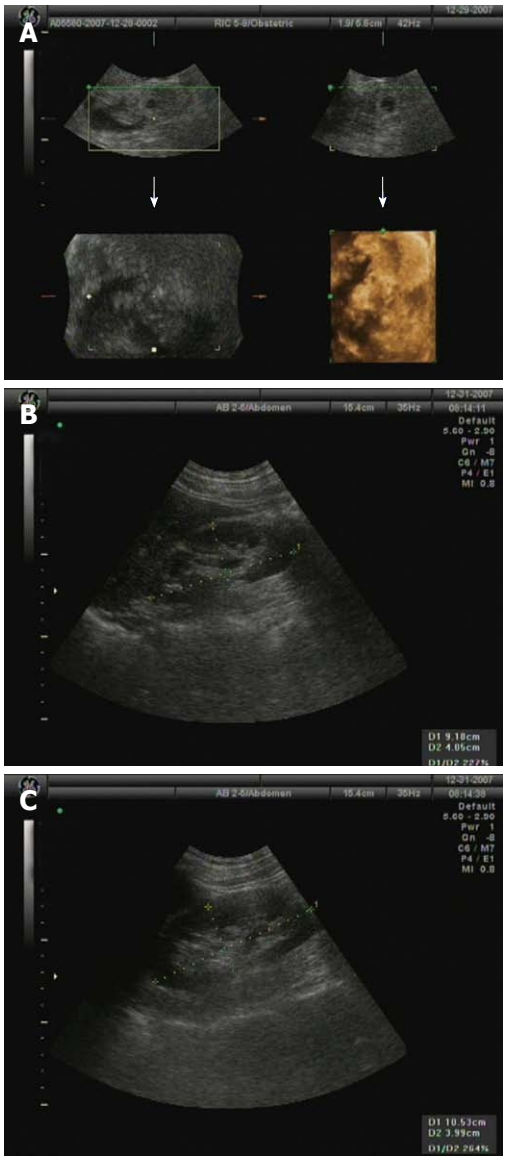

Figure 4 Abdominal and kidney ultrasonography showing no mass and fluid collection (A), left (B) and right (C) normal kidney structure.

Although abdominal separation in our case could not be definitely diagnosed before operation by the radiologist and clinician[8] due to the presence of peritonitis, surgery was performed during which the otherwise rare phenomenon of abdominal separation was discovered, namely a membrane divides the abdominal cavity into two compartments. The anterior compartment contains the colon, while the posterior compartment encapsulates the small intestine, forming an orifice at the end of ileocecal junction. It is at this orifice that a 30 cm ileum is protruded and strangulates the anterior compartment, causing abdominal pain. A CT scan showed an intra-abdominal membrane-like line dividing the two compartments (Figures 2 and 3). Ultrasonography did not show specific intraabdominal evidence (Figure 4). This patient experienced episodes of post prandial abdominal distension, which supported the findings on abdominal CT scan and at operation. His symptoms were relieved after operation. We therefore recommend that abdominal sep-

aration should be differentially diagnosed from acute abdomen. Early diagnosis and operation will greatly reduce the morbidity and mortality of intestinal strangulation.

The intestine has returned to the abdominal cavity and the midgut consisting mainly of the duodenum, jejunum, ileum, ascending colon, and transverse colon has completed its 270-degree loop by weeks 10 and 20 of embryo development, respectively. With the growth of small intestine, the ascending and descending colon are pushing against the body wall in a secondary retroperitoneal location like the pancreas and duodenum[12,13]. Whether the mesentery separates abdomen into two parts of peritonum during the development is not well known. Whether there is any genetic change needs to be further investigated.

In conclusion, abdominal separation with internal hernias is a rare condition that can present as acute abdomen. Such patients can recover if they are properly treated. We strongly recommend that abdominal separation should be differentially diagnosed from acute abdomen.

ACKNOWLEDGMENTS

The authors thank Sangeeta Sharma for the help in manuscript preparation and Jin Xu for preparation of the schema.

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S- Editor Wang JL **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2010 July 14; 16(26): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World Journal of Gastroenterology

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World J Gastroenterol 2010 July 14; 16(26): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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World J Gastroenterol 2010 July 14; 16(26): I-IV
ISSN 1007-9327 (print)

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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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For articles of these sections, original articles, rapid communication and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case

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Figures should be numbered as 1, 2, 3, *etc*., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc*. It is our principle to publish high resolution-figures for the printed and E-versions.

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Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc*., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:
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Volume 16 Number 27
July 21, 2010
World J Gastroenterol
2010 July 21; 16(27): 3347-3474
Online Submissions
www.wjgnet.com/1007-9327office
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WJG World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 27 July 21, 2010

EDITORIAL

3347 Changing face of hepatic encephalopathy: Role of inflammation and oxidative stress
Seyan AS, Hughes RD, Shawcross DL

3358 R0 resection in the treatment of gastric cancer: Room for improvement
Biondi A, Persiani R, Cananzi F, Zoccali M, Vigorita V, Tufo A, D'Ugo D

ORIGINAL ARTICLE

3371 Effect of soy saponin on the growth of human colon cancer cells
Tsai CY, Chen YH, Chien YW, Huang WH, Lin SH

3377 Galangin induces apoptosis of hepatocellular carcinoma cells *via* the mitochondrial pathway
Zhang HT, Luo H, Wu J, Lan LB, Fan DH, Zhu KD, Chen XY, Wen M, Liu HM

3385 8-bromo-7-methoxychrysin-induced apoptosis of hepatocellular carcinoma cells involves ROS and JNK
Yang XH, Zheng X, Cao JG, Xiang HL, Liu F, Lv Y

3394 Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats
Yin YN, Yu QF, Fu N, Liu XW, Lu FG

BRIEF ARTICLE

3402 Sessile serrated adenomas: Demographic, endoscopic and pathological characteristics
Gurudu SR, Heigh RI, De Petris G, Heigh EG, Leighton JA, Pasha SF, Malagon IB, Das A

3406 Can chronic gastritis cause an increase in fecal calprotectin concentrations?
Montalto M, Gallo A, Ianiro G, Santoro L, D'Onofrio F, Ricci R, Cammarota G, Covino M, Vastola M, Gasbarrini A, Gasbarrini G

3411 Pancreatic and pulmonary mast cells activation during experimental acute pancreatitis
Lopez-Font I, Gea-Sorlí S, de-Madaria E, Gutiérrez LM, Pérez-Mateo M, Closa D

3418 Double balloon enteroscopy examinations in general anesthesia
Zubek L, Szabo L, Lakatos PL, Papp J, Gal J, Elo G

3423 Does bilioenteric anastomosis impair results of liver resection in primary intrahepatic lithiasis?
Herman P, Perini MV, Pugliese V, Pereira JC, Machado MAC, Saad WA, D'Albuquerque LAC, Cecconello I

3427 Prevalence of type 2 diabetes in Algerian patients with hepatitis C virus infection
Rouabhia S, Malek R, Bounecer H, Dekaken A, Bendali Amor F, Sadelaoud M, Benouar A

3432 Association of *E-cadherin* (*CDH1*) gene polymorphisms and gastric cancer risk
Al-Moundhri MS, Al-Khanbashi M, Al-Kindi M, Al-Nabhani M, Burney IA, Al-Farsi A, Al-Bahrani B

3437 Transcatheter arterial chemoembolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma
Kasai K, Ushio A, Sawara K, Miyamoto Y, Kasai Y, Oikawa K, Kuroda H, Takikawa Y, Suzuki K

3445 Association of genetic polymorphisms of aldehyde dehydrogenase-2 with esophageal squamous cell dysplasia
Zhou YZ, Diao YT, Li H, Li HQ, Ma Q, Cui J

3450 Radiofrequency ablation in the treatment of small hepatocellular carcinoma: A meta analysis
Liu JG, Wang YJ, Du Z

3457 Impact of human leukocyte antigen mismatching on outcomes of liver transplantation: A meta-analysis
Lan X, Zhang MM, Pu CL, Guo CB, Kang Q, Li YC, Dai XK, Deng YH, Xiong Q, Ren ZM

3465 Precise prediction model and simplified scoring system for sustained combined response to interferon-α
Mao QG, Pan JS, Fang KN, Zhang RM, Hong QY, Song MN, Zhu JP, Huang WQ, Chen LM, Hong MZ

CASE REPORT

3472 Anterograde jejunojejunal intussusception resulted in acute efferent loop syndrome after subtotal gastrectomy
Kwak JM, Kim J, Suh SO

World Journal of Gastroenterology
Volume 16 Number 27 July 21, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
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PUBLISHING
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Beijing 100025, China
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E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
July 21, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3347

World J Gastroenterol 2010 July 21; 16(27): 3347-3357
ISSN 1007-9327 (print)

EDITORIAL

Changing face of hepatic encephalopathy: Role of inflammation and oxidative stress

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Received: March 13, 2010 Revised: April 28, 2010
Accepted: May 5, 2010
Published online: July 21, 2010

Peer reviewers: Dr. Vicente Felipo, Laboratory of Neurobiology, Fundación C.V. Centro de Investigacion Principe Felipe, Avda Autopista del Saler, 16, 46013 Valencia, Spain; Weekitt Kittisupamongkol, MD, Hua Chiew Hospital, Bangkok 10100, Thailand; Xiang-Dong Wang, MD, PhD, Professor, Zhongshan Hospital, Fudan University, Shanghai 230003, China; Dr. Ashraf Dahaba, MD, PhD, MSc, Professor, Department of Anaesthesiology, Medical University of Graz Medical University, Auenbruggerplatz 29, A-8036, Graz, Austria

Seyan AS, Hughes RD, Shawcross DL. Changing face of hepatic encephalopathy: Role of inflammation and oxidative stress. *World J Gastroenterol* 2010; 16(27): 3347-3357 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3347.htm
DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3347

Abstract

The face of hepatic encephalopathy (HE) is changing. This review explores how this neurocognitive disorder, which is associated with both acute and chronic liver injury, has grown to become a dynamic syndrome that spans a spectrum of neuropsychological impairment, from normal performance to coma. The central role of ammonia in the pathogenesis of HE remains incontrovertible. However, over the past 10 years, the HE community has begun to characterise the key roles of inflammation, infection, and oxidative/nitrosative stress in modulating the pathophysiological effects of ammonia on the astrocyte. This review explores the current thoughts and evidence base in this area and discusses the potential role of existing and novel therapies that might abrogate the oxidative and nitrosative stresses inflicted on the brain in patients with, or at risk of developing, HE.

Key words: Hepatic encephalopathy; Ammonia; Inflammation; Oxidative stress; Astrocyte

INTRODUCTION

Hepatic encephalopathy (HE) is a neurocognitive disorder in which brain function is impaired and is associated with both acute and chronic liver dysfunction. HE occurs in the presence of liver injury or when the liver is bypassed in the presence of a portosystemic shunt. In acute liver failure, patients may develop cerebral oedema and increased intracranial pressure. However, recent studies suggest that intracranial hypertension is less frequent than previously described, complicating 25% of acute cases and only 9% of those with sub-acute liver failure[1]. In cirrhosis, it causes a range of neuropsychiatric and motor disturbances spanning a spectrum of abnormalities, which encompass short-term memory impairment, slowing of reaction time, poor concentration, psychomotor retardation, and sensory dysfunction, through to more clinically apparent neurological signs and symptoms. In its most severe form, patients can develop confusion, stupor, and coma[2]. However, abnormalities can be subtle and only become apparent on formal psychometric testing (minimal HE). Minimal HE is thought to be a disorder of executive functioning, primarily leading to impairments in selective atten-

tion, response inhibition, and working memory. This frequently impacts on quality of life[3] and specifically impairs navigation skills[4], which can be demonstrated utilising a driving simulator that correlates impairment with response inhibition and attention[5]. HE has generally been considered to be a reversible process following liver transplantation, although recent studies have suggested that this may not always be the case[6].

The "World Congress of Gastroenterology" in 2002 developed a set of consensus definitions, which has led to the classification of HE into three different types, A-C. (Table 1)[2]. In addition, the clinical presentation of HE was categorised into four main subtypes (Table 2). The heterogeneous nature of the presentation of HE has been the cause of great consternation, and has made the interpretation of comparative studies problematic. The staging of overt HE remains an imprecise art, which is often hampered by its fluctuant course. Thus, more objective methods using electroencephalographic techniques have been developed to assess HE. The effectiveness of using the bispectral index to grade and monitor the course of HE has high discriminative power in patients with both low and high grades of HE, and can be utilised as a simple and objective method of grading HE[7]. It has recently been suggested that we should consider HE as a spectrum of neurocognitive impairment in patients with cirrhosis; the spectrum spanning normal performance to coma[8].

Table 1 Classification of hepatic encephalopathy[2]

Type	Definition
A	Acute and hyperacute liver failure
B	Portosystemic bypass without intrinsic hepatocellular disease
C	Cirrhosis and portal hypertension with portosystemic shunts

A: Acute; B: Bypass; C: Cirrhosis.

Table 2 Clinical presentation of hepatic encephalopathy[2]

Encephalopathy	Definition
Acute	Acute liver dysfunction
Recurrent or episodic	Episodes of mental alteration in a patient with cirrhosis, even in the absence of a known precipitating factor
Persistent	Neurological deficit that persists despite the reversal of liver injury, such as following liver transplantation or the removal of a precipitating factor
Minimal (previously known as subclinical)	No evidence of overt encephalopathy, but subtle cognitive deficits might be detected with a neuropsychological function test battery

THE AMMONIA HYPOTHESIS

Ammonia was first thought to play a major role in the development of HE when studies by Hahn *et al*[9], Nencki *et al*[10] and Nencki *et al*[11] in the 1890s described the "meat intoxification syndrome". By diverting blood away from the liver utilising a surgical shunt from the portal vein into the vena cava of dogs, within 6 wk of the portocaval shunt being constructed, it was observed that the dogs developed symptoms such as aggression, irritability, and convulsions, similar to the symptoms exhibited by patients with cirrhosis and overt HE. The portocaval shunt allows blood to bypass the liver, resulting in a lack of urea metabolism, and arterial ammonia levels were found to be increased. When ammonium salts were administered to the dogs, they rapidly fell into a coma and died[9]. Ammonia was later confirmed as the main causative factor of the "meat intoxification syndrome" in portocaval shunted dogs in 1922[12]. The role of ammonia became increasingly recognised as being important when Gabuzda *et al*[13] and Phillips *et al*[14] attempted to treat patients with ascites with cation exchange resins that absorbed sodium but released ammonium ions, leading to the adverse effect of significant reversible neurological dysfunction, which was indistinguishable from the syndrome we now know as HE. Blood ammonia concentration was subsequently noted to be elevated in patients with liver disease and hepatic coma[15]; the highest values being found in those patients who were comatose[16].

Subsequently, other investigators have shown that ammonia plays a definitive role in the development of HE. Bessman *et al*[17] demonstrated a positive arteriovenous difference in ammonia levels in patients with cirrhosis, suggesting an uptake of free ammonia into the brain. More recently, Ehrlich *et al*[18] demonstrated that by constructing an end-to-side portocaval anastomosis in rats and injecting them with ammonium acetate, the rats demonstrated typical characteristics of HE, such as drowsiness, seizures, and coma in association with elevated blood and brain ammonia concentrations, compared to control rats. Lockwood *et al*[19] were then able to demonstrate the first evidence linking ammonia to HE in humans, using positron emission tomography (PET). A ^{13}N tracer demonstrated that the rate of uptake of ammonia in the brains of patients was greater in those with HE than without. It was postulated that an increased ammonia uptake in the brain was linked to an increased permeability of the blood-brain barrier to ammonia[20]. In acute liver failure, arterial ammonia concentrations of > 150 μmol/L predict a greater likelihood of dying from brain herniation[21], and intracranial hypertension develops in 55% of cases with an arterial ammonia concentration > 200 μmol/L[1]. In cirrhosis, there is no doubt that blood ammonia concentrations are elevated, but there is conflicting evidence regarding the relationship between ammonia concentration and HE severity. Moreover, it is not unusual in clinical practice to see patients with cirrhosis presenting with symptoms of overt HE who have normal or only mildly elevated arterial ammonia concentrations. Indeed, numerous studies have shown that a single test for blood ammonia concentration is a poor method for assessing HE[22]. Furthermore, Ong *et al*[23] studied the blood ammonia levels of patients with chronic liver disease and compared these to their mental states. In patients considered not to have any sign of HE, 60% had ammonia levels higher than normal, whereas there was a high proportion of those with grade 3 or 4

HE with normal or only mildly elevated blood ammonia levels. Whilst there is no denying the involvement of ammonia in the pathogenesis of HE, it seems that there might be other factors involved which are as, if not more, important.

THE ASTROCYTE IN HE

Astrocytes are a type of glial cell found within the central nervous system (CNS), which are involved in maintaining cells within the CNS, including providing nutrients for neurones. Astrocytes are particularly vulnerable to the effects of ammonia in the brain. One reason for this is that the enzyme glutamine synthetase is mainly located within astrocytes. Norenberg *et al*[24] found glutamine synthetase exclusively within astrocytes in rat brains, and none within neurones or other glial cells. It is also important to note that the end-processes of astrocytes surround the capillaries in the CNS. Theoretically, this would ensure that any toxin entering the brain, such as ammonia, is immediately metabolised, protecting other CNS cells from its damaging effects[25]. This theory was tested by Rao *et al*[26], who investigated the effects of ammonia exposure on purely neuronal cultures and co-cultures of neurons and astrocytes. The cultures containing neurons alone showed significant increases in cell death, apoptotic cells, degeneration of neuronal processes, and free radical levels. However, these changes were not detected in the co-cultures, indicating a protective function of astrocytes.

The blood brain barrier remains anatomically intact in HE[27]; however, PET studies have demonstrated an increased permeability-surface area to ammonia with increasing severity of disease[20].

HE in patients with chronic liver disease is characterised neuropathologically by Alzheimer type Ⅱ astrocytosis. This describes morphological changes to astrocytes, which include a large swollen nucleus, prominent nucleolus, and margination of the chromatin pattern. These neuropathological findings have been replicated in the brains of patients with congenital abnormalities of urea cycle enzymes[28], in experimental animal models[29,30], and astrocyte cultures exposed chronically to ammonia[31]. Therefore, it is likely that ammonia taken up into the brain interacts with astrocytes, eventually leading to these characteristic changes.

In acute liver failure, an increased brain ammonia concentration causes astrocyte swelling and patients develop cytotoxic brain oedema[32]. Kato *et al*[32] used electron microscopy to study the cells of patients who died of fulminant hepatic failure. They found brain oedema to be present, with pronounced swelling of astrocytes. Glutamine synthetase catalyses the conversion of ammonia and glutamate to glutamine. As a result, hyperammonemia can lead to excessive levels of glutamine within astrocytes, causing the cells to swell, and therefore explaining the oedema and intracranial hypertension seen with fulminant hepatic failure. Willard-Mack *et al*[33] used rats to investigate whether inducing an acute onset of hyperammonemia caused astrocytes to swell and if inhibiting the action of glutamine synthetase prevented these astrocytic changes. The study found that 8 h after inducing plasma hyperammonemia, changes in astrocyte morphology could be identified. These changes included an increased number of organelles, increased cytoplasmic volume, and an increased nuclear volume. They also found that inhibiting glutamine synthetase attenuated the enlargement of the nuclei and prevented the increase in astrocyte water content seen with hyperammonemia. The results of this study suggest that the production of glutamine by ammonia detoxification, results in water being drawn into astrocytes through osmotic pressure.

There might also be a potential role for vasogenic brain oedema in acute liver failure. This is believed to result from damage to the blood-brain barrier, leading to uncontrolled movement of plasma components and water to extracellular areas of the brain[34]. Consistent with this, animal studies have shown an increased permeability of the blood brain barrier to substances that are normally unable to cross it[35,36]. It has been suggested that perhaps in the early stages of HE, cytotoxic brain oedema predominates, and is enhanced in the later stages by vasogenic brain oedema following damage to the blood brain barrier[37]; however, the ability of mannitol to reduce intracranial hypertension in patients with fulminant hepatic failure indicates that the blood brain barrier remains largely intact[38].

The presence of low-grade astrocyte swelling has been further investigated in human patients with cirrhosis. Córdoba *et al*[39] used magnetic resonance spectroscopy and the magnetisation transfer ratio (a measure of free water in the brain) to assess cirrhotic patients before and after liver transplantation. The results showed a high level of free water in the brain before liver transplantation, which then reduced after transplantation. This correlated with changes in neuropsychological function, suggesting that brain oedema plays a direct role in the changes observed in HE. A further finding of the study was that brain glutamine levels also correlated with the changes in brain water and neuropsychological function, providing further evidence to the theory that hyperammonemia plays an important role in the pathophysiology of HE. Balata *et al*[40] showed that inducing hyperammonemia in patients with cirrhosis leads to an increase in brain glutamine, which results in an increase in brain water, and deterioration in neuropsychological function.

Interestingly, brain oedema and the consequent risk of intracranial hypertension are rarely complications of chronic liver failure, and are more often associated with fulminant hepatic failure. One possible suggestion for this is that in chronic liver disease, cells have more time to use compensatory mechanisms to adapt to the osmotic changes taking place[41].

Ammonia is directly toxic to the brain, and in acute liver failure causes disarray of inhibitory and excitatory neurotransmission[42], impairs brain energy metabolism[43-46], alters expression of several genes that code for important proteins involved in brain function[47,48], and impairs autoregulation of cerebral blood flow[49]. In patients with cirrhosis, there appears to be a shift in the balance between

inhibitory and excitatory neurotransmission towards a net increase in inhibitory neurotransmission.

THE CHANGING FACE OF HE

Although it is widely accepted that ammonia has a key role to play in the pathophysiology of HE, the clinical picture is not always so straightforward. Frequently, the arterial concentration of ammonia can be elevated in the absence of symptoms of HE, and the correlation between the severity of HE and ammonia concentration in patients with cirrhosis can be poor. The theory that several factors could contribute together to the clinical picture of HE was first suggested by Zieve *et al*[50] in 1974, who described the possible synergistic effects of several toxins, with ammonia. Since this first suggestion, it has become increasingly apparent that aspects of the inflammatory response (such as elevation of pro-inflammatory cytokines) in response to infection and/or systemic inflammation, and oxidative stress, participate in a synergistic relationship with ammonia in the pathogenesis of HE[51-53].

THE ROLE OF INFECTION AND INFLAMMATION IN HE

Acute liver failure

Studies in patients with acute liver failure have shown a more rapid progression to severe HE in those patients with evidence of a systemic inflammatory response, supporting a link between inflammation and HE[51]. In addition, in patients with acetaminophen-induced acute liver failure, infection and/or the resulting systemic inflammatory response were shown to be important factors contributing to an increase in the severity of HE[52]. Furthermore, in the advanced stages of HE in acute liver failure, the brain produces a number of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6[54,55]. This relationship is supported by evidence derived from therapeutic interventions, such as moderate hypothermia, that reduce cerebral oedema by reducing cerebral blood flow and inflammatory responses[56,57].

Cirrhosis

In patients with cirrhosis, there is mounting evidence for the role of inflammation in exacerbating the symptoms of HE, thus reinforcing the potential synergistic effects of ammonia and inflammation. Studies have shown this to be the case in patients with minimal HE, and across the whole spectrum of patients with varying degrees of overt HE (Westhaven grades 0-4)[53,58,59]. A recent study confirmed that the presence and severity of minimal HE in cirrhosis is independent of the severity of liver disease and plasma ammonia concentration, but markers of inflammation are significantly higher in those with minimal HE compared to those without[59]. In a further study, significant deterioration of neuropsychological test scores in patients with cirrhosis following induced hyperammonemia during the inflammatory state, but not after its resolution, suggested that inflammation might be important in modulating the cerebral effect of ammonia in liver disease, supporting an inflammatory hypothesis[53].

Synergy with ammonia

As inflammation, infection, and ammonia have been shown to be important in the pathogenesis of HE in cirrhosis, the question has to be raised as to whether infection and inflammation have a synergistic relationship with ammonia[60]. Marini and Broussard used mice with a deficiency in a critical urea cycle enzyme conferring chronic hyperammonemia, to demonstrate an increased sensitivity to inflammation. Furthermore, the hyperammonemic mice developed longer lasting and stronger cognitive defects when exposed to an inflammatory stimulus[61]. In a bile duct ligated (BDL) rat model, Jover *et al*[62] fed an ammonia-containing diet for 2 wk following ligation and compared animals sacrificed 7 d later to those fed a normal chow diet. Ammonia-fed BDL rats had increased cerebral ammonia and demonstrated the presence of type II Alzheimer astrocytosis analogous to patients with cirrhosis presenting with episodic HE. Both BDL groups had evidence of systemic inflammation, but the ammonia-fed BDL rats had increased brain glutamine, decreased brain myoinositol, and a significant increase in brain water compared to BDL controls, alluding to a potential synergistic relationship between ammonia and systemic inflammation. Wright *et al*[27] went on to explore the hypothesis that the inflammatory response induced by lipopolysaccharide (LPS) exacerbates brain oedema in BDL rats. LPS administration increased brain water in ammonia-fed, BDL, and sham-operated animals significantly, but this was associated with the progression to pre-coma only in the BDL animals. LPS induced cytotoxic brain swelling, but the anatomical integrity of the blood brain barrier was maintained. There was evidence of brain and systemic inflammation in BDL rats, which was significantly increased in LPS-treated animals. Nitrosation of proteins in the frontal cortex of BDL and LPS-treated animals was demonstrated. These data provide further evidence that in a background of cirrhosis and hyperammonemia, superimposed inflammation has an important role in the development of HE.

The ammonia-induced nitrosation of astrocytic proteins shown by Wright *et al*[27] has also been demonstrated in isolated astrocytes and astroglial tissue in brain sections of portocaval shunted rats[63]. However, ammonia alone cannot be responsible, because protein nitrosation was not demonstrated in ammonia fed sham-operated and ammonia-fed BDL rats in the absence of an inflammatory stimulus. Therefore, both ammonia and an additional inflammatory insult might need to be present for nitrosation of brain proteins to occur in animals with "subliminal" inflammation, such as that which has been observed in the BDL model[27]. This is further supported by recent work that demonstrated the presence of tyrosine nitration in astrocyte cultures in the presence of concentrations of TNF-α typically observed in patients with acute liver failure[64].

Inflammation and the brain

During an episode of infection, cytokines cannot directly cross the blood brain barrier and are unable to have a direct effect. Nevertheless, the peripheral immune system can still signal the brain to elicit a response during infection and inflammation through the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, both in the periphery and in the brain. Brain signalling may occur by direct transport of the cytokine across the blood brain barrier *via* an active transport mechanism, the interaction of the cytokine with circumventricular organs and activation of afferent neurons of the vagus nerve[65]. Endothelial cells, along with the astrocyte, are major constituents of the blood brain barrier. Endothelial cells are activated during infection, resulting in the release of various mediators into the brain. Activated microglial cells and astrocytes have the ability to produce a full repertoire of cytokines in response to inflammation and injury. One such cytokine is IL-1β, which has been shown *in vitro* to compromise the integrity of the blood brain barrier. This is mediated through the cyclo-oxygenase (COX) pathway within the endothelial cell[66]. In a portocaval shunted rat model that is more akin to a model of minimal HE, Cauli *et al*[67] demonstrated an improved learning ability following the administration of supra-therapeutic doses of the non-steroidal anti-inflammatory drug (NSAID), ibuprofen. This was accompanied by normalisation of COX and inducible NO activity within the cerebral cortex but interestingly also an increase in TNF-α. It is unclear however, how this NSAID specifically interacts with the glutamate-nitric oxide-cGMP pathway and how COX plays a role in the pathogenesis of minimal HE without identification of the specific COX isoform involved and in the absence of neuroanatomical, proteomic and genomic data. Nevertheless, the therapeutic use of NSAID in HE is not novel. Indomethacin (non-selective COX inhibitor) has been shown in patients with acute liver failure[68], and in a portocaval shunted rat model[69], to improve intracranial hypertension and cerebral oedema. Unfortunately, NSAID use is associated with a number of systemic complications, including cardiovascular/renal compromise and cellular prostaglandin metabolism, which impact greatly on not only astroglial function, but also on the development of organ dysfunction, particularly in the context of patients with longstanding liver disease.

TNF-α is released early during infection and can also influence the permeability of the blood brain barrier[70]. Moreover, an association between circulating TNF-α levels in patients with acute[71] and chronic liver failure[72] and the severity of HE, regardless of aetiology, has been recognised. Endothelial cells have receptors for IL-1β and TNF-α which can transduce signals which ultimately culminate in the intracerebral synthesis of NO and prostanoids[73]. Bémeur *et al*[74] investigated the effect of *IL-1β*, *TNF-α* and interferon-α (*IFN-α*) gene deletions on the onset of HE. Deletion of the *IFN-γ* gene had no effect on brain water levels or neuropsychiatric status. On the other hand, *IL-1β* and *TNF-α* gene deletions significantly delayed the onset of HE and brain oedema.

The relationship between the brain and inflammation is not one way. Molecular and neurophysiological studies during the past decade have suggested that pro-inflammatory responses are controlled by evolutionary neural circuits that operate reflexively[75,76]. The afferent arc of the reflex consists of nerves that sense injury and infection. This activates efferent neural circuits including the cholinergic anti-inflammatory pathway, which modulate immune responses and the progression of inflammatory disease. It might therefore be possible to target neural networks for the treatment of inflammation. This novel and fascinating body of work has recently been reviewed by Tracey[77].

Innate immune dysfunction

Innate immune dysfunction occurs in both acute and chronic liver failure, and up to 50% of admissions to hospital in patients with cirrhosis are likely to be related to the development of infection. In response to infection, the body initiates the innate immune response with phagocytic cells, such as monocytes and neutrophils. This response is particularly relevant to the liver, as the liver is the first organ to encounter bacteria or other toxins absorbed in the gut from the portal vein. Bacterial translocation of organisms from the gut in patients with cirrhosis and portal hypertension results in chronic endotoxemia. This culminates in a local milieu of pro-inflammatory cytokines/chemokines which can upregulate adhesion receptors and activate neutrophils[78]. There is significant literature on the immune response to infection in liver disease, which involves an important role of phagocytes and release of inflammatory cytokines. Patients with cirrhosis are functionally immunosuppressed and have impairment of several host defence mechanisms. The hemodynamic derangement of cirrhosis resembles that produced by endotoxin, and bacteremia can greatly exacerbate this state[79].

Neutrophils are a key component of the innate immune response. Ammonia has been shown to induce neutrophil dysfunction by inducing cell swelling, impaired phagocytosis, and increased oxidative burst in normal neutrophils *ex vivo*, in ammonia-fed rats and in patients with cirrhosis given an ammonia load[80]. Not only does this make patients potentially vulnerable to developing bacterial and fungal infections, but induces oxidative stress, and may ultimately culminate in a "sepsis-like" immune paralysis[81] and a reduction in monocyte HLA-DR expression[82].

OXIDATIVE STRESS

The evidence for the role of oxidative stress in the pathogenesis of HE is incontrovertible. Animal studies have shown significant reductions in the activities of glutathione peroxidase and superoxide dismutase enzymes, both in the liver and brain of rats exposed to ammonium acetate. Superoxide levels, in submitochondrial particles, were found to be elevated in ammonia-exposed rats[83] and lipid peroxidation has been shown to be increased, further demonstrating that hyperammonemia induces oxidative stress[84].

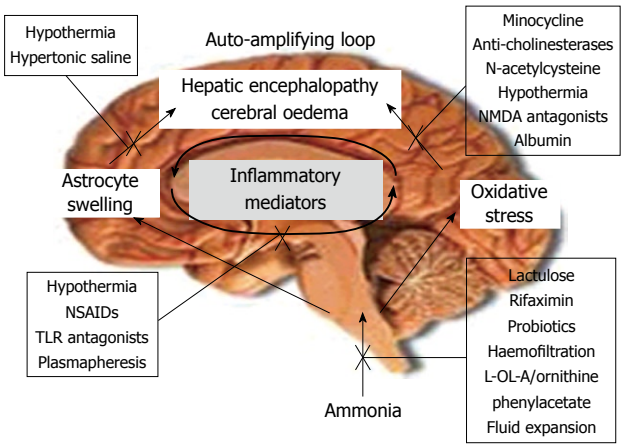

Figure 1 The "Two-hit" hypothesis. In a background of liver injury and hyperammonemia, a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia can drive further astrocyte swelling, oxidative stress and lead to a rapid deterioration in neurocognitive function. The close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop". The sites of action of potential therapies are indicated on the Figure. L-OL-A: L-ornithine L aspartate; NMDA: N-methyl D-aspartate; NSAID: Non-steroidal anti-inflammatory; TLR: Toll-like receptor.

N-methyl D-aspartate (NMDA) receptors play a key role in the production of free radicals and an NMDA antagonist can prevent the calcium-mediated increase in oxidative stress[85]. *In vivo* excessive ammonia-induced NMDA receptor activation reduces antioxidant enzyme activity and results in increased production of superoxide anions[86]. It is, however, extremely difficult to differentiate whether it is oxidative stress that influences astrocyte swelling or whether astrocyte swelling itself induces oxidative stress through NMDA receptor and calcium-dependent mechanisms[87]. Either way, whether one considers that "the chicken came before the egg or vice versa", it would imply that the close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop" which promotes the development of HE[88] (Figure 1).

The production of reactive oxygen species (ROS) can arise in a number of different ways. Aside from ROS arising from neutrophil activation[80] and local and systemic inflammation/infection, ammonia and hypo-osmotic swelling-induced nitric oxide synthesis, the activation of NADPH oxidase[89], and mitochondrial glutamine uptake all generate ROS[90-92]. From these data we can propose a "two-hit hypothesis" in the pathogenesis of HE. Liver dysfunction leads invariably to hyperammonemia, which leads to astrocyte swelling, and in the longer term, structural changes to astrocytes (Alzheimer's type II astrocytosis). After this initial "hit", a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia in a patient with cirrhosis can drive further astrocyte swelling, oxidative stress, and lead to a rapid deterioration in neuropsychological function (Figure 1 and Table 3).

Table 3 Factors precipitating hepatic encephalopathy[2]

Precipitating factors	Ammonia load e.g. upper gastrointestinal bleed or portocaval shunt
	Inflammation/oxidative stress
	Infection
	Dehydration
	Hyponatremia
	Sedative drugs e.g. benzodiazepines

Uptake of ammonia by astrocytes leads to the production of glutamine through the action of glutamine synthetase. Glutamine exposure in cultured astrocytes increases oxidative stress[91]. Mitochondrial glutamine uptake and subsequent cleavage of glutamine by phosphate-activated glutaminase elevates mitochondrial ammonia, which stimulates ROS production *via* induction of the mitochondrial permeability transition (MPT)[93]. However, cultured astrocytes exposed to ammonia produce ROS and begin swelling almost immediately, whereas MPT induction and glutamine accumulation occur thereafter.

Although astrocytes are relatively resistant to oxidative and nitrosative stress, neighbouring neurones are vulnerable to free radical attack. This can compromise brain energy metabolism and neurotransmission in patients with HE. Furthermore, ammonia, TNF-α, benzodiazepines, and hyponatremia can all trigger nitric oxide-dependent mobilisation of zinc which can augment GABAergic neurotransmission[94].

The mechanism through which free radical production is increased is currently not fully understood. One suggestion is based on findings that link an increase in calcium release to hyperammonemia. Rose *et al*[95] exposed cultured mice astrocytes to ammonium chloride. They observed a transient increase in the concentration of calcium ions from intracellular stores. The use of a calcium chelator (BAPTA) prevented the ammonia-induced production of free radicals[25]. Another possibility is that ROS are produced through activation of NMDA receptors[96].

One other area of research interest involves oxidation of RNA. It has been shown that in patients with Alzheimer's disease, there is significant RNA oxidation, which might result in impairments in protein synthesis and, consequently, cognitive function in patients[97]. Görg *et al*[98] reported the effects on cultured rat astrocytes and rat brain *in vivo* of ammonia exposure. Ammonia exposure was associated with a rapid, reversible oxidation of RNA (thought to involve NMDA receptor activation and calcium release). Consistent with this theory is the fact that some substrates required for learning and memory require protein synthesis[96]. Disruption of this protein synthesis *via* RNA oxidation might therefore interfere with cognitive function.

THERAPEUTIC STRATEGIES IN HE

To date, most therapeutic strategies in HE have been focused on lowering arterial concentrations of ammonia

and modulating inter-organ ammonia metabolism, but these remain largely ineffective. Treatments based on the hypothesis that the colon is the primary organ responsible for the generation of ammonia have ranged from dietary protein restriction, to the use of non-absorbable disaccharides, non-absorbable antibiotics, and colectomy[99]. However, Córdoba *et al*[100] showed that diets with normal protein content can be administered safely to patients with cirrhosis with episodic HE and that protein restriction does not have any beneficial effect for cirrhotic patients during an episode of HE and indeed, might even be detrimental in a patient with an underlying catabolic state.

It has been demonstrated that lactulose administered to patients with minimal HE in an unblinded open label study[101] might be of benefit and another open label randomised placebo controlled study in patients with a previous history of overt HE suggested that lactulose might delay the onset of a recurrent episode of HE[102]. However, in a recently published systematic review[103], which had very few high quality studies to base its findings on, lactulose was not found to have any impact on mortality in patients with cirrhosis presenting acutely with overt HE.

The use of non-absorbable antibiotics had been largely abandoned after concern that long-term administration of neomycin might lead to problems with nephrotoxicity and ototoxicity, and with metronidazole might lead to peripheral neuropathy. However, support for this strategy has been recently reinvigorated with the publication of the largest double blind placebo controlled study (n = 299) by Bass *et al*[104], which compared rifaximin (which has no known long term toxicity) favourably with placebo for the secondary prophylaxis of HE.

Benzodiazepine antagonists such as flumazenil also emerged as a potential therapy for HE patients. An analysis of six randomised controlled trials showed that 27% patients treated with flumazenil showed a clinical improvement, whilst 19% of treated patients showed an electroencephalographic improvement[105].

In the sickest cohorts, direct ammonia removal by hemofiltration in the intensive care unit is effective, but unfortunately by this stage multiorgan dysfunction and bacteremia might have superseded. Likewise, albumin dialysis in patients with acute-on-chronic liver failure improves HE grade[106], but the improvement is independent of changes in ammonia or cytokines[107] and remains controversial[108].

To address the issue of inter-organ ammonia metabolism, recent studies in patients with cirrhosis have shown that other than the gut, kidneys and muscle might be important targets[99]. Volume expansion produces significant increases in renal ammonia excretion resulting in a reduction in plasma ammonia concentration. This was shown to improve mental state, supporting the notion that the kidneys can be manipulated favourably[109]. During the hyperammonemic state, muscle detoxifies ammonia through conversion to glutamine[110,111]. L-ornithine L-aspartate (LOLA), which is a mixture of two amino acids, provides intermediates that increase glutamate availability for synthesis of glutamine and illustrates the concept that muscle can detoxify ammonia. Administration to animals with acute liver failure resulted in reduced brain water[112], but a recent study in patients with acute liver failure did not have any impact on brain dysfunction or survival[113]. When given to patients with cirrhosis and HE, administration of LOLA resulted in an improvement in HE compared with placebo-treated controls[114], although a recent meta-analysis concluded that it had little effect in patients with minimal HE[115]. Jalan *et al*[116] have hypothesised that this inefficacy might result from an accumulation of glutamine resulting in a rebound rise in circulating ammonia. By utilising a strategy which enables the excretion of glutamine, Davies *et al*[117] have demonstrated a synergy between L-ornithine and phenylacetate in reducing arterial ammonia in BDL rats.

However, in this review we have already convincingly demonstrated that ammonia, although central in the development of HE, is not solely responsible for its development. Infection/inflammation and oxidative stress are key determinants and indeed act synergistically with ammonia. Although ammonia could potentially be responsible for the development of neutrophil dysfunction, a patient with cirrhosis presents independently as a model of chronic endotoxemia that has direct implications on the innate and adaptive immune systems. We must therefore also look to therapies that directly or indirectly target the proinflammatory milieu.

Potential therapeutic strategies might include NMDA antagonists[84], leukodepletion[118], antagonism of pro-inflammatory cytokines[119], antioxidants [N-acetylcysteine (NAC)[120] and albumin[107,121]], anti-inflammatories (COX inhibitors[67] and minocycline[122,123]), probiotics[124] and hypothermia[125]. Excitement surrounds the prospect of small molecules that modulate toll-like receptor (TLR)-4 signalling, which can potentially down regulate neutrophil activation and other cellular responses. Early data indicate that TLR-4 antagonists can reduce LPS-stimulated cytokine release in healthy volunteers and results from phase 3 clinical trials are awaited. Inhibition of TLR-2, 4, and 9 prevented the increase in neutrophil oxidative burst induced from plasma from patients with alcoholic hepatitis. Furthermore, albumin, an endotoxin scavenger, prevented the deleterious effect of patients' plasma on neutrophil phagocytosis, spontaneous oxidative burst, and TLR expression[121]. This might also explain the beneficial role of albumin dialysis on HE[107,126].

When administered early after an overdose of acetaminophen, intravenous NAC prevents hepatic necrosis by replenishing stores of glutathione[127]. In patients with acute liver failure secondary to an overdose of acetaminophen, and in patients with acute liver failure secondary to other causes, NAC has been shown to increase oxygen delivery and consumption associated with increases in mean arterial pressure, cardiac index[128], and cerebral perfusion pressure[120]. These beneficial hemodynamic effects have been shown to be mediated by enhanced activity of the nitric oxide/soluble cGMP system[129] and suggest that NAC could have a beneficial role in the treatment of patients with cirrhosis who have developed overt HE.

As the role of central pro-inflammatory mechanisms are believed to be important in the pathogenesis of HE, then another novel therapeutic candidate drug to be considered is minocycline, which has been shown in two very recent studies by Jiang *et al*[122,123] to have anti-inflammatory effects in rats with acute liver failure. Minocyline treatment prevented both microglial activation [CD11b/c (OX-42) expression on immunohistochemistry] as well as the upregulation of IL-1β, IL-6, TNF-α, heme-oxygenase-1, eNOS, iNOS mRNA and protein expression with a concomitant attenuation of the progression of HE and brain edema, and at least in part, by reduction of oxidative/nitrosative stress. Thus, minocycline might also have promise in patients with acute and chronic liver failure cirrhosis and HE, and could be taken forward into randomised placebo controlled trials.

Modulation of intestinal microbiota is an emerging strategy to reduce the bacterial translocation of LPS and other bacterial activators of TLRs. Probiotics have been shown to reduce bacterial translocation and were shown to improve liver function and prevent the development of infection and HE in patients with cirrhosis[124]. Furthermore, probiotics have been shown to restore neutrophil phagocytic capacity in patients with alcoholic cirrhosis, possibly by reducing endogenous levels of IL-10 and TLR-4 expression[130].

Recent studies show that hypothermia is efficacious in patients with uncontrolled intracranial hypertension that are undergoing liver transplantation[56,125]. Hypothermia displays many beneficial effects on brain water and intracranial hypertension relating to decreased brain ammonia, cerebral blood flow, mediators of inflammation, and oxidative stress[131]. The sites of action of potential therapies for HE is shown in Figure 1.

CONCLUSION

HE is a dynamic neuropsychological spectral disorder that develops after liver injury. The pathophysiological mechanisms behind the development of HE are still not fully understood, but ammonia and the downstream consequences of ammonia uptake by astrocytes remain fundamental to the process. Ammonia not only leads to astrocyte swelling, but also alters neurotransmission, mitochondrial function, and induces oxidative stress. Astrocyte swelling and oxidative stress are closely related and result in "an auto-amplifying" loop. The presence of local and systemic inflammation and the release of ROS further exacerbate the cerebral effects of ammonia. Anti-inflammatory and anti-oxidative strategies may abrogate these effects and offer real treatment options to patients with HE in the future.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3358

World J Gastroenterol 2010 July 21; 16(27): 3358-3370
ISSN 1007-9327 (print)

EDITORIAL

R0 resection in the treatment of gastric cancer: Room for improvement

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Received: February 10, 2010 Revised: March 18, 2010
Accepted: March 25, 2010
Published online: July 21, 2010

Abstract

Gastric carcinoma is one of the most frequent malignancies in the world and its clinical behavior especially depends on the metastatic potential of the tumor. In particular, lymphatic metastasis is one of the main predictors of tumor recurrence and survival, and current pathological staging systems reflect the concept that lymphatic spread is the most relevant prognostic factor in patients undergoing curative resection. This is compounded by the observation that two-thirds of gastric cancer in the Western world presents at an advanced stage, with lymph node metastasis at diagnosis. All current therapeutic efforts in gastric cancer are directed toward individualization of therapeutic protocols, tailoring the extent of resection and the administration of preoperative and postoperative treatment. The goals of all these strategies are to improve prognosis towards the achievement of a curative resection (R0 resection) with minimal morbidity and mortality, and better postoperative quality of life.

Key words: Gastric cancer; R0 resection; Total gastrectomy; Lymph node dissection; Adjuvant therapy; Preoperative therapy

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Biondi A, Persiani R, Cananzi F, Zoccali M, Vigorita V, Tufo A, D'Ugo D. R0 resection in the treatment of gastric cancer: Room for improvement. *World J Gastroenterol* 2010; 16(27): 3358-3370 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3358.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3358

INTRODUCTION

Despite an incidence rate that has steadily declined over the past few decades, gastric carcinoma is one of the most frequent malignancies worldwide. An estimated 934 000 new cases are diagnosed each year, with the highest incidence rate in Northeast Asia, intermediate incidence rates in Europe and South America, and the lowest incidence rates in North America, Africa, South Asia, and Oceania[1,2].

Early dissemination of the disease through the lymphatic system, blood, and peritoneum has limited optimal surgery as a cure, except in patients with early-stage cancers. In Japan and Korea, the introduction of screening for gastric cancer has been shown to improve early detection, and almost half of newly diagnosed patients are detected at an early stage[3-6]. Due to the lower disease incidence rate, this strategy has not been deemed cost-

effective in Europe or North America. Consequently, two-thirds of gastric cancers in the Western world present at an advanced stage, with lymph node metastasis at the time of diagnosis[7].

The mainstay of treatment is radical surgery, but even with optimal surgical resection, the prognosis remains dismal in Western countries. Numerous attempts have been undertaken to improve clinical outcomes. To date, most therapeutic efforts are directed toward an individualization of therapeutic protocols, tailoring the extent of surgery and integrating it with the administration of preoperative and/or postoperative treatment. The goal of such strategies is to improve prognosis towards the achievement of a curative resection with minimal morbidity and mortality and better postoperative quality of life.

R0 RESECTION: DEFINITIONS

Curative resection refers to the absence of tumor after surgical treatment, and in the Western world, it meets the R0 resection definition provided by Hermanek *et al*[8] more than 15 years ago. R0 resection indicates a microscopically margin-negative resection, in which no gross or microscopic tumor remains in the primary tumor bed. R1 resection indicates the removal of all macroscopic disease, but microscopic margins are positive for tumor. R2 indicates gross residual disease with gross residual tumor that was not resected (primary tumor, regional nodes, and macroscopic margin involvement).

If this definition holds, R0 resection should represent a surgical cure, with a high survival rate and low recurrence. Considering the low survival rate after R0 surgical treatment in the Western case-mix, it is clear that the R0 definition needs to be revised, especially in locally advanced cases[9-11]. It is likely that there is a tendency to misclassify a number of cases as R0 resection, which inexorably will recur, which suggests that a curative treatment was not actually achieved.

The reason that the definition of Hermaneck is not in accordance with this scenario may be because it is mainly concerned with the primary tumor site, and not examining in detail the three pathways of tumor dissemination: portal blood stream to the liver, peritoneal surfaces and lymphatic dissemination. With these methods of dissemination, it is often beyond the surgeon's ability to achieve loco-regional control of the cancer. It may be difficult or impossible for the surgeon to reduce the incidence of metastases to the liver, as well as to contain the peritoneal seeding of cancer cells, or the removal of all extra-regional metastatic lymph nodes.

In the eastern world, Japanese guidelines have given a different definition to the curative gastric resection based on both surgical and histopathological details[12]. Resection A: no residual disease, with a high cure probability. It implies resections satisfying all of the following conditions: tumor without serosal invasion; N0 treated by D1, D2, or D3 lymph node dissections, or tumor with first-level lymph node treated by D2 or D3 resection; no distant, peritoneal or liver metastases, negative cytological examination of peritoneal fluid and proximal and distal margins > 10 mm. Resection B: no histopathologic residual disease but not fulfilling criteria for resection A. Resection C: definite residual disease.

These strict criteria emphasize that once the tumor penetrates the serosa or invades adjacent organs, it begins to spread by routes other than the regional lymphatic system. Specifically, tumor metastasis can occur through the peritoneum, extra-regional lymph nodes and the portal-hepatic blood, which consequently diminishes the probability of a cure. Such a definition would imply that more than two-thirds of patients are considered non-curatively treated by surgery in the Western world, which underestimates the role of surgery at these stages.

Today, both definitions seem inadequate: they merely indicate the absence or presence of residual tumor cells in the tumor bed after surgical treatments or provide an estimation of the probability of cure with surgery. In reality, the surgeons must consider themselves responsible not only for resection of the large mass of the primary cancer and overt lymph node metastases in the tumor bed, but also for dealing with microscopic and distant residual disease.

R0 RESECTION AND PREOPERATIVE IMAGING: WHAT CAN WE ANTICIPATE?

Although surgical pathology provides the most accurate information on tumor extent, clinical preoperative staging is crucial to select the appropriate treatment strategy. Today, clinical staging has been improved by technical enhancement in endoscopic ultrasound (EUS), computed tomography (CT), positron emission tomography (PET), combined PET-CT scan, magnetic resonance imaging (MRI) and laparoscopic staging. Presently, EUS and CT are widely used for preoperative staging[13].

Although the accuracy of T staging has been much improved for EUS (current range: 78%-92%)[14-20] and CT (current range: 69%-89%)[17,21-27], N staging accuracy is still poor (63%-78% in EUS[14-20], 51%-78% in CT[17,21-27]). MRI has had limited use in the staging of gastric cancer, primarily as a result of difficulties with motion artifacts, cost, time required for examination, and lack of an appropriate oral contrast agent[28,29]. However, in recent studies, overall T staging accuracy has been reported to be between 71.4% and 82%, which is similar to CT[29]. In N staging, several studies have shown that the accuracy of MRI nodal staging is inferior to CT staging with both techniques tending to understage nodal status[28,29]. Moreover, MRI has showed a greater sensitivity than CT in detecting liver, bone, and peritoneal dissemination[29].

Generally, PET is not routinely performed in the clinical staging of gastric cancer. From clinical studies focusing on PET, it is concluded that, for N staging, PET has a significantly higher specificity (92%) but lower sensibility (56%) compared to CT in the detection of local lymph node involvement[30-32]. Recent reports have confirmed the limited role of PET in the preoperative staging of gastric cancer, but it must be pointed out

that combined PET-CT can significantly improve overall staging accuracy compared to PET and CT alone[33,34].

Due to the inaccuracy of CT for the detection of ≤ 5 mm macrometastases on the peritoneal surface or liver, staging laparoscopy is recommended as the next step in the evaluation of patients with locoregional disease. Staging laparoscopy can detect metastatic disease or modified preoperative therapeutic strategy in 23%-54% of patients, thus confirming its crucial role in staging gastric carcinoma[35-37]. Moreover, there is some evidence that laparoscopy permits a more accurate staging of extraserosal tumors, whereas EUS might sometimes lead to misinterpretation of T3 invasion, when edema distorts the interface between the stomach and adjacent tissues[18,38,39].

In addition, staging laparoscopy facilitates cytological examination of abdominal lavage fluid. Cytology of peritoneal fluid or lavage may reveal the presence of free intraperitoneal gastric cancer cells, which identifies patients with an otherwise occult microscopic carcinomatosis. Recent evidence has suggested that patients with positive findings on peritoneal cytology have a poor prognosis, similar to that of patients with macroscopic stage Ⅳ disease[40].

SURGICAL DEBATES OF R0 RESECTION

R0 resection: Total vs subtotal gastrectomy, what else?

Some issues about the extent of gastric resection seem to have been settled. Total gastrectomy should be avoided if adequate free resection margins can be obtained with subtotal gastrectomy: a gross surgical margin of at least 5 cm for the intestinal type or 8-10 cm for the diffuse type[41-44]. Many authors agree on the necessity of total gastrectomy if the cancer encroaches on an imaginary line between the angula incisura of the lesser curvature and the "bare" area on the greater curvature between the gastroepiploic vessels and the short gastric vessels[44]. This is because the lymph drainage from such a tumor feeds into the splenic hilum and flows along the splenic artery, as well as passing proximally and distally.

Proximal tumors and tumors of the gastroesophageal junction (GEJ) deserve different considerations. These tumors are traditionally classified according to the Siewert classification system, which takes into account the center of the tumor and the variable involvement of the esophagus and stomach: type Ⅰ, esophageal adenocarcinoma of the distal esophagus, with the center located between 1 and 5 cm above the GEJ; type Ⅱ, true adenocarcinoma of the cardia located within 1 cm above and 2 cm below the GEJ; and type Ⅲ, subcardial adenocarcinoma located between 2 and 5 cm below the GEJ. Surgical treatment of these tumors usually requires an extended total gastrectomy with resection of variable portions of the distal esophagus. The extent of resection of the distal esophagus depends on the extent of the tumor spread[45].

Generally, patients with type Ⅰ tumors are best treated by esophagectomy with gastric pull-up to the neck or by esophagogastrectomy (transthoracic or transhiatal). Type Ⅱ and Ⅲ tumors can be resected by gastrectomy with frozen-section-guided resection of the distal esophagus (transhiatally extended gastrectomy)[46]. Although total gastrectomy has been the procedure of choice in these tumors, some authors have advocated proximal gastrectomy as a surgical option, and in a retrospective study conducted by the Memorial Sloan Kettering Cancer Center, proximal gastrectomy has been reported to have similar mortality rate, hospital stay, and recurrence and survival rates[47]. Even if the R0 resection rate does not differ between groups, other authors have reported poor functional and quality of life results in patients undergoing proximal resection[48-50]. Although it is difficult to make definitive conclusions in the absence of a prospective randomized trial, it does appear that total gastrectomy remains the procedure of choice in these patients.

R0 resection: The "circumferential/lateral" margin

The progression of the cancer through the stomach wall to the adjacent structures makes one aware of the concept of circumferential/lateral margins and provides the rationale for conservative and extended surgery.

If diagnosed at an early stage, it may be possible to obtain a margin-negative resection without traditional gastrectomy (subtotal, proximal or total gastrectomy). When margin-free resection is warranted, the only limiting factor is the risk of lymph node metastasis. For patients with a well- to moderately well-differentiated tumor of less than 2 cm in size, with no submucosal invasion or lymphangioinvasion, local excision by endoscopic mucosal resection (EMR) has been the preferred treatment in Japan for the past 15 years, since the risk of lymph node metastases is thought to be very low[51].

Although a prospective randomized trial is lacking in the literature, results of a systematic review of cohort studies have shown that EMR has favorable disease-specific survival, incidence of local recurrence and complications, compared with surgery[52,53]. Endoscopic submucosal dissection (ESD) is a newly developed technique that can remove large tumors in one piece. In a comparison with EMR, resection that removes tumors in one piece was more frequent in an ESD group and resulted in a better 3-year recurrence-free rate, despite a higher complication rate[54,55].

Currently, indications for ESD, according to Japanese guidelines, are only for well-differentiated intramucosal (T1a) tumors. However, a large-scale study analyzing lymph node metastasis of early cancer has expanded the criteria for endoscopic treatment of early gastric cancer, which is based on tumor characteristics with a very low risk of lymph node metastasis[56]. This study showed that patients with intramucosally or submucosally well-differentiated tumors of less than 3 cm and poorly intramucosally differentiated tumors of less than 2 cm have a very low risk of lymph node metastasis.

The results of both the United Kingdom Medical Research Council and the Dutch trials, along with more recently randomized controlled trials, large retrospective series and meta-analysis[57-63] have reported a significantly worse prognosis, higher mortality, higher complication

rate, and longer hospital stay in patients who have undergone gastrectomy with prophylactic splenectomy or pancreaticosplenectomy.

Theoretically, in patients with T4 gastric adenocarcinoma, extended resection is required to improve the R0 resection rate. With careful patient selection, gastrectomy with additional organ resection can be done with acceptable morbidity and low mortality. Improvements in preoperative evaluation to confirm T3 and T4 disease are needed because postoperative histopathological examination has revealed that multi-organ resections are often performed for pT3 tumors, with a relatively small proportion of pT4 tumors[64,65]. Independent factors of a worse prognosis, such as N3 tumors and large diameter tumors (> 10 cm), have to be excluded before performing extended resection[66,67]. Based upon these issues, the cautious clinical behavior is to reconsider any clinically defined T4 tumor on a case by case basis before planning extended multi-organ resection.

R0-resection: When can the lymph node dissection be considered margin-negative?

The extent of lymphadenectomy continues to represent the main area for surgical research in gastric cancer, and the surgical strategy of choice is still a matter for debate. Lymphatic metastasis is one of the main predictors of tumor recurrence, and survival and current pathological staging systems reflect the concept that lymphatic spread is the most relevant prognostic factor in patients resected with curative intent[68-73]. Recurrence rates attributed to residual lymph node metastasis around the celiac artery have led to the concept that complete clearance of the metastatic lymph nodes by extended dissection (D2) may prolong survival. In Japan, where gastric cancer is far more common than in Western countries, a standardized lymph node dissection has been developed over the past 40 years and is used nationwide with therapeutic benefit and long-term survival rates of $\geqslant$ 60% after 5 years. Retrospective studies from Japan, and later from Korea[74], involving more than 10 000 patients, have suggested that extended lymph node dissection combined with gastrectomy increases 5-year survival rate from 50% to 62%, compared to a 5-year survival rate of 15%-30%, as a result of limited resections in the United States[75-79].

The importance of adequate lymph node dissection as part of a potentially curative resection has led to the development and publication of "The General Rules for the Gastric Cancer Study in Surgery and Pathology", which was definitively published in English in 1996[12]. Several Western reports have confirmed that extended lymphadenectomy, similar to that recommended in the General Rules, can be safely performed with improvements in survival[80-85].

In the Western world, the challenge has been to show whether these results could be generalized for unselected patients. To date, four prospective randomized trials of Japanese-defined D1 *vs* D2 lymph node dissection and two meta-analysis studies have been conducted[86-92].

All of these studies have documented limited survival benefits with unacceptable morbidity and mortality that is probably associated with pancreaticosplenectomy, low case volume, and a lack of specialist training[93,94]. Moreover, some authors have suggested that extended lymph node dissection combined with rigorous pathological evaluation results in improved staging rather than therapeutic benefit. Through accurate staging, patients with advanced stage cancer are well categorized, and any comparisons with series of non-standardized lymph node dissection, or under-staged patients, are therefore inaccurate. These results have made many Western surgeons reluctant to perform extended lymph node dissection routinely in an effort to obtain better regional disease control, and possibly, some survival advantage. Nevertheless, there is some evidence that extended lymph node dissection may offer a definite chance for a cure in a subset of patients with pN2 disease[88], even if these patients cannot be distinguished preoperatively.

At the same time, in the eastern World, where D2 lymph node dissection is not a matter of debate, the challenge has been to demonstrate that super-extended lymph node dissection offers a better chance of a cure in gastric cancer treated with curative intent. The Taipei single-institution study that has compared D1 and D3 dissection has demonstrated a significant overall survival benefit in extended lymph node dissection, but no significant improvements in disease-free survival or in per-protocol analysis[90]. Moreover, the study showed that the morbidity of extended lymphadenectomy, although not lethal, is substantial even in experienced hands[95]. Finally, a multi-institutional, randomized and controlled trial by the Japan Clinical Oncology Group (JCOG-9501) has failed to demonstrate a survival benefit when super-extended (D2 + para-aortic node) lymph node dissection was performed. Moreover, in this randomized trial, the rate of postoperative morbidity in patients with a body mass index of $\geqslant$ 25 kg/m^2 and age > 65 years was a notable concern[96].

Geographical differences notwithstanding, all of these results agree with Cady's paradigm "...the therapeutic effect of cancer surgery is akin to that of a drug with a threshold or plateau effect: dose response up to a certain plateau, and then no further therapeutic effect beyond this point, only more complications"[97].

From a practical point of view, it is hard to believe that unresected overt nodal metastases in the tumor bed will not worsen prognosis. Likewise, it is hard to believe that resection of more negative lymph nodes will improve it. Tailoring lymph node dissection on the basis of actual lymph node involvement could be a key point for performing appropriate lymph node dissection and avoiding high rates of postoperative morbidity.

In the late 1980s, Kampschöer *et al*[98] developed software that was designed to match cases with characteristics similar to a given case. With seven demographic and clinical inputs, all identifiable preoperatively or intraoperatively, the program was able to predict the statistical likelihood of nodal disease for each of the 16 main nodal stations around the stomach[98-100]. The so-called "Maruyama Index of Unresected Disease" (MI), when retrospectively

used, was able to quantify the adequacy of lymphadenectomy. Such a novel measure was defined as the sum of Maruyama Program predictions for lymph node stations (Japanese stations 1-12) left *in situ* by the surgeon[101,102]. In a large United States adjuvant chemoradiation study, MI proved to be a strong predictor of survival that was independent from the level of lymph node dissection[103]. Furthermore, a blinded retrospective analysis of Dutch D1 *vs* D2 trial data has suggested that low-MI surgery is associated with significantly increased survival, regardless of lymph node dissection[104]. The MI aims to define an R+ lymph node dissection, and it appears that surgeons might have a better impact on single patient survival by pursuing a low MI operation (low probability of lymph node metastases left *in situ*) instead of relying exclusively on D-level guidance.

When the probability of lymph node metastasis is considered low, sentinel node dissection can be considered as another approach to customize lymph node dissection[105-107]. The sentinel nodes are the first sites of lymph node metastasis from a primary tumor and theoretically predict the involvement of more distant lymph nodes. To date, selective sentinel node dissection, detectable using the injection of either dyes or radioactive tracers, represents a standard procedure for melanoma and breast cancer with low probability of lymph node metastasis. In early gastric cancer, the risk of lymph node metastases is 2%-5% for patients with mucosal cancer and 11%-20% for those with submucosal cancer[108]. Sentinel node mapping results in gastric cancer have been variable since the lymphatic drainage from the stomach is very complicated and multidirectional, with an incidence of skip metastasis ranging from 5% to 10%[109]. Moreover, early reports have demonstrated that the loco-regional lymph node station contains truly positive nodes, even when the sentinel biopsy is negative. These anatomical peculiarities have led to the concept of a "sentinel lymphatic basin"[110], which indicates the lymph node stations to which sentinel nodes belong. Dissection of these stations can provide an acceptable safety net for the clinical application of these procedures, and minimize the possibility of leaving metastasis behind. Preliminary studies have shown that these sentinel node techniques are an acceptable procedure for pathological T1 tumors with a diameter of < 40 mm, although long-term follow-up data are still required[111-114].

R0 RESECTION: IS IT MERELY A SURGICAL TARGET?

Along with these classical surgical topics, in the past 20 years, three different modalities of adjuvant (pre- and postoperative) therapy have been proven to be effective by large-scale randomized trials. These include postoperative chemoradiation therapy (Unites States INT-0116 trial)[115], postoperative single-drug chemotherapy (Japanese ACTS-GC trial)[116] and perioperative three-drug combination chemotherapy (European MAGIC trial)[117]. Since the publication of these trials, surgery alone is no longer considered the standard treatment for patients with resectable locally advanced forms of gastric cancer, and the concept of radical resection needs to take into account the fact that R0 resection is not an exclusively surgical target.

Postoperative therapy: Recovery of R0 resection

Many studies and several meta-analyses with a focus on adjuvant postoperative chemotherapy have been conducted[118-127]. Summarizing their results, we can state that there is insufficient evidence, at present, to recommend postoperative chemotherapy as standard adjuvant treatment in Western patients. At present, these results should be cautiously managed, since these studies included very different patient populations, surgical procedures, and non-standardized timing and regimens of adjuvant therapy that are now considered as outdated[128]. At the same time, results from pivotal studies on postoperative chemoradiotherapy are inconclusive and conflicting because of the relatively small number of patients recruited[129-133].

In the United States, between 1991 and 1998, a study from the SWOG-Intergroup 0116 trial randomly assigned 556 patients to surgery only and surgery plus postoperative chemoradiotherapy: 45 Gy radiotherapy at 1.8 Gy/d, given 5 d/wk for 5 wk, with modified doses of fluorouracil and leucovorin on the first 4 d and last 3 d of radiotherapy. Two 5-d cycles of fluorouracil and leucovorin were given after, and one cycle was given before chemoradiotherapy[115]. Although clinically significant toxicity was recorded after chemoradiotherapy, the overall and relapse-free survival results of the surgery-alone arm were significantly worse than those of the adjuvant chemoradiotherapy arm. Chemoradiotherapy significantly improved median survival from 27 to 36 mo. Distant relapse was the most common pattern of recurrence in the adjuvant group (33% *vs* 18%), whereas local recurrence was more common in the surgery-only group (29% *vs* 19%). In this trial, < 10% of patients received formal D2 dissection, whereas 54% underwent D0 dissection. A common interpretation of these results is that adjuvant therapy may be useful in high-risk patients treated with inadequate lymph node dissection, because, through radiotherapy, it can eliminate residual lymph node metastasis, which would have been removed by D2 resection. A Korean non-randomized study[134] recently has shown that chemoradiotherapy after Japanese D2 resection improves survival. Currently, promising results from a randomized study conducted by the same group (SMC-IRB 2004-08-10 trial) are anticipated[135].

In 2007, the most convincing evidence on the benefits of adjuvant therapy was reported by the Japanese ACTS-GC trial (Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer)[116]. In this trial, 1059 patients with stage Ⅱ or Ⅲ gastric cancer who had undergone curative D2 gastrectomy were randomized to observation or 1-year administration of oral S-1. The study was terminated at the first interim analysis due to a highly significant difference in survival that favored chemotherapy. The incidence rate of loco-regional, lymphatic and peritoneal relapse was significantly lower in the chemotherapy arm than in the surgery-alone arm, although the rate of distant metastases

did not differ between the two arms. This study reported a survival-associated advantage with adjuvant chemotherapy within the context of surgery performed according to Japanese standards.

New ongoing trials investigating adjuvant therapy (CLASSIC trial, SMC-IRB 2004-08-10, CALGB-80101) are expected to show the true efficacy and survival benefits in the near future[135-137].

In the past 30 years, Japanese and Korean researchers have performed a number of trials that have investigated the use of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. A variety of immunotherapeutic agents, such as protein-bound polysaccharide (polysaccharide K extracted from mycelia of *Coriolus versicolor*; PSK)[138,139], *Streptococcus pyogenes* preparation (OK-432)[140,141], polysaccharide sizofiran[142], *Nocardia rubra* cell wall skeleton[143], Bacillus Calmette-Guerin (BCG)[144] and polyadenylic-polyuridylic acid[145] have been used in addition to chemotherapy.

Results from randomized trials that have compared adjuvant chemo-immunotherapy with surgery alone or with other chemotherapeutic schedules have been contradictory because of a lack of robust evidence in clinical practice[146]. However, interesting results have been derived from two recent meta-analyses about OK-432 and PSK[147,148].

The benefit of combined adjuvant chemotherapy and immunotherapy with OK-432 (a lyophilized preparation of a low virulence group A *S. pyogenes*), in patients with curatively resected gastric cancer was assessed by Sakamoto *et al*[147] in a meta-analysis of data derived from 1522 patients enrolled in six randomized clinical trials. In these trials, adjuvant chemotherapy, usually consisting of induction with mitomycin C plus long-term oral fluordinated pyrimidines, was compared with the same chemotherapy plus OK-432. The 3-year survival rate for all eligible patients in the six trials was 67.5% in the chemo-immunotherapy group *vs* 62.6% in the chemotherapy-only group. The 3-year overall survival odds ratio was 0.81 (95% CI: 0.65-0.99). The beneficial treatment effect was shown to be statistically significant ($P < 0.044$). The results of this meta-analysis were interpreted by the authors to suggest that chemo-immunotherapy after surgery with OK-432 can improve the survival of patients with successfully resected gastric cancer.

The effect of adjuvant immunochemotherapy with PSK after curative resection of gastric cancer by means of a meta-analysis of eight randomized trials has been assessed by Oba *et al*[148]. In this analysis, the estimated overall HR was 0.88 (95% CI: 0.79-0.98, $P = 0.018$) with no significant heterogeneity between the treatment effects observed in different studies. The authors have concluded that the addition of PSK to standard chemotherapy offers significant advantages in survival over chemotherapy alone for patients with curative resections of gastric cancer.

Also for postoperative chemo-immunotherapy, there is a necessity for clear evidence in future studies; particularly, the clinical use of immunostimulating factors should be tested in large randomized trials.

Pre-/perioperative therapy: Induction of R0 resection

The rationale for preoperative therapy is based on several theoretical assumptions. Preoperative antiblastic therapy might reduce the risk of proliferation and allow for *in vivo* chemosensitivity tests, thus facilitating the choice of the most appropriate postoperative regimen. Furthermore, the preoperative approach has two distinct advantages: increased compliance due to an undoubtedly better performance status in patients who are not burdened with surgical complications, nutritional impairment, or damaged vascularization of the tumor bed. The twofold goal of eliminating hidden micrometastases along with tumor down-staging might increase the probability of a truly curative complete resection with delayed surgery.

Investigation of the efficacy and possible uses of chemotherapy in patients with advanced gastric cancer began in the late 1970s[149-151]. Encouraging results, however, were not reported until the early 1990s, when two independent studies in patients with non-resectable disease found that chemotherapy led to subsequent resection in 40%-50% of patients, with an increase in total median survival of 18 mo, compared with unresected patients[152,153]. These preliminary observations encouraged the introduction of preoperative chemotherapy protocols for potentially resectable, locally advanced gastric cancer (Table 1)[117,154-166]. However, the results of these first trials are questionable, mainly because of their methodological limitations. By following an inaccurate preoperative staging process, several authors have recruited patients on non-homogeneous criteria, commonly recruiting patients with locally advanced gastric cancer and others with disease of unclear stages, without a fixed distinction between resectable and non-resectable tumors. In addition to non-homogeneous methods of recruitment, other sources of bias in early trials included the use of different chemotherapeutic regimens, non-standardized surgery or surgery of questionable quality, and missing or poorly detailed response criteria.

In 1993, the Dutch Gastric Cancer Group started the first randomized controlled trial of exclusively preoperative chemotherapy for gastric cancer (cardia tumors were excluded)[161]. The regimen used was FAMTX (fluorouracil, doxorubicin, and methotrexate), which was, at that time, the gold standard of treatment for adenocarcinoma of the stomach. This trial had many accrual problems and was prematurely stopped after an interim analysis showed that FAMTX was unlikely to achieve the goal of a 15% increase in curative resectability after preoperative chemotherapy. Several biases have been outlined for this study, particularly the inaccuracy of the staging procedure with optional use of CT and laparoscopy, and inadequate extension of lymphadenectomy. The investigators reported a high rate of tumor progression during treatment (36%) along with a reduction in curative resections (56% *vs* 62%) and a decreased median survival (18 mo *vs* 30 mo), compared with untreated patients. Even if all of the statistical differences in this study were insignificant, both the short-term and long-term results were discouraging[161,167].

Since the late 1990s, ambitious European phase Ⅲ

Table 1 Trials of preoperative chemotherapy in gastric cancer

Author	Phase	Selection criteria	Preoperative	Postoperative	Pts	R0 (%)[1]	Pathological CR (%)	Median survival (mo)
Ajani *et al*[154], 1991	Ⅱ	M0 *Resectable* (+ GEJ)	EFP × 2	EFP × 3	25	72	0	15
Leichman *et al*[155], 1992	Ⅱ	M0 *Resectable*	FPL × 2	IP FUDR + P cisplatin × 2	38	88	8	> 17
Kang *et al*[156], 1992	Ⅲ RCT	M0 *Loc. advanced*	EFP × 3	EFP × 3-6	53	79	8	43
			None		54	61	-	30
Ajani *et al*[157], 1993	Ⅱ	M0 *Resectable*	EAP × 3	EAP × 2	48	90	0	16
Rougier *et al*[158], 1994	Ⅱ	M0 *Loc. advanced* (+ GEJ)	FP × 6	None	30	78	0	16
Kelsen *et al*[159], 1996	Ⅱ	M0 *Loc. advanced*	FAMTX × 3	IP FP + F	56	77	NS	15
Crookes *et al*[160], 1997	Ⅱ	M0 *Resectable* (+ GEJ)	FPL × 2	IP FUDR + IP cisplatin × 2	59	71	9	52
Songun *et al*[161], 1999	Ⅱ RCT	T2-T4; M0	FAMTX × 4	None	27	75	NS	18
			None		29	75	-	30
Schuhmacher *et al*[162], 2001	Ⅱ	Ⅲ-Ⅳ; M0 (+ GEJ)	EAP	None	42	86	0	19
D'Ugo *et al*[163], 2006	Ⅱ	T3-T4 anyN; T ≤ 2 N+; M0	EEP × 3 or ECF × 3	EEP × 3 or ECF × 3	34	82	3	> 28
Cunningham *et al*[117], 2006	Ⅲ RCT	Ⅱ-Ⅳ; M0 (+ GEJ)	ECF × 3	ECF × 3	250	74	NS	18
			None	None	253	68	-	30
Boige *et al*[164], 2007	Ⅲ RCT	*Resectable* (+ GEJ)	FP × 3	FP × 3	113	84	NS	NS
			None	None	111	73	-	
Schuhmacher *et al*[165], 2009	Ⅲ RCT	*Loc. advanced* T3-T4NxM0	FP × 2	None	72	81.9	NS	> 36
			None		72	66.7		
Kinoshita *et al*[166], 2009	Ⅱ	Schirrous *Resectable*	TS-1 × 2	None	55	80.8	0	NS

[1]The "R0" resection rate was calculated only among resection procedures. Pts: Number of patients recruited R0, curative (R0) resections; CR: Complete response; GEJ: Gastroesophageal junction; EFP: Etoposide, fluorouracil, and cisplatin; FPL: Fluorouracil, cisplatin, and leucovorin; IP: Intraperitoneal; FUDR: 5-fluoro-2'-deoxyuridine; RCT: Randomized controlled trial; EAP: Etoposide, doxorubicin, and cisplatin; FP: Fluorouracil and cisplatin; FAMTX: Fluorouracil, doxorubicin, and methotrexate; F: Fluorouracil; NS: Not stated; EEP: Etoposide, epirubicin and cisplatin; ECF: Epirubicin, cisplatin, and fluorouracil.

trials have been designed to provide a definitive demonstration of the efficacy of preoperative treatments. The adoption of strict selection criteria made the selection of patients so difficult that some studies were stopped prematurely (EORTC 40954 and SWS-SAKK-43/99 trials)[165,168]. Only the MAGIC trial (started in the United Kingdom in 1994) and the FFCD 9703 trial (started in France in 1996) have been completed[117,164]. These two studies have yielded substantial evidence supporting the efficacy of perioperative chemotherapy for an increased survival rate (36% *vs* 23%, estimated at 5 years for MAGIC, 38% *vs* 24% estimated at 5 years for FFCD 9703, Table 1), along with a significantly higher curative resection rate in the treated group *vs* the surgery-alone group (79% *vs* 70%, $P = 0.03$ for MAGIC, 84% *vs* 73% in arm 2, $P = 0.04$ for FFCD 9703) without an increase in perioperative morbidity or mortality.

The possible increase in the actual R0-resection rate has been an important goal of preoperative chemotherapy. In a phase Ⅱ study of a perioperative chemotherapy protocol, the achievement of R0 resection in response to preoperative chemotherapy was shown to be the most significant prognostic indicator by univariate and multivariate analysis. Furthermore, R0 resection was the only independent variable in determining the probability of long-term survival in locally advanced gastric carcinoma. The overall survival for all curatively resected patients is higher when compared to historical series treated with surgery alone for locally advanced gastric cancer[163,169].

Based on the results of the SWOG 9008/INT-0116 trial[115], the integration of chemotherapy with radiation applied in the preoperative phase has gained much interest. Some benefits of preoperative radiotherapy for gastric cancer have been reported by a pivotal randomized single-center Chinese study by Zhang *et al*[170]. This study recruited 317 patients with adenocarcinoma of the cardia that were randomly assigned to radiotherapy followed by surgery, or surgery alone. This study indicated a significant 5-year survival benefit for patients treated with neoadjuvant radiotherapy as compared with surgery alone (30.1% *vs* 19.8%, respectively), with an improved rate of complete curative resection after radiotherapy (80% *vs* 62%). Recently, published phase Ⅱ studies have verified the efficacy of chemoradiotherapy in terms of complete pathological response (up to 30% in some series) and increased long-term survival without an increase in morbidity or mortality[171-174].

All of the above results suggest that R0 resection is not an exclusive surgical target in locally advanced gastric cancer, but that it can be facilitated or achieved by preoperative therapy (induction of R0 resection).

Many answers are expected from ongoing trials exploring ways of improving preoperative treatment strategies for resectable gastric cancer: the MAGIC B trial (United Kingdom National Cancer Research Institute ST03 trial) of perioperative epirubicin, cisplatin, and capecitabine, with or without the endothelial growth factor antibody, bevacizumab[175]; the CRITICS trial (Chemo-Radiotherapy after Induction chemoTherapy In Cancer of the Stomach), a phase Ⅲ study that is randomizing between preoperative chemotherapy (three courses of epirubicin/cisplatin/capecitabine) and gastric surgery with

limited lymph node dissection followed by postoperative chemotherapy (another three courses of epirubicin/cisplatin/capecitabine) or chemoradiotherapy[176]; and the JCOG trial 0501 (Japan Clinical Oncology Group Study 0501 trial) and KYUH-UHA-GC04-03 Kyoto trial, which are testing preoperative oral fluoropyrimidine S-1 together with cisplatin *vs* postoperative oral fluoropyrimidine S-1[177].

CONCLUSION

In gastric cancer, radical resection (R0 resection) offers the best chance for a cure because it is defined as the complete surgical removal of any residual cancer cells in the tumor bed. However, distant and loco-regional failure rates in most radically resected patients with positive lymph nodes or involvement of the serosa contradict this statement.

All current therapeutic efforts in resectable gastric cancer are directed toward the individualization of therapeutic protocols, which tailors the extent of resection and the administration of pre- and postoperative treatment. A paradigm shift has rapidly advanced in the past 10 years: three pivotal studies in three different areas of the world (United States, Europe and Japan) have demonstrated that multimodal treatments improve the prognosis for patients with resectable gastric cancer. The common target of all of these strategies is to improve prognosis towards the achievement of a true curative resection (R0 resection) with minimal morbidity and mortality.

In gastric cancer, surgical research has always proceeded slowly, and standardization is still far from being settled. Geographical differences in epidemiology and treatment approaches and a lack of surgical gold standards have diverted attention from the pursuit of a multimodal approach. In other solid neoplasms worldwide, the multimodal approach has already been validated. In the near future, we expect the same to occur for gastric cancer, provided that the published evidence that is needed to reach this goal is further improved and developed. The result of treatment for locally advanced gastric cancer is the sum of the effect of local tumor control by surgery, with or without radiotherapy and/or systemic chemotherapy. The role of each treatment modality varies according to the stage of the disease, individual patient risk, surgical volume, available chemotherapy regimens and quality of radiotherapy. Evidence of the effect of different combinations of treatments should be established for each clinical circumstance, and surgeons should play a key role in this.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3371

World J Gastroenterol 2010 July 21; 16(27): 3371-3376
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Effect of soy saponin on the growth of human colon cancer cells

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Supported by National Scientific Council Scientific Project Grant, No. 94-2313-B-038-003
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Received: January 26, 2010 Revised: April 10, 2010
Accepted: April 17, 2010
Published online: July 21, 2010

Abstract

AIM: To investigate the effect of extracted soybean saponins on the growth of human colon cancer cells.

METHODS: WiDr human colon cancer cells were treated with 150, 300, 600 or 1200 ppm of soy saponin to determine the effect on cell growth, cell morphology, alkaline phosphatase (AP) and protein kinase C (PKC) activities, and P53 protein, c-Fos and c-Jun gene expression.

RESULTS: Soy saponin decreased the number of viable cells in a dose-dependent manner and suppressed 12-O-tetradecanol-phorbol-13-acetate-stimulated PKC activity ($P < 0.05$). Cells treated with saponins developed cytoplasmic vesicles and the cell membrane became rougher and more irregular in a dose-dependent manner, and eventually disassembled. At 600 and 1200 ppm, the activity of AP was increased ($P < 0.05$). However, the apoptosis markers such as c-Jun and c-Fos were not significantly affected by saponin.

CONCLUSION: Soy saponin may be effective in preventing colon cancer by affecting cell morphology, cell proliferation enzymes, and cell growth.

Key words: Soy saponin; Colon cancer; Apoptosis; Cell proliferation; Cell differentiation

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Tsai CY, Chen YH, Chien YW, Huang WH, Lin SH. Effect of soy saponin on the growth of human colon cancer cells. *World J Gastroenterol* 2010; 16(27): 3371-3376 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3371.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3371

INTRODUCTION

Colon cancer is one of the major causes of cancer mortality worldwide, which results from interactions of different factors such as aging, family history and dietary style. It has been suggested that consumption of higher levels of soy foods can lead to a lower incidence of acquiring colon cancer[1,2]. The most discussed compounds related to colon cancer in soy are isoflavone and saponins[3]. Saponins have been found to have biological benefits and have been utilized pharmaceutically[4]. Soy saponins are amphiphilic compounds and categorized as triterpenoic saponins. They are able to interact with the cancer cell membranes that are rich in phospholipids and cholesterol and with the hydroxyl groups on the aglycone moiety[5]. Research has found that steroid saponins extracted from fenugreek reduce the number of aberrant crypt foci in azoxytethane-induced rat colon cancer, and induce apoptosis of HT-29 human colon cancer cells[6]. However, whether soy saponins affect the growth of cancer cells by causing apoptosis or necrosis is still not clear.

In this study, we investigated the *in vitro* physical and biological effects of soy saponins on WiDr colon cancer cells, the same cell line as HT-29[7] (American Type Culture Collection, Rockville, MD, USA; Catalogue 1988), by

examining the number of living cells, cell morphology, alkaline phosphatase (AP) and protein kinase C (PKC) activities, and the expression of c-Jun, c-Fos, and P53 protein, and cell apoptosis.

MATERIALS AND METHODS

Soy saponin preparation and analysis

Saponin extraction was performed according to the method of Berhow *et al*[8]. The purity of crude saponin extracted was examined by high performance liquid chromatography (HPLC) (TSP, Germany) using commercial soy saponin as a standard (Wako, Japan). The HPLC conditions were as follows: C18 column (Vercopak, ODS-3, 4.6 mm × 250 mm); UV absorbance: 190-350 nm; analyzing temperature 30℃; flow rate: 1 mL/min; gradient solvent system: solvent A, 0.05% trifluoroacetic acid in water, solvent B, acetonitrile; 63% A to 52% A in 38 min.

Cell culture and viability

Cells were cultivated in minimal essential medium that contained 10% fetal bovine serum, sodium bicarbonate (1.5 g/L), and 1.0 mmol/L sodium pyruvate at 37℃ and 5% CO_2. Cells were subcultured into a new medium (100 mm diameter dish) when they reached a high density, at a series of dilutions from 1:3 to 1:6. When they reached 2×10^3 cells/well, cells were cultivated in each well of a 96-well plate. After stable attachment in the medium (day 0), cells were treated with five different concentrations (0, 150, 300, 600, 1200, 2400 ppm) of soy saponins (16 wells/concentration). CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA) was used to measure the viability of cells every 24 h for 3 d.

Cell morphology observation

After the cells were treated with different concentrations of soy saponins for 24 h, they were examined by electron microscopy. Scanning electron microscopy (SEM; S-2400; Hitachi, Japan) was performed to observe the differences in cell morphology. Transmission electron microscopy (TEM; H600; Hitachi) was used to investigate intracellular morphology.

Cell proliferation/differentiation measurement

Cells were cultivated in a 10-cm Petri dish. After the cells were stable, fresh medium with 0, 150, 300, 600, and 1200 ppm of soy saponins was added. Cells were cultivated for another 72 h before being subjected to tests for proliferation and differentiation. Sodium butyrate (2.5 mmol/L) was used as a positive control for detecting AP activity. The level of differentiation was measured using Alkaline Phophatase Liquicolor (Human, Germany). The activity of PKC was measured using Peptag® Assay (Promega, USA). c-Jun, c-Fos, and wild-type P53 protein expression in WiDr cells was analyzed using SDS-PAGE and western blotting. A 12% resolving gel and a 5% stacking gel were applied. β-actin (43 kDa) was used as the internal control. The antibodies used were rabbit anti-c-Jun polyclonal antibody (Stressgen, Canada), rabbit anti-c-Fos polyclonal antibody, mouse anti-P53 monoclonal antibody, and mouse anti-β-actin monoclonal antibody (Sigma, USA).

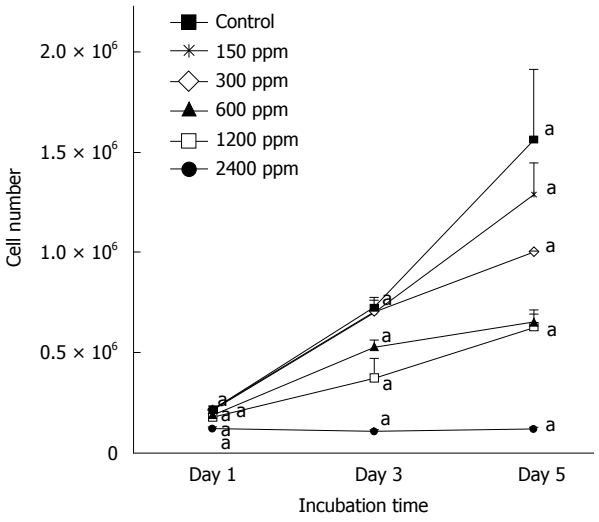

Figure 1 Effect of extracted crude soybean saponins on the growth of WiDr cells. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Values are mean ± SD. Points with letter "a" represent significant differences at the $P < 0.05$ level.

Statistical analysis

Data were expressed as mean ± SD. One-way analysis of variances and Fisher's least significant difference were performed using SAS 8.13. Differences were significant at $P < 0.05$.

RESULTS

Soy saponins and cell count

Figure 1 illustrates the dose-dependent inhibitory effect of soy saponins on the number of WiDr cells. At the end of day 1, the cell count was significantly lower in the group treated with 2400 ppm saponins compared to that in the control group ($P < 0.05$). The percentage inhibition was 40.7%. At the end of day 3, compared to the control group, the number of cells in the groups treated with 600, 1200 and 2400 ppm was significantly lower ($P < 0.05$), with percentage inhibition of 27.4%, 56.6% and 84.8%, respectively. At the end of day 5, the groups treated with 300, 600, 1200 and 2400 ppm of soy saponins had a lower cell count than the control group ($P < 0.05$), with percentage inhibition of 36.0%, 57.9%, 59.7% and 92.2%, respectively. Under light microscopic observation at the end of day 5, it was shown that cell density in the medium decreased in parallel with the increase of soy saponins in the medium (Figure 2). Figure 3 shows that under treatment with soy saponins (150-2400 ppm), the percentage cell survival was decreased in a reversed dose-dependent manner ($P < 0.05$) at each time point.

SEM of WiDr cells

Figure 4A-D shows the SEM observation of WiDr cells treated with 0, 300, 600, 1200 and 2400 ppm of soy saponins. When the dose of soy saponins increased, the

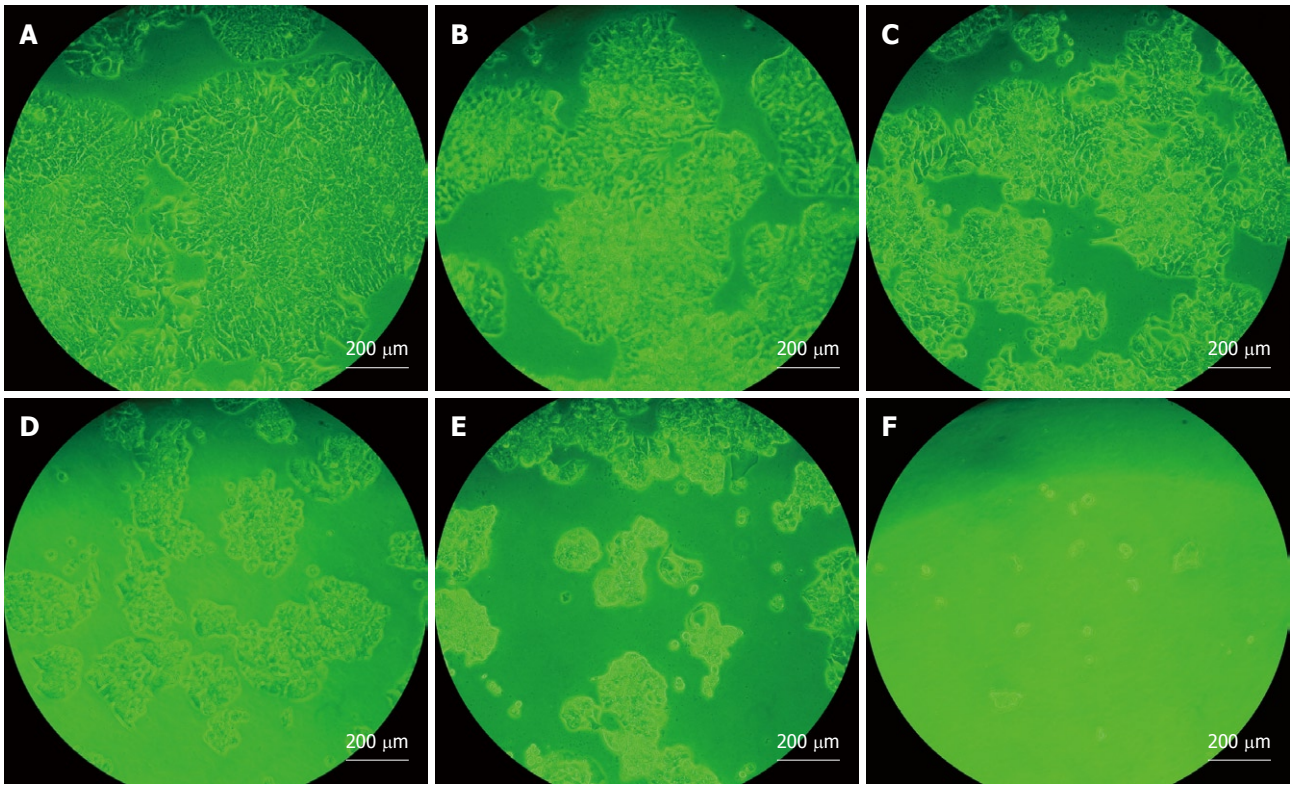

Figure 2 Cell number and morphological effects of extracted crude soybean saponins on WiDr cells. A: Untreated control culture for 5 d; B: Culture exposed to 150 ppm extracted crude soybean saponins for 5 d; C: 300 ppm; D: 600 ppm; E: 1200 ppm; F: 2400 ppm.

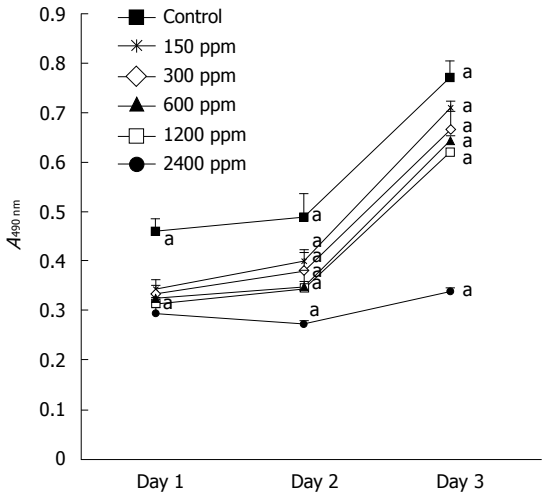

Figure 3 Effect of extracted crude soybean saponins on viability of WiDr cells. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Values are mean ± SD. Points with letter "a" represent significant differences at the $P < 0.05$ level.

surface of WiDr cells became rougher, and the cell shape changed from round to irregular. As the dose reached 1200 ppm (Figure 4C), breaks were seen on the surface of WiDr cells. At 2400 ppm soy saponin, complete deformation of WiDr cells was observed (Figure 4D).

Activity of AP

Figure 5 shows that soy saponins induced AP activity in WiDr cells in a dose-dependent manner ($P < 0.05$). The WiDr cell line is one of the colon cancer cell lines without AP activity. The control sample with sodium butyrate showed increased AP activity, while the one without sodium butyrate did not. The activated AP indicated that WiDr cancer cells might slow down the proliferation process but shift toward the differentiation process.

Activity of PKC

The effect of soy saponin on PKC activity is shown in Figure 6. 12-O-tetradecanoyl phorbol-13-acetate (TPA) was added to the medium to induce PKC activity. The medium without TPA showed no PKC activity. As the dose of saponins in the medium increased, the inhibitory effect on PKC increased.

Expression of P53, c-Jun, and c-Fos

There was no significant difference in the expression of P53 and c-Fos proteins between the groups with/without soy saponin treatment (data not shown). On the other hand, Figure 7 shows a trend towards an inhibitory effect of saponins on the expression of c-Jun after 3 d of incubation.

DISCUSSION

In this study, we investigated the effects of soy saponin on cell growth, proliferation/differentiation-related enzyme activities, and the expression of apoptosis-related proteins of WiDr human colon cancer cells. We found that soy saponins effectively inhibited the growth rate and survival

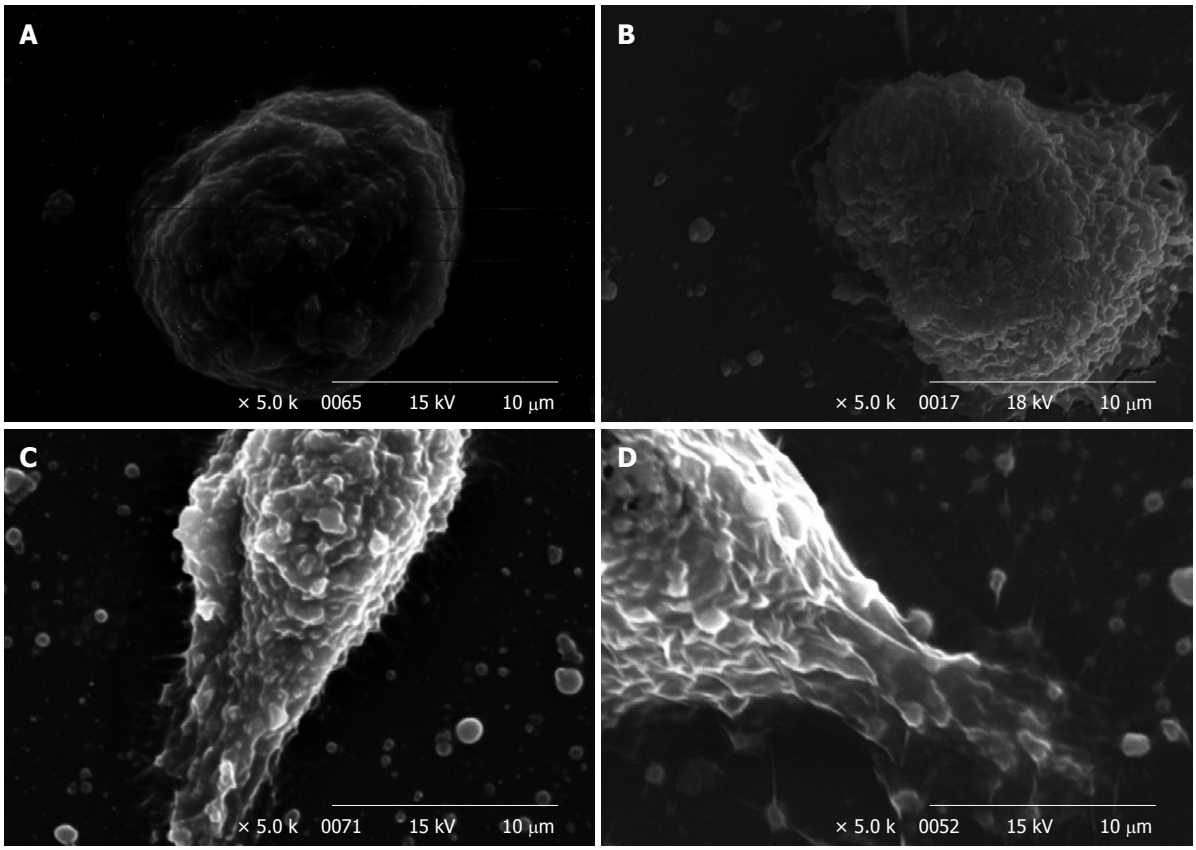

Figure 4 Scanning electron microscopy electron micrographs of WiDr cells. Cells were treated with 0 (A), 300 (B), 1200 (C) and 2400 ppm (D) soy saponin, for 1 d.

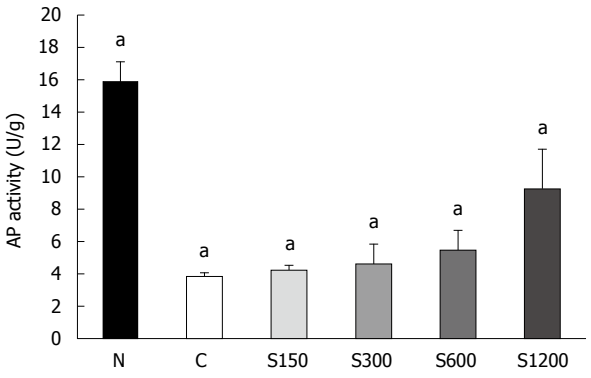

Figure 5 Effect of extracted crude soybean saponins on alkaline phosphatase activity of WiDr cells. N: Culture exposed to 2.5 mmol/L sodium butyrate for 3 d; C: Untreated control culture for 3 d; S150: Culture exposed to 150 ppm extracted crude soybean saponins for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Different concentrations of saponin at each incubation time were compared using one way analysis of variances and Fisher's least significant difference test. Values are mean ± SD. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

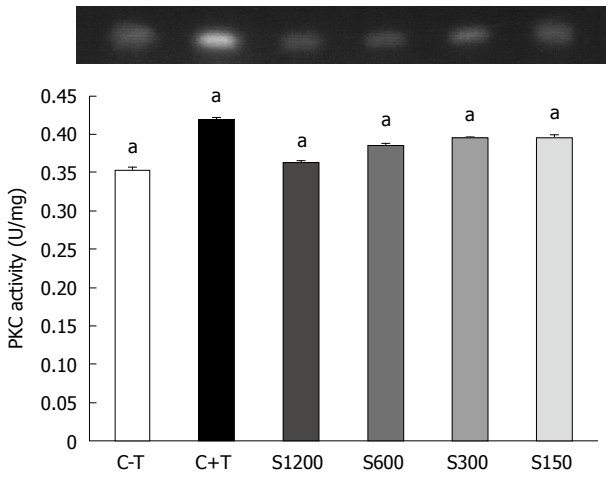

Figure 6 Effect of extracted crude soybean saponins on protein kinase C activity of WiDr cells. C-T: Untreated control culture for 3 d; C+T: Control culture + 100 ng/mL tetradecanoyl phorbol-13-acetate (TPA); S150: Culture exposed to 150 ppm extracted crude soybean saponins + 100 ng/mL TPA for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Values are mean ± SD. Different concentrations of saponin at each incubation time were compared using one way analysis of variances and Fisher's least significant difference test. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

of human colon cancer cells in a dose-dependent manner. Soy saponins are amphiphilic compounds that can be used as bio-surfactants. They are structurally similar to oleanolic acid and ursolic, which have been shown to be glucocorticoid receptors with anti-carcinogenic activity[9,10].

PKC is one of the markers for cell proliferation. PKC activity increases as the cells undergo the proliferation process. As shown in our study, the addition of soy saponin effectively inhibited the activity of PKC in a dose-dependent manner. On the other hand, P53 protein is responsible for regulating some reactions such as the cell cycle, DNA repair, and apoptosis[11,12]. The relationship between P53 protein and the HT-29 cell death is still not clear[13]. Shen *et al*[14] have found that 2'-OH flavanone inhibits the growth of HT-29 cells *via* increasing the expres-

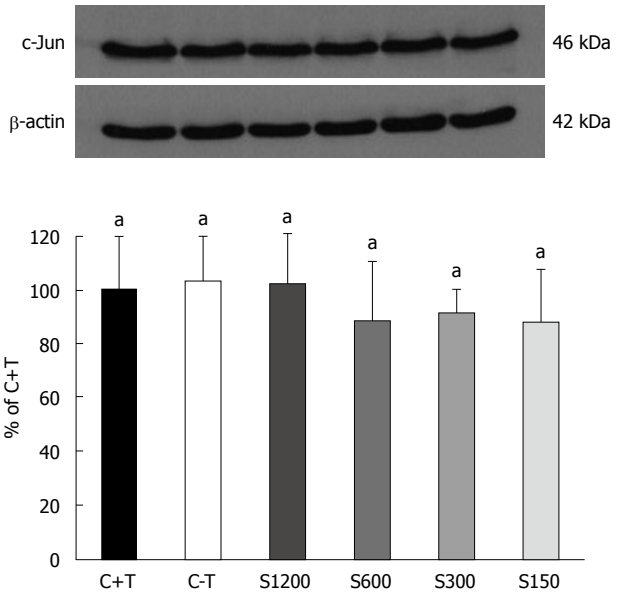

Figure 7 Effect of crude soybean saponins extracted on c-Jun (46 kDa) expression of WiDr cells. C+T: Control culture + 100 ng/mL tetradecanoyl phorbol-13-acetate (TPA); C-T: Untreated control culture for 3 d; S150: Culture exposed to 150 ppm extracted crude soybean saponins + 100 ng/mL TPA for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Values are mean ± SD. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

sion of P21, but it has no effect on P53 protein. In our study, we did not find any inhibitory effect of soy saponin on the P53 protein of WiDr cells.

Under normal conditions, cells proliferate to a certain level and then differentiate to different kinds of cells. If the cells are stimulated by some exogenous factors, for example, carcinogens, cells may not differentiate, proliferate abnormally, and form tumors. In our study, compared to the control group, the cell number was decreased and AP activity was increased by addition of soy saponins, which is an indication of cell differentiation. It has been shown that materials such as vitamin D3, with membranolic actions, can regulate the transportation of Ca^{2+} ions through the membrane and induce cell differentiation[15]. Our SEM results showed that cell morphology was changed by saponins, with a similar membranolic effect. Soy saponins may also promote cell differentiation if the cell membrane has not been destroyed by too high a concentration.

Programmed cell death can be categorized into two types, type I apoptosis and type Ⅱ autophagic death[16-18]. The major differences in these two types are that, in apoptosis, cells die individually, and phagocytes are necessary for cell degradation. On the other hand, in autophagic death, cells die in groups through a lysosomal mechanism, in which vacuoles are observable in cells[17]. In our SEM study, cell membranes became rough and wrinkled when treated with high concentrations of saponins. In addition, under TEM observation (Figure 8), vacuoles appeared in the cells that had been treated with higher concentrations of saponins, which may be an indication of type Ⅱ autophagic death. It has been found that the level of microtubule-associated protein light chain 3 is increased after

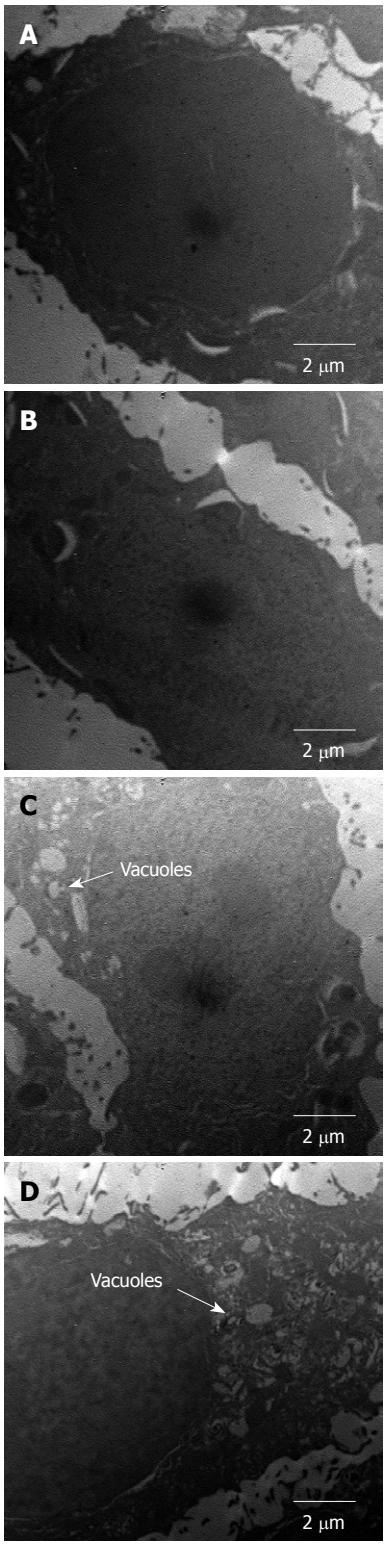

Figure 8 Transmission electron microscopy electron micrograph of WiDr cells treated with 150 (A), 300 (B), 600 (C), and 1200 (D) ppm of extracted crude soybean saponins for 1 d. At 600 and 1200 ppm of saponin, vacuoles were observable.

saponin treatment[19], which is an indication of type Ⅱ autophagic death. For these reasons, the inhibitory effect of soy saponins on WiDr cells may not be apoptosis, but rather autophagic death at higher concentrations.

In conclusion, we found that soy saponins changed the membrane structure and affected the growth of WiDr

cells in two different ways; by increasing the AP activity while reducing PKC activity to induce cell differentiation at lower concentrations, or by inducing type Ⅱ autophagic death at higher concentrations. This may need further investigation.

COMMENTS

Background

Colon cancer is one of the major causes of cancer mortality worldwide. Soy saponins are categorized as amphiphilic compounds, and may be able to react with the phospholipids and cholesterol on the membrane of cancer cells, and with the hydroxyl groups on the aglycone moiety.

Research frontiers

Steroid saponins extracted from fenugreek reduced the number of colon aberrant crypt foci in azoxymethane-induced rat colon cancer and induced apoptosis of HT-29 human colon cancer cells. However, how soy saponins affect the growth of cancer cells is still not clear. In this study, the authors investigated the *in vitro* physical and biological effects of soy saponins on WiDr colon cancer cells.

Innovations and breakthroughs

Recent studied have suggested that saponins affect the growth of colon cancer cells. This is believed to be the first thorough study that has focused on the relationship between biomarkers of apoptosis, such as expression of c-Jun, c-Fos, and P53 protein, and cell morphology, proliferation, and differentiation.

Applications

By understanding how soy saponins affect colon cells, this study may represent a future strategy for prevention or treatment of colon cancer.

Peer review

The authors investigated the inhibitory effects of soy saponins on colon cancer cells. Soy saponins inhibited the growth of colon cancer cells by reducing protein kinase C activity, while the features of type Ⅱ programmed cell death (autophagic death) was observed. It is a well written paper with promising results that may be the basis of forthcoming research in cancer biology and therapy.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3377

World J Gastroenterol 2010 July 21; 16(27): 3377-3384
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Galangin induces apoptosis of hepatocellular carcinoma cells *via* the mitochondrial pathway

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Supported by The administration of traditional Chinese medicine of Guangdong province, China, No. 1050047
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Received: March 21, 2010 Revised: April 23, 2010
Accepted: April 30, 2010
Published online: July 21, 2010

Abstract

AIM: To investigate the mechanism by which galangin, a polyphenolic compound derived from medicinal herbs, induces apoptosis of hepatocellular carcinoma (HCC) cells.

METHODS: The 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay was used to measure cell viability. Apoptosis was evaluated by *in situ* uptake of propidium iodide and Hoechst 33258 and was then detected by fluorescence microscopy. Protein expressions were detected by Western blotting. To confirm the apoptotic pathway mediated by galangin, cells were transfected by *bcl-2* gene to overexpress Bcl-2 or siRNA to down-regulate Bcl-2 expression.

RESULTS: Galangin (46.25-370.0 μmol/L) exerted an anti-proliferative effect, induced apoptosis, and decreased mitochondrial membrane potential in a dose and time-dependent manner. Treatment with galangin induced apoptosis by translocating the pro-apoptotic protein Bax to the mitochondria, which released apoptosis-inducing factor and cytochrome c into the cytosol. Overexpression of Bcl-2 attenuated galangin-induced HepG2 cell apoptosis, while decreasing Bcl-2 expression enhanced galangin-induced cell apoptosis.

CONCLUSION: Our data suggests that galangin mediates apoptosis through a mitochondrial pathway, and may be a potential chemotherapeutic drug for the treatment of HCC.

Key words: Hepatocellular carcinoma; Galangin; Mitochondria; Bcl-2; Apoptosis

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Zhang HT, Luo H, Wu J, Lan LB, Fan DH, Zhu KD, Chen XY, Wen M, Liu HM. Galangin induces apoptosis of hepatocellular carcinoma cells *via* the mitochondrial pathway. *World J Gastroenterol* 2010; 16(27): 3377-3384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3377.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3377

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in China. Surgical therapy, chemotherapy, and radiation have been used for the treatment of HCC. However, HCC remains one of the

more difficult cancers to treat. Although chemotherapy is a common therapeutic strategy after surgery, its use has been toxic to normal tissues and limits their use. Therefore, it is important to screen for new anti-cancer drugs.

Recent studies show that a number of dietary compounds possess anti-cancer properties[1], including curcumin, genistein, quercitin, resveratrol, piceatannol and pterostilbene. These dietary compounds may enhance the efficacy of chemotherapeutic agents by modifying the activity of specific targets that control cell proliferation and apoptosis such as Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis protein[2], Akt[3], c-myc, neuronal apoptosis inhibitory protein, c-IAP-2, nuclear factor-κB[4], survivin, p21WAF1 and p53[5].

Galangin (4H-1-Benzopyran-4-one, 3,5,7-trihydroxy-2-phenyl-), a flavonoid, is a polyphenolic compound derived primarily from medicinal herbs, including Alpinia officinarum Hance, Alnus pendula Matsum, Plantago major L, and Scutellaria galericulata L. (S. scrodifolia Fisch.). Eaton *et al*[6] demonstrated that galangin could inhibit the methoxyresorufin *O*-demethylase activity of CYP1A2, CYP1A1 and P-form phenolsulfotransferase. These observations suggest galangin may be a potential chemopreventive agent in sulfation-induced carcinogenesis.

Furthermore, Moon has reported that galangin had cancer preventive properties and induced apoptosis in several cancer cell lines[7]. Another study showed that galangin could induce a G0-G1 cell cycle arrest, modulate the expression of cycline/cdk, and decrease Bcl-2 levels, which leads to apoptosis of imatinib-resistant Bcr-Abl expressing chronic myelogenous leukemia cell lines[8].

Based on these previous reports, it was suggested that galangin could inhibit cell proliferation and induce apoptosis. Therefore, galangin may be a potential anti-tumor agent. However, the mechanism by which galangin exert its anti-tumor activity is unknown. In this study, we demonstrate the effects of galangin on HCC cells and elucidate the mechanism by which galangin induces apoptosis.

MATERIALS AND METHODS

Cell culture

Three human liver cancer cell lines (HepG2, Hep3B and PLC/PRF/5) were obtained from American type culture collection (Rockville, MD, USA) and kept in Institute of Biochemistry and Molecular Biology, Guangdong Medical College. All cell lines were cultured in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) containing penicillin (100 μg/mL) and streptomycin (100 μg/mL) and supplemented with 10% fetal bovine serum (Sijiqing Laboratories, Hangzhou, China) at 37℃ in a humidified atmosphere with 5% CO_2.

Agents and chemicals

Galangin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the culture medium below 0.1% (v/v). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Hoechst 33258, propidium iodide, rhodamine 123 [2-(6-amino-3-imino-3*H*-xanthen-9-yl) benzoic acid methyl ester] were purchased from Sigma (St. Louis, MO, USA). Rabbit or goat polyclonal antibodies against Bax, caspase-3, caspase-9, cytochrome c, apoptosis-inducing factor (AIF), PAPR, Vox Ⅳ, GAPDH and Bcl-2 were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA).

MTT viability assay

HCC cells (5.0×10^3) were seeded and treated with different concentrations of galangin or different times in 96-well plates. The number of viable cells in each well was determined by adding 10 μL 5 mg/mL MTT solution. The cells were dissolved with 100 μL of solution that contained 20% SDS and 50% dimethyl formamide after cells had been incubated for 4 h at 37℃. The optical densities were quantified at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Varioskan Flash Reader spectrophotometer (THERMO, MA, USA)[9].

Cell apoptosis analysis by fluorescence staining

The HCC cells were cultured in 6-well plates (3.0×10^5 cells/well) and treated with different concentrations of galangin at 37℃ in a humidified atmosphere with 5% CO_2 for 24 h. Cell apoptosis was evaluated by *in situ* uptake of propidium iodide and Hoechst 33258 as described by McKeague *et al*[10]. Briefly, galangin-treated cells were washed with phosphate buffered saline (PBS), and incubated in PBS containing 40 μg/mL propidium iodide and 2.5 μg/mL Hoechst 33258 for 10 min. Five hundred microliters of methanol: acetic acid (3: 1) fixative were then added directly to each well. Cells were viewed under fluorescence microscopy (Nikon Eclipse ET2000-E, Japan). The apoptotic index was calculated from the number of apoptotic nuclei *vs* total number of nuclei at each visual field.

Measurement of mitochondrial membrane potential[11]

HCC cells were treated with different concentrations of galangin for different times. Cells were then treated with rhodamine 123 with a final dye concentration of 10 μg/mL at 37℃ for 15 min, 5% CO_2 atmosphere prior to examination. Mitochondrial membrane potential was determined by flow cytometry. The change of fluorescent intensity of rhodamine 123 indicated the change in mitochondrial membrane potential.

Overexpression and knockdown of Bcl-2

The HCC cells were transfected with different plasmids [pcDNA3.1(+)-*Bcl-2*, pcDNA3.1(+)Vectors] using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Bcl-2 siRNA sequence was synthesized according to Fu's report[12], sense sequence: 5'-CGGAGGCUGGGAUGCCUUUdTdT-3', antisense sequence: 3'-dTdTGCCUCCGACCCUACGGAAA-5'. Before transfection, cells were seeded in 6 well plates or 60 mm tissue culture dishes containing DMEM medium without antibiotics for 24 h. Cells were transfected using lipofectamine 2000 with 2 μg plasmid or 100 pmol siRNA in each well. Bcl-2 protein level was measured by immu-

noblot analysis 24 h post-transfection. Cells were treated with galangin for another 24 h, and viability was determined by MTT and fluorescence staining methods.

Preparation of proteins in the mitochondrial and cytosolic fractions

Twenty four hours after treating with different concentrations of galangin, cells were washed twice in ice-cold PBS and resuspended in five volumes of ice-cold extract buffer (20 mmol/L Hepes-KOH, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 0.1 mmol/L PMSF, pH 7.5). The resuspended cells were homogenized and centrifuged at 750 *g* for 10 min at 4℃. The supernatants were centrifuged at 13000 *g* for 15 min at 4℃ to obtain the mitochondrial pellets. The remaining supernatants were centrifuged to obtain the cytosolic fractions. The protein concentrations of the resulting supernatants and mitochondrial fractions were measured.

Western blotting

The cells were loaded with cell decomposition buffer (pH 8.0) that contained 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 0.05% phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin (Sigma, USA), and 2 μg/mL leupeptin (Sigma, USA). The proteins were determined as described previously by Western blotting[9] using the antibody (Santa Cruz Biotechology, Santa Cruz, CA, USA), and Western blotting luminal reagent (Amersham Biosciences, Uppsala, Sweden).

Statistical analysis

The values given are presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance with LSD test. In all cases, $P < 0.05$ was considered as significant.

RESULTS

Galangin inhibits proliferation of HCC cells

We used the MTT assay to determine the effects of galangin on the proliferation of HCC cells. Using galangin at concentrations of 46.25, 92.5, 185 and 370 μmol/L, we observed an anti-proliferative effect on HCC cells that was dose-dependent (Figure 1A). Additionally, galangin could also inhibit HCC cell proliferation in a time-dependent manner (Figure 1B). The IC_{50} of galangin to HCC cells (HepG2, Hep3B, and PLC/PRF/5) 24 h post-treatment were 134.0, 87.3 and 79.8 μmol/L, respectively.

Galangin induces apoptosis of HCC cells

To determine whether galangin-treated HCC cells undergo apoptosis, we stained cells using Hoechst 33258/PI. As shown in Figure 2A, we observed a significant increase in the number of cells that exhibited nuclear condensation when treated with galangin for 24 h. This observation was similarly found in all three HCC cell lines tested. Our data also showed that the apoptotic index of the three HCC cells increased in a dose-dependent manner treated by galangin (Figure 2B).

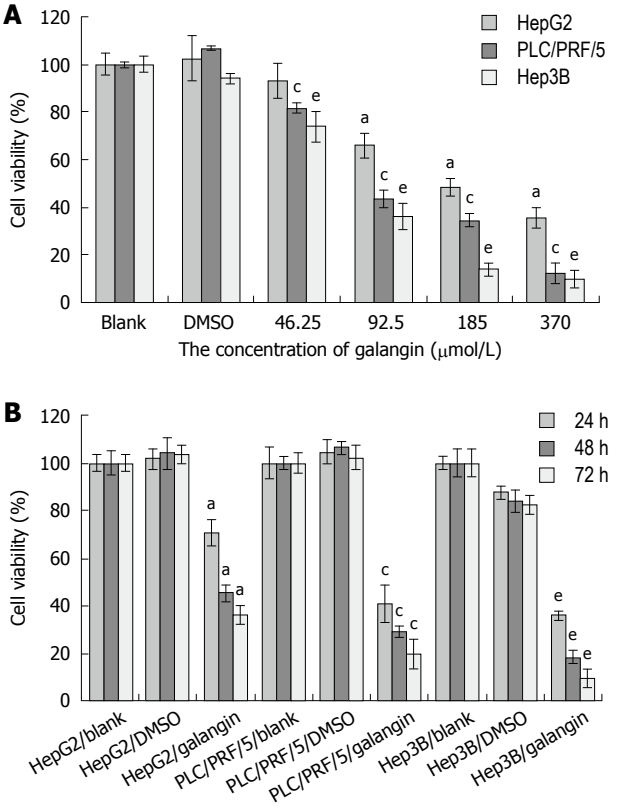


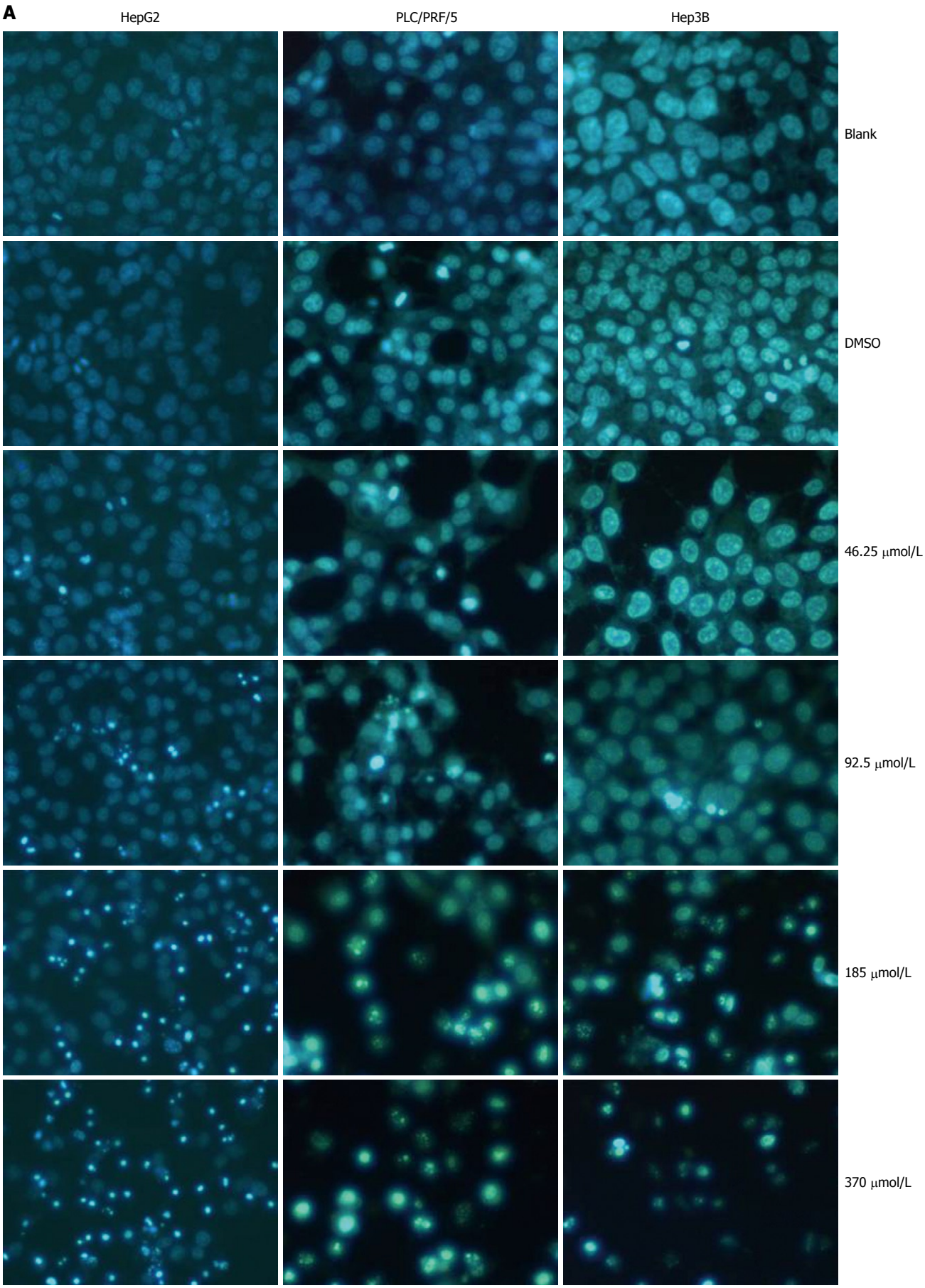
Figure 1 Effects of galangin on cell viability of three hepatocellular carcinoma cell lines. A: Three hepatocellular carcinoma (HCC) cell lines were treated with 46.25, 92.5, 185, and 370 μmol/L galangin for 24 h. The IC_{50} of galangin to HepG2, Hep3B, and PLC/PRF/5 cells were 134.0, 87.3 and 79.8 μmol/L, respectively; B: Three HCC cell lines were treated with 92.5 μmol/L galangin for 24, 48 and 72 h. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^cP < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^eP < 0.05$ *vs* Hep3B/DMSO cell/DMSO-treatment group.

Galangin reduces the mitochondrial membrane potential of HCC cells

We observed a reduction in the mitochondrial membrane potential of all three HCC cell lines after treating with galangin (Figure 3). The mitochondrial membrane potential of the HCC cells decreased following treatment with galangin and this occurred in a dose- and time-dependent manner.

Galangin affects protein levels involved in the apoptosis pathway of HCC cells

In Figure 4, we evaluated the effects of galangin on protein expression involved in apoptosis of the HCC cell lines. Translocation of Bax to the mitochondria can alter the outer mitochondrial membrane permeability. Some pro-apoptotic proteins, including cytochrome c and AIF, are released into the cytosol from the mitochondria. Our data shows that Bax levels in the mitochondrial fraction of galangin-treated HCC cells increased in a dose-dependent manner, suggesting that the translocation of Bax into the mitochondria was involved in cell death induced by galangin (Figure 4A). Furthermore, we observed that the levels of cytochrome c and AIF in the cytosol of galangin-treated HCC cells increased in a dose-dependent manner. This suggests that the mitochondrial release of


A
HepG2
PLC/PRF/5
Hep3B
Blank
DMSO
46.25 μmol/L
92.5 μmol/L
185 μmol/L
370 μmol/L

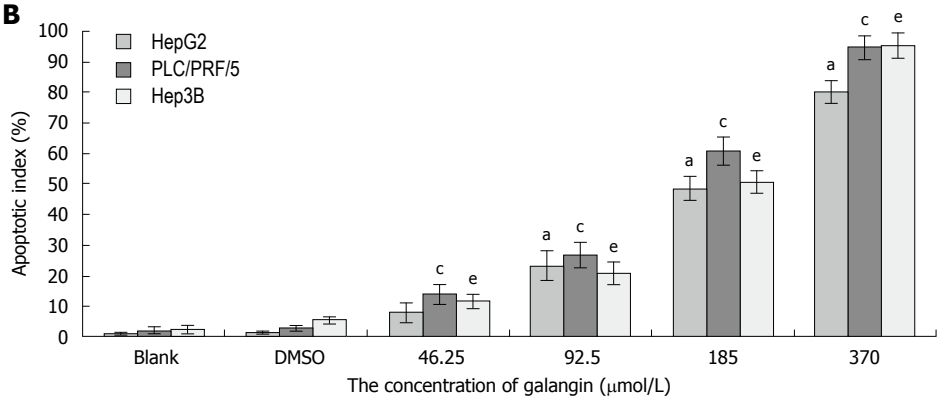


Figure 2 Hepatocellular carcinoma cells apoptosis induced by galangin. A: Morphology of apoptotic cells, pictures were taken under a 20 × objective; B: Cells were treated with different concentrations of galangin for 24 h and stained with Hoechst 33258/PI to measure apoptosis. mean ± SD. $n = 4$. $^{a}P < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^{c}P < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^{e}P < 0.05$ *vs* Hep3B/DMSO cell/DMSO-treatment group.

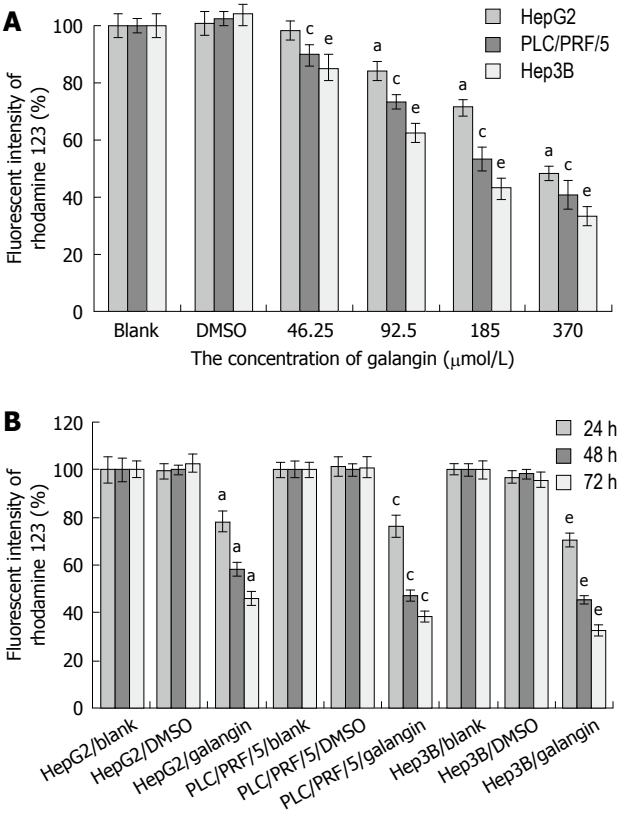


Figure 3 Effect of galangin on the mitochondrial membrane potential of hepatocellular carcinoma cell lines. A: Three hepatocellular carcinoma (HCC) lines were treated with 46.25, 92.5, 185, and 370 μmol/L galangin for 24 h; B: Three HCC were treated with 92.5 μmol/L galangin for 24, 48 and 72 h. mean ± SD. $n = 4$. $^{a}P < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^{c}P < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^{e}P < 0.05$ *vs* Hep3B/DMSO-treatment group.

cytochrome c and AIF into the cytosol may play a role in induction of cell apoptosis by galangin (Figure 4B). We also analyzed the effects of galangin on caspase-3 and caspase-9 activation, and poly(ADP-ribose) polymerase (PARP) cleavage by Western blotting. We showed caspase-9 activation in a dose-dependent manner, and observed a concomitant increase of caspase-3 activation and PARP cleavage (Figure 4C).

Bcl-2 protein level affects galangin-induced HepG2 cell apoptosis

To determine the role of Bcl-2 in galangin-treated HepG2 cells, we overexpressed and downregulated Bcl-2 by transfecting cells with pcDNA3.1-Bcl-2 and siRNA targeting Bcl-2, respectively. In Figure 5A, we show that we were able to overexpress and knockdown Bcl-2 sufficiently. As shown in Figure 5B and C, HepG2 cells overexpressing Bcl-2 were more resistant to galangin-induced apoptosis than control cells ($P < 0.05$). However, Bcl-2-knockdown HepG2 cells were more sensitive to galangin leading to decreased cell survival ($P < 0.05$) (Figure 5D). Taken together, these observations suggest that galangin induces apoptosis of HepG2 cell apoptosis *via* the mitochondrial pathway.

DISCUSSION

Studies have showed that most flavonoids exhibit anti-proliferative effects against tumor derived cell lines including leukemia[13], melanoma[14], colon[15], breast carcinoma[16], lung, and prostate[17]. Some reports have demonstrated that galangin is a naturally occurring non-toxic flavonoid with chemopreventive and anti-proliferative effects[6-8,18,19].

In this study, we demonstrated that galangin inhibited the proliferation of HCC cells and induced apoptosis at concentrations as low as 46.25 μmol/L and in excess of 185 μmol/L, respectively. Galangin-induced apoptosis was characterized by analyzing the effects on caspase-3 activation, PARP cleavage, and DNA condensation in HCC cells.

The mitochondrial pathway is commonly involved in the death stimuli. There are primarily two major events involved in apoptosis *via* the mitochondrial pathway. The first event is a change in mitochondrial membrane permeability, which leads to decreased mitochondrial membrane potential. Our data demonstrated reduced mitochondrial membrane potential as indicated by rhodamine 123 staining following treatment with galangin at different concentrations. The second event in the mitochondria-induced apoptotic pathway is the release of cytochrome c and AIF from the intermembrane space of the mitochondria into the cytosol. Here, we also showed that galangin increased

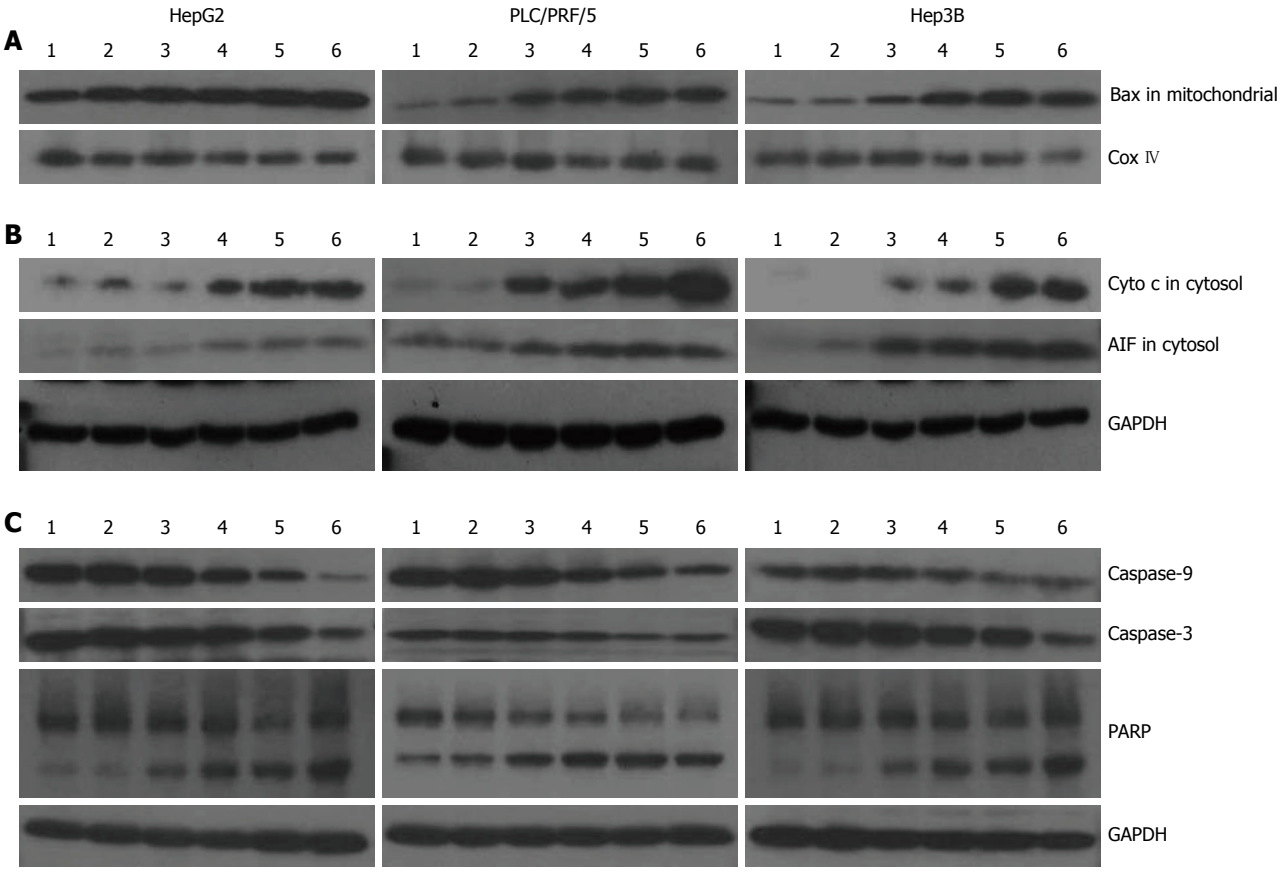


Figure 4 Effects of galangin-treated hepatocellular carcinoma cells on protein expression involved in the mitochondria pathway. A: The changes of Bax in the mitochondria; B: The changes of cytochrome c and apoptosis-inducing factor (AIF) in the cytosol; C: Caspase-3 and caspase-9 activation, and Poly(ADP-ribose) polymerase (PARP) cleavage induced by galangin. 1: Blank control group; 2: Dimethyl sulfoxide-treatment groups; 3: Cells were treated with 46.25 μmol/L galangin for 24 h; 4: Cells were treated with 92.5 μmol/L galangin for 24 h; 5: Cells were treated with 185 μmol/L galangin for 24 h; 6: Cells were treated with 370 μmol/L galangin for 24 h.

the release of cytochrome c and AIF in the cytosol. Thus, our data indicates that galangin-induced apoptosis of HCC cells occurs through the mitochondrial pathway.

The Bcl-2 family of proteins is involved in the mitochondrial apoptotic pathway by inducing the release of cytochrome c from the mitochondrial intermembrane space. Cytochrome c cooperates with Apaf-1 (Apoptotic protease activating factor 1) to induce caspase activation, leading to cell apoptosis[20]. The Bcl-2 family proteins are categorized into three groups based on the four Bcl-2 homology domains (BH1-4 domains). Bcl-2, Bcl-w, Bcl-xL and Mcl-1, which contain BH domains 1-4 and are localized to the outer mitochondrial membrane, are anti-apoptotic Bcl-2 proteins[21]. These proteins can directly bind and inhibit the proapoptotic Bcl-2 family in the mitochondria pathway of apoptosis. The proapoptotic proteins of Bcl-2 family members are functionally divided into two classes. One class is the effector molecule, which includes Bak and Bax, and permeabilizes the outer mitochondrial membrane to release cytochrome c into the cytosol. The other class is the BH3-only proteins including Bad, Bid, Bik, Bim, Bmf, bNip3, Hrk, Noxa and Puma, which promote cell apoptosis through protein-protein interactions with other Bcl-2 family members[21].

Our data indicates that galangin causes Bax translocation to the mitochondria in HCC cells. In non-apoptotic cells, Bax is located in the cytosol or loosely bound to the outer membrane of the mitochondria in monomeric forms. However, Bax translocates to the mitochondrial membrane and homodimerizes in the presence of a death signal. As a result, the outer mitochondrial membrane is permeable to release cytochrome c and AIF into the cytosol. The release of cytochrome c, which is an important protein in the electron transfer chain, can lead to reduced mitochondria membrane potential and adenosine triphosphate (ATP) synthesis. The results of our experiments showed that galangin induces cytochrome c release and decreases mitochondrial membrane potential.

In the cytosol, cytochrome c can bind to Apaf-1, which is a cytosolic protein. Apaf-1 undergoes a conformational change upon binding to dATP or ATP, leading to the formation of the apoptosome complex. The apoptosome recruits procaspase-9, resulting in caspase 9-caspase 3 activation. This caspase cascade is responsible for the hydrolysis of key cytoplasmic proteins and for the cleavage of genomic DNA nucleosomes into 180 bp fragments *via* caspase-activated DNase, such as PARP. Caspase-3 is an executioner of apoptosis that subsequently cleaves many important intracelluar substrates, leading to chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine, and formation of apoptotic bodies[22]. Our data also shows galangin-treated HCC cells did indeed cause caspase-9 and caspase-3 activation, and PARP cleavage.

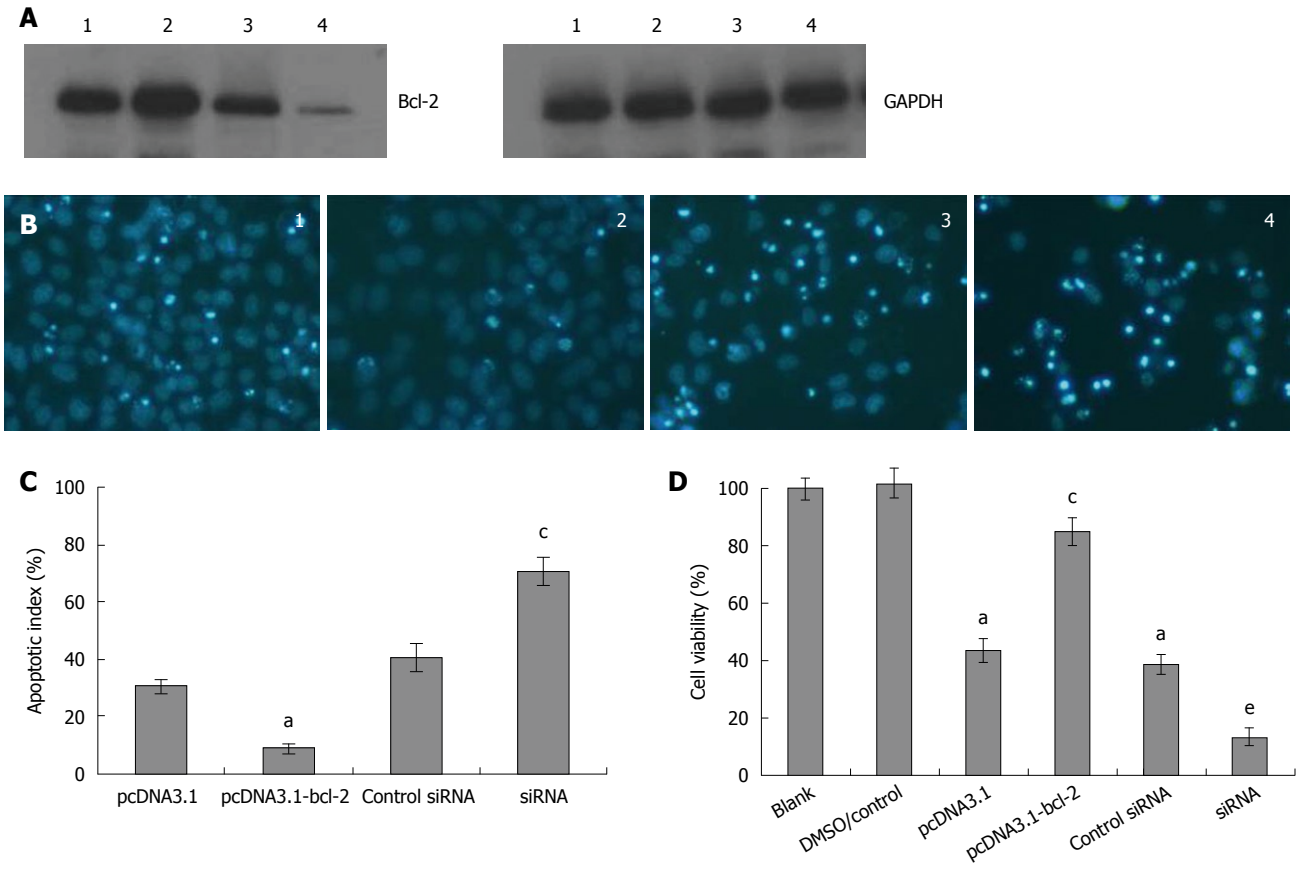


Figure 5 Effects of Bcl-2 on galangin-induced HepG2 cell apoptosis. A: Bcl-2 protein level was measured in HepG2 cells by Western blotting after cells were transfected; B: Morphology of apoptotic cells. Cells were treated with 185 μmol/L galangin for 24 h and stained with Hoechst 33258/PI to measure apoptosis. Pictures were taken under a 20 × objective. 1: Control vector group; 2: pcDNA3.1-bcl-2-transfected group; 3: Control siRNA group; 4: Bcl-2 siRNA-transfected group; C: Bcl-2 level affect apoptotic index of HepG2 cell induced by galangin. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* pcDNA3.1 group; $^cP < 0.05$ *vs* control siRNA group; D: The effect of Bcl-2 level on cell viability induced by galangin. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide-treatment group; $^cP < 0.05$ *vs* pcDNA3.1-transfected group; $^eP < 0.05$ *vs* control siRNA group.

In our study, overexpression of Bcl-2 could suppress the apoptotic effects of galangin on HCC cells. Bcl-2 is an important anti-apoptotic protein that suppresses different drug-induced activation of the mitochondria-apoptotic pathway, such as etoposide[23], berberine[24], safatoposide[25], epigallocatechin-3-gallate[26], curcumin[27] and anti-inflammatory drugs[28]. The Bcl-2 protein can block the oligomerization of Bax and Bak and inhibit the apoptotic program[29,30]. Moreover, our data also showed that Bcl-2 decrease could enhance HCC cell sensitivity to galangin. These results show that Bcl-2 can modulate the effects of galangin on HCC cells and indicate that galangin induces apoptosis *via* the mitochondrial pathway.

We demonstrated that AIF is released from mitochondria into the cytosol in HCC cells upon galangin treatment. AIF migrates into the nucleus and induces high-molecular-mass DNA fragmentation and marginal chromatin condensation independent of caspases[31,32]. Therefore, HCC cells undergoing apoptosis may be due to a combination of caspase activation and AIF release.

In summary, we demonstrate that galangin induces HCC cell apoptosis *via* the mitochondrial pathway. Our data demonstrated that (1) galangin induces HCC cell apoptosis by triggering Bax translocation to the mitochondria; (2) galangin-treated HCC cells causes the release of AIF and cytochrome c into the cytosol from the mitochondria; and (3) overexpression of Bcl-2 attenuated galangin-induced HepG2 cells apoptosis, while down-regulated Bcl-2 expression enhanced galangin to induce cell apoptosis.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in China. However, HCC remains one of the more difficult cancers to treat. It is important to screen for new anti-cancer drugs. Dietary compounds possess anti-cancer properties.

Research frontiers

A number of dietary compounds possess anti-cancer properties. These dietary compounds may modify the activity of specific targets that control cell proliferation and apoptosis. Galangin could inhibit the methoxyresorufin *O*-demethylase activity of CYP1A2, CYP1A1 and P-form phenolsulfotransferase. Galangin induced apoptosis in several cancer cell lines and arrested the cell cycle, modulated the expression of cycline/cdk, and decreased Bcl-2. It was suggested that galangin may be a potential anti-tumor agent. However, the mechanism by which galangin exerts its anti-tumor activity is unknown.

Innovations and breakthroughs

In this study, the authors demonstrate the effects of galangin on HCC cells and elucidate the mechanism by which galangin induces apoptosis. This is the first study to report that galangin mediates apoptosis through a mitochondrial pathway. Galangin may be a potential chemotherapeutic drug for the treatment of hepatocellular carcinoma cells.

Applications

Understanding the mechanism by which galangin induces apoptosis may lead

to its use as an anti-cancer treatment of HCC. This study may represent a future potential chemotherapeutic drug in the treatment of HCC with galangin.

Terminology

The mitochondrial pathway is an important apoptotic pathway involved in the mitochondrial membrane permeability change and the release of cytochrome c and apoptosis-inducing factor (AIF) from the intermembrane space of the mitochondria into the cytosol. Bcl-2 is an important anti-apoptotic protein that suppresses different drug-induced activation of the mitochondria-apoptotic pathway.

Peer review

In this study, Zhang *et al* demonstrated that galangin, a polyphenolic compound from herbs, induced apoptosis in hepatocellular carcinoma cells through the mitochondrial pathway. Galangin treatment led to inhibition of cell growth, nuclear fragmentation, mitochondrial membrane potential collapse, caspase activation, Bax mitochondrial translocation, and release of cytochrome c and AIF. Modulation of Bcl-2 by overexpression or knockdown altered galangin-induced apoptosis. The results are convincing and the study is informative. The manuscript could be improved by making several changes.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3385

World J Gastroenterol 2010 July 21; 16(27): 3385-3393
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

8-bromo-7-methoxychrysin-induced apoptosis of hepatocellular carcinoma cells involves ROS and JNK

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Supported by Hunan Provincial Natural Science Foundation of China, No. 03JJY5009
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Received: February 2, 2010 Revised: April 23, 2010
Accepted: April 30, 2010
Published online: July 21, 2010

Abstract

AIM: To investigate whether the apoptotic activities of 8-bromo-7-methoxychrysin (BrMC) involve reactive oxygen species (ROS) generation and c-Jun N-terminal kinase (JNK) activation in human hepatocellular carcinoma cells (HCC).

METHODS: HepG2, Bel-7402 and L-02 cell lines were cultured *in vitro* and the apoptotic effects of BrMC were evaluated by flow cytometry (FCM) after propidium iodide (PI) staining, caspase-3 activity using enzyme-linked immunosorbent assay (ELISA), and DNA agarose gel electrophoresis. ROS production was evaluated by FCM after dichlorodihydrofluorescein diacetate (DCHF-DA) probe labeling. The phosphorylation level of JNK and c-Jun protein was analyzed by Western blotting.

RESULTS: FCM after PI staining showed a dose-dependent increase in the percentage of the sub-G1 cell population ($P < 0.05$), reaching 39.0% ± 2.8% of HepG2 cells after 48 h of treatment with BrMC at 10 μmol/L. The potency of BrMC to HepG2 and Bel-7402 (32.1% ± 2.6%) cells was found to be more effective than the lead compound, chrysin (16.2% ± 1.6% for HepG2 cells and 11.0% ± 1.3% for Bel-7402 cell) at 40 μmol/L and similar to 5-flurouracil (33.0% ± 2.1% for HepG2 cells and 29.3% ± 2.3% for Bel-7402 cells) at 10 μmol/L. BrMC had little effect on human embryo liver L-02 cells, with the percentage of sub-G1 cell population 5.4% ± 1.8%. Treatment of HepG2 cells with BrMC for 48 h also increased the levels of active caspase-3, in a concentration-dependent manner. z-DEVD-fmk, a caspase-3-specific inhibitor, prevented the activation of caspase-3. Treatment with BrMC at 10 μmol/L for 48 h resulted in the formation of a DNA ladder. Treatment of cells with BrMC (10 μmol/L) increased mean fluorescence intensity of DCHF-DA in HepG2 cells from 7.2 ± 1.12 at 0 h to 79.8 ± 3.9 at 3 h and 89.7 ± 4.7 at 6 h. BrMC did not affect ROS generation in L-02 cells. BrMC treatment failed to induce cell death and caspase-3 activation in HepG2 cells pretreated with N-acetylcysteine (10 mmol/L). In addition, in HepG2 cells treated with BrMC (2.5, 5.0, 10.0 μmol/L) for 12 h, JNK activation was observed. Peak JNK activation occurred at 12 h post-treatment and this activation persisted for up to 24 h. The expression of phosphorylated JNK and c-Jun protein after 12 h with BrMC-treated cells was inhibited by N-acetylcysteine and SP600125 pre-treatment, but GW9662 had no effect. SP600125 substantially reduced BrMC-induced cell death and caspase-3 activation of HepG2 cells. N-acetylcysteine and GW9662 also attenuated induction of cell death and caspase-3 activation in HepG2 cells treated with BrMC.

CONCLUSION: BrMC induces apoptosis of HCC cells by ROS generation and sustained JNK activation.

Key words: Hepatocellular carcinoma; 8-bromo-7-methoxychysin; Chrysin; Reactive oxygen species; Jun N-terminal kinase

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Yang XH, Zheng X, Cao JG, Xiang HL, Liu F, Lv Y. 8-bromo-7-methoxychrysin-induced apoptosis of hepatocellular carcinoma cells involves ROS and JNK. *World J Gastroenterol* 2010; 16(27): 3385-3393 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3385.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3385

INTRODUCTION

Epidemiological and intervention studies in both humans and animals have shown that regular consumption of fruits, vegetables, and tea is associated with decreased risk of cancer[1]. Fruits, vegetables, and tea provide essential nutrients and many diet-derived phenolics, in particular flavonoids, which have been demonstrated to exert potential anticarcinogenic activities[2]. Chrysin (5,7-dihydroxyflavone, ChR), a natural and biologically active flavone extracted from many plants, honey, and bee propolis, has been shown to inhibit cell proliferation and induce apoptotic cell death in a variety of cancer cells. Investigations into the molecular mechanisms underlying the inhibition of cell proliferation and induction of apoptosis by ChR have shown that ChR inhibited the growth by downregulating expression of proliferating cell nuclear antigen in HeLa cells[3], induced apoptosis through caspase activation and Akt inactivation in leukemia cells[4-7], and induced cell cycle arrest in human colon carcinoma cells, human esophageal adenocarcinoma OE33 cells and human lung adenocarcinoma cells[8-10]. Nevertheless, poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer chemotherapeutic agents[11]. It has been reported that ChR halogenated derivatives had stronger bioactivities than the lead compound[12]. The higher hepatic metabolic stability and intestinal absorption of the methylated polyphenols make them more favorable than the unmethylated polyphenols for development as potential cancer chemopreventive agents[13]. Our previous study showed that the effect of 8-bromo-7-methoxychrysin (BrMC) on the inhibition of proliferation and induction of apoptosis in a colon cancer cell line, HT-29, and a gastric cancer cell line, SGC-7901, was stronger than that of ChR[14-17]. However, the molecular mechanisms of induced apoptosis in human hepatocellular carcinoma cells (HCC) by BrMC were not clear.

Although flavonoids are generally considered as antioxidants, they can also generate reactive oxygen species (ROS) depending on their structure and molecular environment[18]. A number of flavonoids exert direct and indirect pro-oxidant effects by inhibiting the mitochondrial respiratory chain complexes I by inducing glutathione (GSH) depletion[19-23]. In addition, Kachadourian *et al*[19-24] have reported that chrysin is a potent inducer of ROS generation and GSH depletion in A549, HL-60, and PC-3 cells. We here demonstrate that BrMC induces apoptosis of human HCC at least partly by promoting generation of ROS, and ROS-dependent sustained activation of c-Jun N-terminal kinase (JNK).

MATERIALS AND METHODS

Cell culture and reagents

Human HCC HepG2 [AFP(+), no tumorigenicity in immunosuppressed mice], Bel-7402 [AFP(+),with high frequency of tumorigenicity], and human embryo liver L-02 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin, and incubated in a humidified atmosphere of 5% CO_2 at 37℃. BrMC was synthesized as described previously[14]. ChR was purchased from the Sigma Chemical Co. (St Louis, MO, USA). BrMC and ChR were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1% in media. N-acetylcysteine (NAC), and GW9662 were obtained from Sigma. Caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), and the caspase-3 specific inhibitor Z-Asp-Glu-Val-Asp-CH2F (Z-DEVD-fmk), were obtained from Calbiochem (La Jolla, CA, USA). Dichlorodihydrofuorescein diacetate (DCHF-DA) was from Molecular Probes Inc. (Eugene, OR, USA). Rabbit anti-human total JNK was from Cell Signaling Technology (Beverly, MA, USA); mouse anti-human phospho-JNK, phospho-c-Jun, total c-Jun and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology. The commercial anti-hepatoma agent 5-flurouracil (5-FU) was obtained from Sigma Chemical Co. and was used as an apoptotic inducer positive control whereas 0.1% DMSO was used as a negative control.

Flow cytometry with propidium iodide staining

As previously described[25], cells were treated with serum-free medium for 24 h, followed by treatment with media containing different concentrations of test agents for 48 h. Cells were collected and prepared as a single cell suspension by mechanical blowing with phosphate-buffered saline (PBS), washed with cold PBS twice, fixed with 700 mL/L alcohol at 4℃ for 24 h, stained with propidium iodide (PI), and cell apoptosis was detected using flow cytometry (FCM; American BD Company, FACS 420).

DNA agarose gel electrophoresis

As previously described[26], cells were treated with serum-free medium for 24 h, followed by treatment with media containing different concentrations of test agents for 48 h. Cells were washed twice with PBS and DNA was extracted with Apoptotic DNA Ladder Detection Kit (Bodataike Company, Beijing) according to the manufacturer's in-

structions. The extracted DNA was kept at 4℃ overnight. Then 8.5 μL of DNA sample was mixed with 1.5 μL of 6 × buffer solution, electrophoresed on 20 g/L agarose gel containing ethidium bromide at 40 V, and observed through the DBT-08 gel image analysis system.

Caspase-3 activity assay

To evaluate caspase-3 activity, cell lysates were prepared after treatment with test agents. Assays were performed in 96-well microtiter plates by incubating 20 μg cell lysates in 100 μL reaction buffer (1% NP-40, 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 10% glycerol) containing the 5 μmol/L caspase-3 substrate (DEVD-pNA). Lysates were incubated at 37℃ for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type). In the caspase inhibitors assay, cells were pretreated with a caspase-3 specific inhibitor (10 μmol/L, Z-DEVD-fmk) for 1 h prior to addition of test agents.

Determination of ROS

Intracellular ROS accumulation was measured by FCM using the fluorescent probe DCHF-DA. Briefly, cells were incubated with 10 μmol/L of DCHF-DA for 30 min at 37℃ in the dark after treatment with various concentrations of test agents. After incubation, the cells were washed with PBS and analyzed within 30 min by FCM equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to DCHF-DA were collected with a 525 nm band pass filter. As a rule, 10000 cells were counted in each determination.

Western blotting analysis

As previously described[27], cells were collected, washed 3 times with PBS, lysed in cell lysate containing 0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL PMSF, and then centrifuged at 13000 × *g* for 10 min at 4℃. Extracted protein sample (25 μg total protein/lane) was added in the same volume of sample buffer solution and subjected to denaturation at 100℃ for 10 min, then electrophoresed on 100 g/L or 60 g/L sodium dodecyl sulfate polyacrylamide gel electrophoresis at 100 mA for 3 h, and finally transferred onto polyvinylidene fluoride membrane (PVDF) (Millipore). The PVDF membrane was treated with Tris-Buffered Saline Tween-20 (TBST) containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the first antibodies phospho-JNK, total JNK, phospho-c-Jun, total c-Jun and β-actin (1:1000 dilution), respectively, at 37℃ for 2 h. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA). Total JNK, total c-Jun and β-actin (1:1000 dilution) were used as an internal control. Images were scanned, followed by densitometry analysis with UN-SCAN-IT software (Silk Scientific).

Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA) for analysis. Data were represented as mean ± SD. The means of multiple groups were compared with one-way analysis of variance, after the equal check of variance, and two-two comparisons of the means were performed using the least significant difference method. Statistical comparison was also performed with two-tailed *t*-test when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of BrMC on apoptosis in human HCC

To determine whether BrMC selectively induces apoptosis of human HCC, the human HCC lines HepG2 and Bel-7402 and human embryo liver L-02 cells were treated with increasing concentrations of BrMC for 48 h. Apoptotic cell death was examined by: (1) cell population with sub-G1 contents of DNA using FCM after PI staining; (2) caspase-3 activity determined by enzyme-linked immunosorbent assay; and (3) DNA fragmentation observed by DNA agarose gel electrophoresis. Figure 1A shows that there is a dose-dependent increase in the percentage of sub-G1 cell population ($P < 0.05$), reaching 39.0% ± 2.8% of HepG2 cells after 48 h of treatment with BrMC at 10 μmol/L. The potency of BrMC in HepG2 and Bel-7402 (32.1% ± 2.6%) cells was found to be more effective than the lead compound, chrysin (ChR, 16.2% ± 1.6% for HepG2 cells and 11.0% ± 1.3% for Bel-7402 cells) at 40 μmol/L and similar to 5-FU (33.0% ± 2.1% in HepG2 cells and 29.3% ± 2.3% in Bel-7402 cells) at 10 μmol/L. BrMC had little effect in human embryo liver L-02 cells, with the percentage of the sub-G1 cell population 5.4% ± 1.8%. Compared with HepG2 cells, Bel-7402 cells were less sensitive to BrMC. Parallel to the cell lethal effect and the enhanced caspase-3 activity, treatment of HepG2 cells with BrMC for 48 h increased the levels of active caspase-3, in a concentration-dependent manner (Figure 1B). Requirement of caspase activity for BrMC-induced apoptosis was examined using a caspase-3-specific inhibitor, z-DEVD-fmk. The data showed that z-DEVD-fmk was able to prevent activation of caspase-3 (Figure 1B). Similarly, treatment with BrMC at 10 μmol/L for 48 h resulted in the formation of a DNA ladder (Figure 2). These results indicate that BrMC selectively induced apoptotic cell death of HCC in a caspase-dependent fashion.

Effects of BrMC on ROS generation in HepG2 cells

Because oxidative damage plays an important role in anticancer effects of ChR[19], we subsequently examined the level of intracellular ROS in HepG2 and L-02 cells after treatment with BrMC using an oxidation-sensitive fluorescent probe DCHF-DA, which is oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Figure 3A shows that treatment of cells with BrMC (10 μmol/L) increased mean fluorescence intensity of DCF in HepG2 cell from 7.2 ± 1.12 at 0 h to 79.8 ± 3.9 at 3 h and 89.7 ±

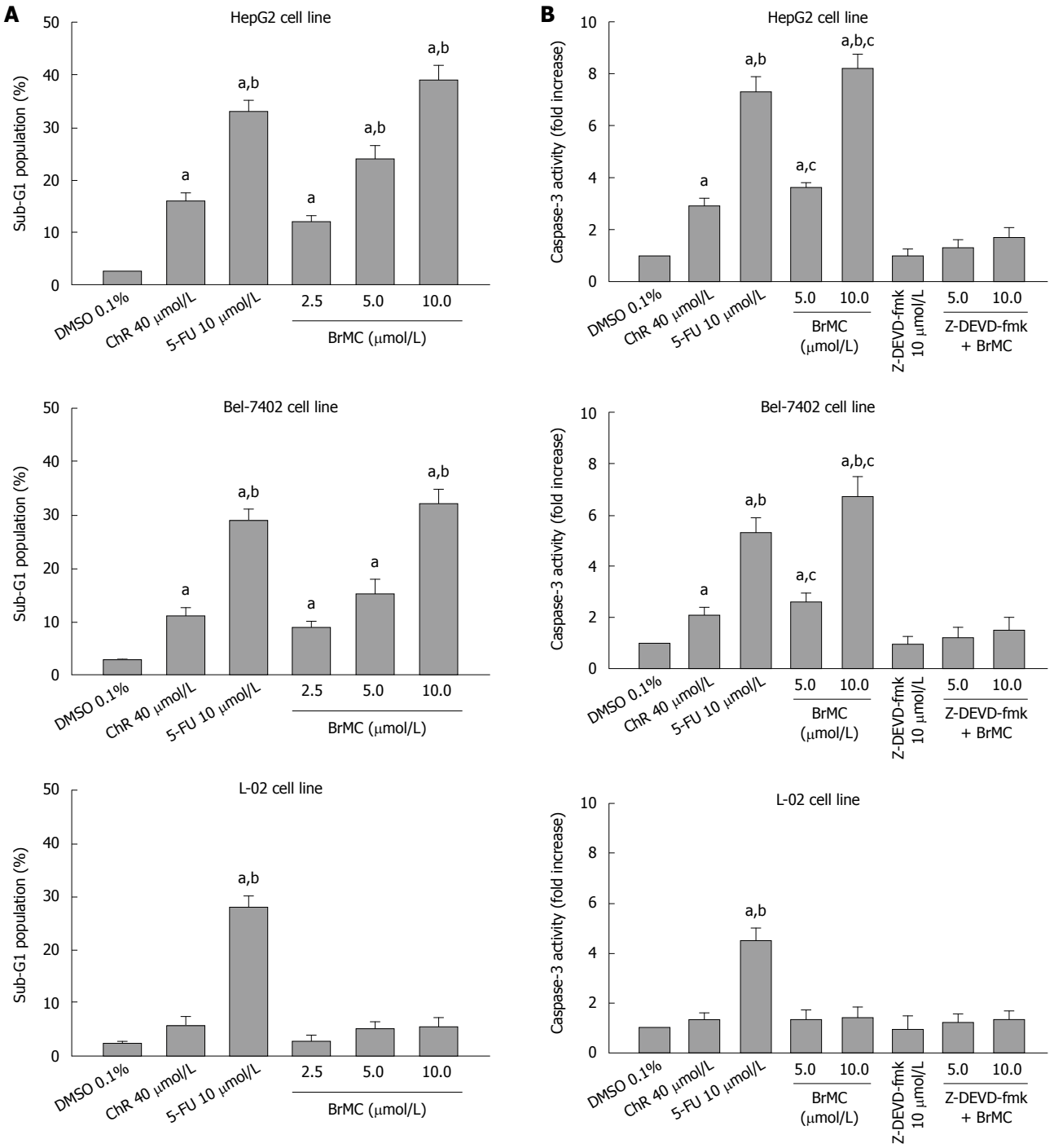

Figure 1 Effects of 8-bromo-7-methoxychrysin on the percentage sub-G1 cell population (A), caspase-3 activity (B) in human hepatocellular carcinoma HepG2, Bel-7402 and human embryo liver L-02 cells. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{b}P < 0.05$ *vs* treatment with chrysin (ChR); $^{c}P < 0.05$ *vs* treatment with Z-Asp-Glu-Val-Asp-CH2F (Z-DEVD-fmk) plus BrMC. 5-FU: 5-flurouracil.

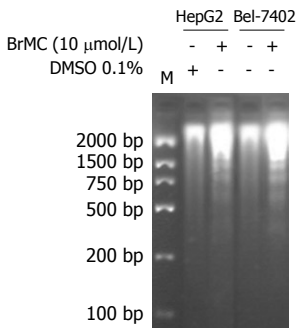

Figure 2 Effects of 8-bromo-7-methoxychrysin on DNA fragmentation in human hepatocellular carcinoma HepG2 and Bel-7402 cells. DMSO: Dimethyl sulfoxide.

4.7 for 6 h. However, BrMC did not affect ROS generation in L-02 cells. BrMC treatment failed to induce ROS generation in HepG2 cells pretreated with 10 mmol/L NAC. We next investigated whether generation of ROS induced by BrMC was accompanied by apoptotic cell death after BrMC treatment. To determine a link between elevation of the intracellular ROS level and apoptotic cell death in BrMC-treated cells, HepG2 cells were pre-incubated with the thiol-containing antioxidant NAC before treatment with BrMC. BrMC treatment failed to induce

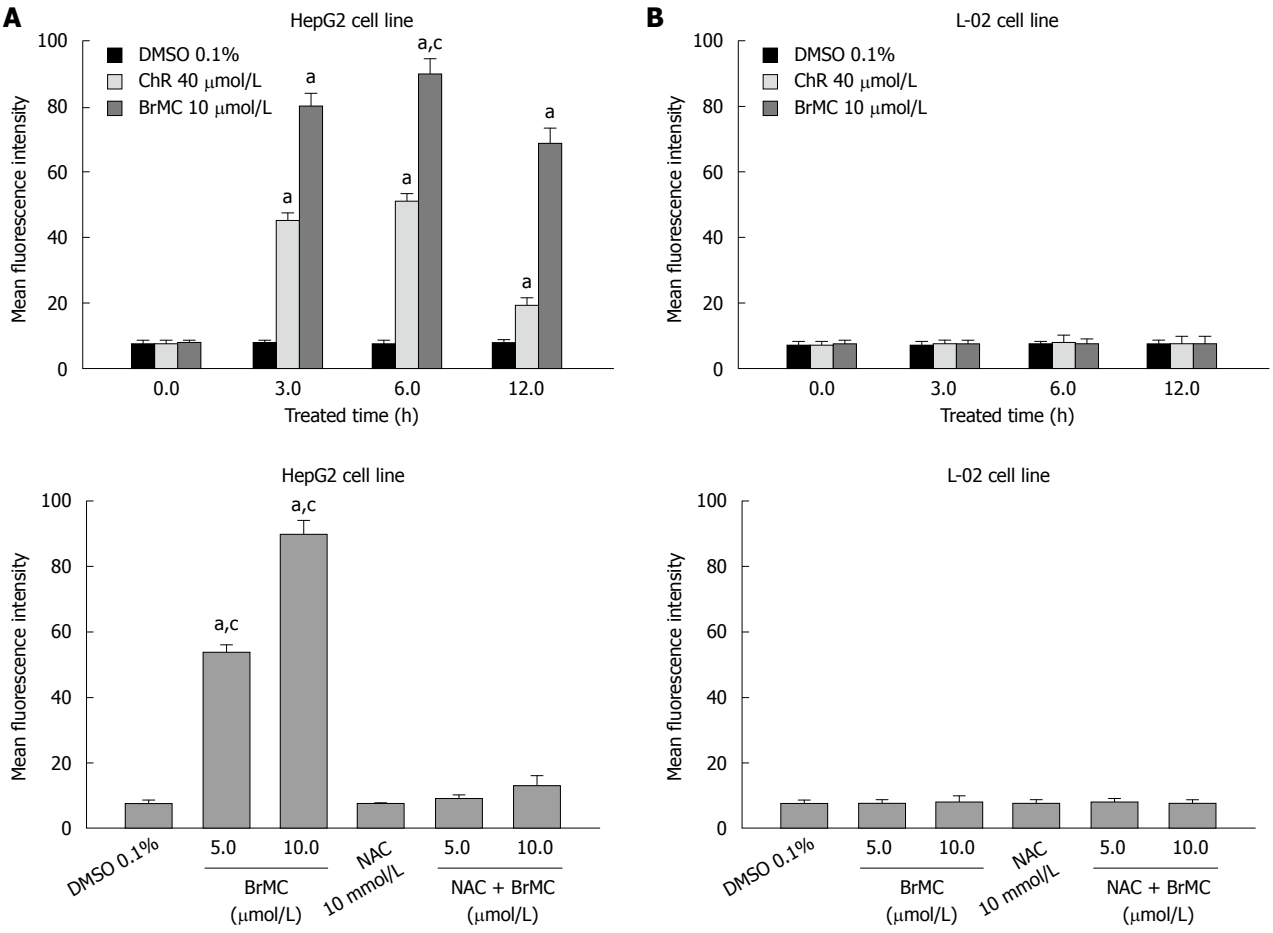

Figure 3 Effects of 8-bromo-7-methoxychrysin on reactive oxygen species generation in human hepatocellular carcinoma HepG2 (A) and human embryo liver L-02 cells (B). [a]*P* < 0.05 *vs* baseline or treatment with dimethyl sulfoxide (DMSO); [c]*P* < 0.05 *vs* treatment with N-acetylcysteine (NAC) plus 8-bromo-7-methoxychrysin (BrMC).

cell death and caspase-3 activation in cells pretreated with 10 mmol/L NAC (Figure 4). These observations suggest that a selective increase in the intracellular ROS level after BrMC treatment in HCC is required in the cell death pathway, accompanied by activation of caspase-3.

Effects of BrMC on JNK activation in HepG2 cells

It is well known that many therapeutic agents trigger apoptosis *via* activation of stress-related signaling pathways including JNK-mediated ones[28]. JNK plays distinct roles in cell death. Transient activation of JNK is believed to be antiapoptotic whereas persistent activation is pro-apoptotic[29,30]. Here we examined the effect of BrMC-induced JNK activation. JNK activation was measured by Western blotting analysis of phosphorylated JNK and its downstream target c-Jun. In HepG2 cells treated with BrMC (2.5, 5.0, 10.0 μmol/L) for 12 h, JNK activation was observed (Figure 5A). Time course experiments revealed peak JNK activation at 12 h post-treatment and this activation persisted for up to 24 h (Figure 5B).

Effects of BrMC-stimulated JNK activation on induction of apoptosis and caspase-3 activation in HepG2 cells

It has been reported that ChR derivatives induce apoptosis of human HCC and human gastric cancer cells by activating peroxisome proliferator-activated receptor-γ (PPARγ)[17,26]. One of the important components in ROS signaling is JNK activation[31]. These results prompted us to investigate whether NAC, an antioxidant, and GW9662, a blocker of PPARγ, and JNK inhibitor SP600125 affected the phosphorylated JNK protein level in HepG2 cells treated with BrMC. Figure 6A shows that the expression of phosphorylated JNK protein after 12 h with BrMC-treated cells was inhibited by NAC and SP600125 pretreatment, but GW9662 had no effect. To examine the effects of BrMC-stimulated JNK activation on induction of apoptosis and caspase-3 activation in HepG2 cells, we used the JNK inhibitor SP600125 to investigate the role of JNK in BrMC-induced cell death and caspase-3 activation. SP600125 substantially reduced BrMC-induced cell death and caspase-3 activation of HepG2 cells (Figure 6B-D). In addition, NAC and GW9662 also inhibited induction of cell death and caspase-3 activation in HepG2 cells treated with BrMC (Figure 6B-D). These results suggest that ROS production and JNK activation are required for BrMC-induced cell death and caspase-3 activation in HepG2 cells.

DISCUSSION

Our previous study showed that the effect of BrMC on

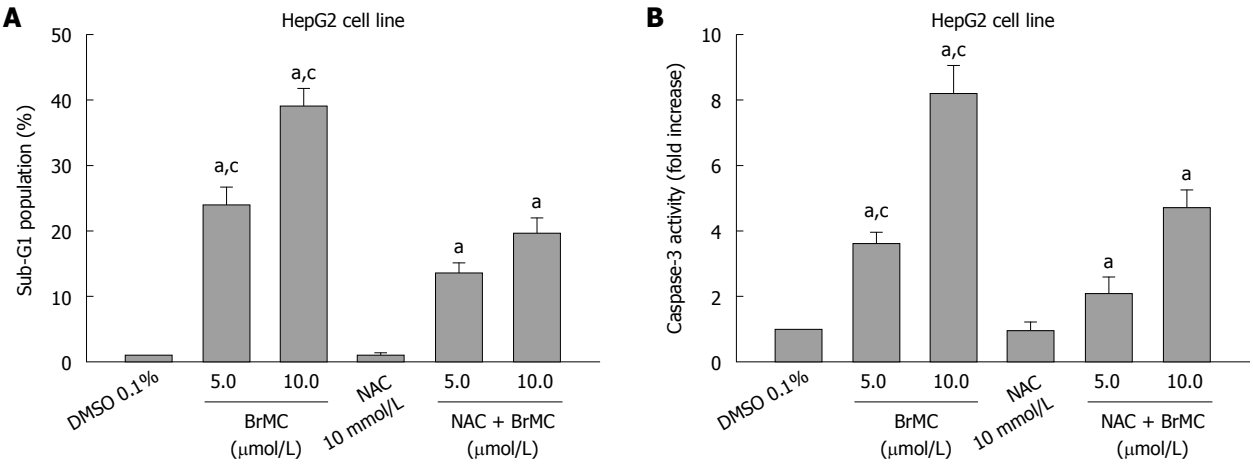

Figure 4 Effects of N-acetylcysteine on 8-bromo-7-methoxychrysin-induced apoptosis rate (A) and caspase-3 activity (B) in HepG2 cells. $^{a}P < 0.05$ *vs* treatment with medium (0 h) or dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with N-acetylcysteine (NAC) plus 8-bromo-7-methoxychrysin (BrMC).

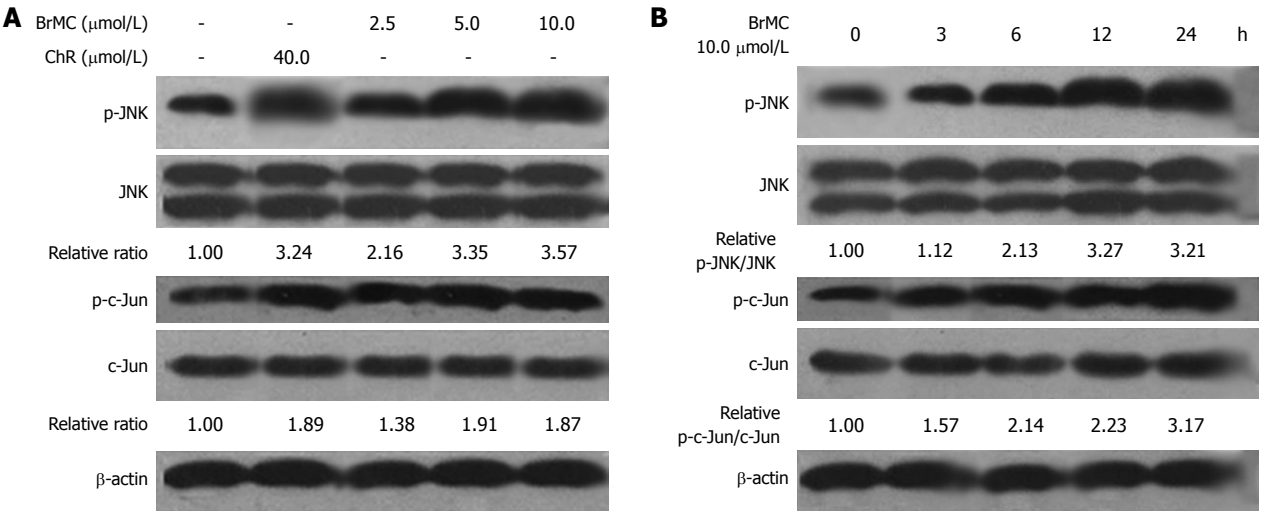

Figure 5 Effects of 8-bromo-7-methoxychrysin on the level of phosphorylated Jun N-terminal kinase and phosphorylated c-Jun in HepG2 cells (Western blotting, mean ± SD, *n* = 3). 8-bromo-7-methoxychrysin (BrMC) elevated the level of phosphorylated Jun N-terminal kinase (JNK) and phosphorylated c-Jun in a concentration-dependent manner (A) and in a time-dependent manner (B). The ratio of p-JNK/JNK or p-c-Jun/c-Jun was normalized to 0 h or the untreated group.

the inhibition of proliferation and induction of apoptosis in a colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 was stronger than that of ChR[14,16]. In addition, it has been reported that ChR is a potent inducer of ROS generation and GSH depletion in A549, HL-60, and PC-3 cells[19,20]. In this study, we firstly showed that BrMC selectively induced apoptotic cell death of human HCC in a caspase-dependent fashion, with little effect on human embryo liver L-02 cells (Figures 1 and 2). The potency of BrMC in HepG2 and Bel-7402 cells was found to be greater than ChR and similar to 5-FU. Secondly, we indicated that BrMC selectively induced apoptosis of HepG2 cells and was accompanied by ROS generation. However, BrMC did not affect ROS generation of L-02 cells (Figures 3 and 4). Furthermore, we demonstrated that BrMC induced sustained activation of JNK in HepG2 cells in a ROS-dependent manner (Figures 5 and 6).

ROS have been associated with carcinogenesis but also, paradoxically, with mitochondrial-mediated cell death in cancer cells. The overproduction of ROS as a central event in mitochondrial-mediated apoptosis is now well documented[32-35]. The antioxidant properties of flavonoids have been associated with their cardioprotective and neuroprotective properties, yet such an association is much less certain concerning their cancer preventive properties. In the case of flavonoids, however, their chemopreventive properties may rather rely on eliminating precancerous cells because of their prooxidant properties *in vivo*. This is likely the case of apigenin and ChR, where their cytotoxicity may result from a combination of interference with the mitochondrial respiratory chain and multidrug resistance protein-mediated GSH depletion[36,37]. It is worth noting that the bee product propolis, which is known to exert antimicrobial, antiviral, and cancer preventive properties, contains ChR, a poor antioxidant, as one of its major components[38]. Intracellular ROS mediate multiple cellular responses, including protein kinase activation[39], cell cycle progression[40], myeloid cell differentiation[41,42], and apoptotic and necrotic cell death[43]. It has been reported in several studies that depletion of intracellular GSH plays

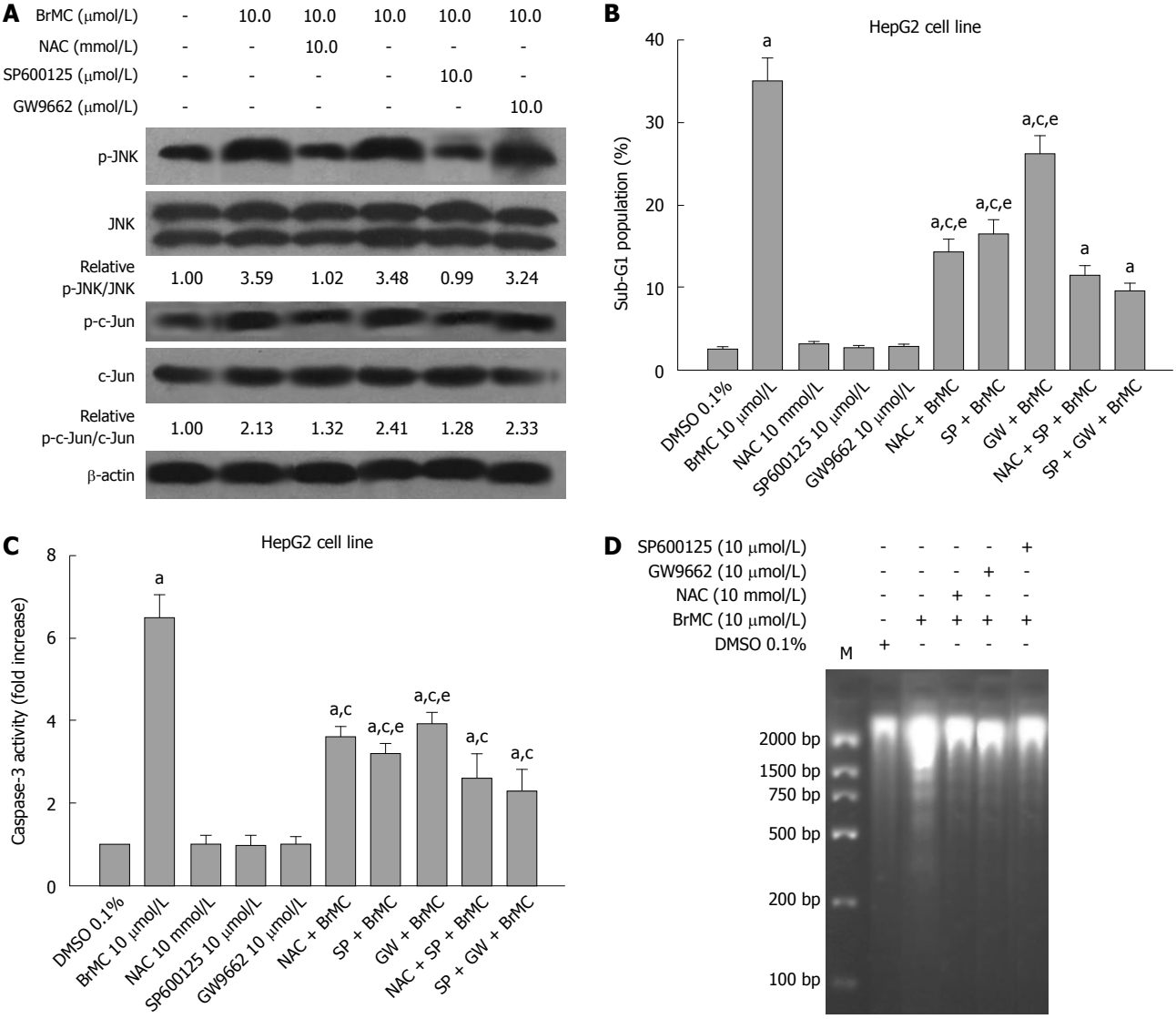

Figure 6 Effect of N-acetylcysteine, an antioxidant, and GW9662, a blocker of peroxisome proliferator-activated receptor-γ, and Jun N-terminal kinase inhibitor SP600125 on 8-bromo-7-methoxychrysin-induced activation of Jun N-terminal kinase (A), apoptosis (B), activation of caspase-3 (C), and DNA fragmentation (D) in HepG2 cells. The ratio of p-Jun N-terminal kinase (JNK)/JNK or p-c-Jun/c-Jun was normalized to 0 h or the untreated group. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 8-bromo-7-methoxychrysin (BrMC) alone; $^{e}P < 0.05$ *vs* treatment with N-acetylcysteine (NAC) in combination with SP600125 and BrMC or GW9662 in combination with SP600125 and BrMC.

a critical role in initiating apoptosis by ChR[10,19]. This is likely caused by the reversible interaction between ChR and GSH. In present study, we found that the ChR derivative, BrMC promoted accumulation of ROS products in a concentration-dependent manner in HepG2 cells but not in L-02 cells (Figure 3). NAC is an antioxidant agent and mainly known as a ROS scavenger. It reduces ROS generation and protects the cells from oxidative stress. BrMC-induced apoptosis of HepG2 cells was accompanied by ROS generation. It has been reported that arsenic trioxide-induced ROS generation was inhibited by NAC treatment[44]. We used NAC as an antioxidant to investigate the ROS generation induced by BrMC. NAC treatment not only reduced ROS generation but also attenuated induction of apoptosis in HepG2 cells (Figures 3 and 4). All together, these results indicate that induction of ROS generation contributes to BrMC-induced apoptosis in HepG2 cell line.

In this study, we investigated the signaling pathways affected by BrMC in HepG2 cells. We show that BrMC persistently activates JNK and induces apoptosis of HepG2 cells (Figures 5 and 6). BrMC-activated signaling pathways appear to activate executioner caspases because caspase 3 activity was enhanced in cells exposed to BrMC (Figure 1). JNK is implicated in mediating endoplasmic reticulum stress-induced apoptosis[45]. It has been shown that triterpenoids activate JNK leading to apoptosis[46,47]. In the present study, we detected both JNK activation and apoptotic cell death in cells exposed to BrMC. In addition, the presence of the JNK inhibitor SP600125 attenuated BrMC-induced apoptosis of HepG2 cells, indicating that BrMC-induced apoptosis is also JNK dependent (Figures 5 and 6). Collectively, we conclude that JNK activation mediates BrMC-induced apoptosis in the HepG2 cell line. We noted that SP600125 inhibited BrMC-induced c-Jun phosphorylation completely, but only partially prevented induction of apop-

tosis by BrMC in HepG2 cells (Figure 5). Thus, we cannot role out the possibility that other mechanisms also participate in BrMC-induced apoptosis in the HepG2 cell line.

It has been documented in several studies that depletion of intracellular GSH plays a critical role in initiating apoptosis by chrysin[10,19]. Zou *et al*[46]recently showed that triterpenoids deplete intracellular GSH, resulting in JNK-dependent apoptosis in human lung cancer A549 cells. In this study, we found that the presence of NAC blocked the effects of BrMC not only in generating ROS but also in activating JNK and triggering apoptotic cell death (Figure 6).

In summary, the present study has shown that BrMC promotes accumulation of intracellular ROS, resulting in sustained activation of JNK, leading to apoptosis in human HCC but not in human embryo liver L-02 cells. While further investigation is required to provide evidence for the efficacy of this HCC therapy in a nude mouse model and whether it reaches an effective dose *in vivo*, these results highlight a new mechanism responsible for BrMC-induced apoptosis, and raise the possibility that BrMC may be promising as a candidate for human HCC therapy.

COMMENTS

Background

Chrysin (5,7-dihydroxyflavone), a natural and biologically active flavone extracted from many plants, honey, and propolis, has been shown to inhibit cell proliferation and induce apoptotic cell death in a variety of cancer cells. Nevertheless, poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer chemopreventative agents. The authors have synthesized 8-bromo-7-methoxyhysin (BrMC), a novel chrysin analogue. BrMC has been demonstrated to inhibit proliferation and induction of apoptosis in a colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 and its effect was stronger than that of chrysin.

Research frontiers

Epidemiological and intervention studies in both humans and animals have shown that regular consumption of fruits, vegetables, and tea is associated with decreased risk of cancer. Fruits, vegetables, and tea provide essential nutrients and many diet-derived phenolics, in particular flavonoids, which have been demonstrated to exert potential anticarcinogenic activities. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones, flavanols, and flavans. Chrysin is a natural flavonoid contained in many plant extracts, honey, and propolis. Several studies in recent years have shown that chrysin and its derivatives have multiple biological activities, such as anti-inflammatory, anti-cancer, and anti-oxidative effects. However, the cellular and molecular mechanisms underlying chrysin and derivatives induced apoptosis of cancer cells are not clearly understood.

Innovations and breakthroughs

The authors firstly showed that BrMC, a novel chrysin analogue induced apoptotic cell death of human hepatocellular carcinoma cells (HCC) in a caspase-dependent fashion. BrMC-induced apoptosis of HepG2 cells was accompanied by ROS generation. Induction of reactive oxygen species (ROS) generation contributes to BrMC-induced apoptosis in a HepG2 cell line. In addition, they demonstrated that BrMC induced sustained activation of Jun N-terminal kinase (JNK) in HepG2 cells in a ROS-dependent manner.

Applications

The present study has shown that BrMC promotes accumulation of intracellular ROS, resulting in sustained activation of JNK, leading to apoptosis in human HCC. These results suggest that BrMC is a promising candidate for human HCC therapy.

Peer review

This is an original article by Jian-Guo Cao's group that investigated the effect of BrMC on HCC. They have found that BrMC induces apoptosis in HepG2 and Bel-7402 cells by generation of ROS, JNK activation, and activation of caspase-3. Overall the experiments were conducted appropriately, and the content is interesting.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3394

World J Gastroenterol 2010 July 21; 16(27): 3394-3401
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats

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Received: March 8, 2010 Revised: April 30, 2010
Accepted: May 7, 2010
Published online: July 21, 2010

Abstract

AIM: To compare the effects of four *Bifidobacteria* strains (*Bifidobacteria L66-5*, *L75-4*, *M13-4* and *FS31-12*, originated from normal human intestines) on weight gain, lipid metabolism, glucose metabolism in an obese murine model induced by high-fat diet.

METHODS: Forty-eight Sprague-Dawley rats were randomly divided into six groups. Control group received standard chow, model group received high-fat diet, and intervention groups received high-fat diet added with different *Bifidobacteria* strains isolated from healthy volunteers' fresh feces. All rats were executed at the 6th weekend. Body weight (BW), obese indexes, oral glucose tolerance test, serum and liver lipid and serum insulin (INS) were tested. Liver lipid deposition was classified pathologically.

RESULTS: Compared with the model group, *B. M13-4* improved BW gains (264.27 ± 26.91 *vs* 212.55 ± 18.54, $P = 0.001$) while *B. L66-5* induced a decrease in BW (188.47 ± 11.96 *vs* 212.55 ± 18.54, $P = 0.043$). The rest two strains had no significant change in BW. All the four strains can reduce serum and liver triglyceride and significantly alleviate the lipid deposition in liver. All strains showed a trend of lowing serum and liver total cholesterol while *B. L66-5* and *B. FS31-12* did so more significantly. In addition, all the four strains showed no significant differences in serum INS and glucose level.

CONCLUSION: The response of energy metabolism to administration of *Bifidobacteria* is strain dependent. Different strains of *Bifidobacteria* might drive different directions of fat distribution.

Key words: *Bifidobacterium*; Obesity; Serum lipid; Body weight

Peer reviewers: Laura E Matarese, MS, RD, LDN, FADA, CNSD, Thomas E. Starzl Transplantation Institute, UPMC Montefiore, 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, United States; Antonio Gasbarrini, MD, Professor, Department of Internal Medicine, Gemelli Hospital, Catholic University of Rome, Largo A. Gemelli 8, 00168 Rome, Italy

Yin YN, Yu QF, Fu N, Liu XW, Lu FG. Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats. *World J Gastroenterol* 2010; 16(27): 3394-3401 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3394.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3394

INTRODUCTION

Obesity is becoming a global epidemic, and a major contributor to increased incidence of serious chronic diseases such as type 2 diabetes, cardiovascular diseases, hepatic and skeletal muscle insulin resistance, and certain forms of cancer[1]. Several strategies have been used to

treat obesity, including diet control, exercise, behavior therapy, medications and surgery. However, the diet control and exercise are too hard to be strictly carried on. Also, undesirable side effects of drugs have restricted their therapeutic use. Fortunately, some recent interesting researches on obesity are helping to validate a new approach to control this medical disorder.

Recent researches have demonstrated that obesity may lead to the composition shift of gut microbiota in both mice and humans. Fewer *Bacteroidetes* and more *Firmicutes* are colonized in the gut of obese people and animals, shown by some 16S-rRNA-gene-sequence-based comparative surveys of gut bacteria[2,3]. Weight loss makes the ratio of *Bacteroidetes* to *Firmicutes* up-regulated in humans[3]. Dietary inclusion of *Lactobacillus*, which belongs to *Bacteroidetes* and/or *Bifidobacterium*, can improve obesity both in murine model and humans[4-6]. The intentional manipulation of community structure of gut microbiota may be a novel strategy to treat obesity.

Bifidobacterium is one of the most numerous "probiotic" in mammalian gut among commensal bacteria, which belongs to *Actinomycetes* and also a kind of lactic acid bacteria. It can help *Bacteroides* degrade polysaccharides[7] and inhibit exogenous cholesterol absorption from the small intestine[8]. Although most researches focus on the hypocholesteremia effect of *Lactobacillus*[4,9-11], a study showed that a strain of *Bifidobacterium longum* exhibited a more significant effect in lowering serum total cholesterol than a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp.*bulgaricus* (SL) both in rats and humans[6]. In contrast, probiotics VSL#3 (a combination of *Streptococcus thermophilus* and several species of *Lactobacillus* and *Bifidobacteria*) was found to increase liver fat with no significant changes in comprehensive metabolic panel or in body weight (BW) in a clinical research[12]. These results suggested that different strains may have their specificities in colonization or function. Furthermore, *Bifidobacterium longum* can induce an expansion of *Bacteroides. Thetaiotaomicron*'s substrates range under co-colonized condition, while another *Bifidobacterium* species, *B. animalis* showed no significant impact[7]. *Bifidobacteria* preparations are safe, widely-used, and well-tolerant. Thus, some specific strains of *Bifidobacteria* related to lipid metabolism and BW may be a potential therapeutic candidate for management of obesity.

In order to screen out more efficient strains in obese management among different strains of *Bifidobacteria* originated from normal human intestines, we established an obese model in rats induced by high-fat diet, and compare their effects on weight gain, lipid metabolism and glucose metabolism. Several strains of *Bifidobacteria* showed dependant effects in obese control in this study, thus would act as potential therapeutic candidates.

MATERIALS AND METHODS

Preparation of bacterial cultures

Four *Bifidobacteria* strains were isolated from healthy volunteers' fresh feces in our facility, and identified according to biochemical characteristics (API 20A biochemical strip, BioMerieux sa). The four strains were named *B. L66-5*, *B. L75-4*, *B. M13-4* and *B. FS31-12*, respectively, and maintained at -80℃ in our laboratory. The strains were all grown in MRS medium under anaerobic condition. When measured at 600 nm (A_{600}), the exponential and stationary growth reached an optical density of 1-2 and 1-4.5, respectively. The correspondence between absorbance and bacterial counts was established (1 mL of culture at A_{600} = 1 contains about 10^8 colony-forming units, CFU). The number of CFU administered was routinely verified by plating. Strains were harvested by centrifugation at 2000 × *g* for 20 min, washed twice with neutral saline, and resuspended at 1 × 10^8 CFU/mL concentration in neutral saline, and 0.4 mL bacterial solution was administered to each rat by intragastric gavage.

Animals and diet

Forty-eight 3-wk-old male Sprague-Dawley (SD) rats were purchased from Slaccas Lab Animal Ltd, Shanghai, China, weighing 50-70 g. The animals were housed in individual stainless steel cages under standard conditions (20-22℃, 50%-55% humidity, 12/12 h dark/light cycle). The rats were fed with a solid standard chow [DongChuang Lab Animal Ltd., Hunan, China, including 17.53% (wt/wt) protein, 6.08% (wt/wt) fat, and 59.98% (wt/wt) carbohydrate, calories (1250 kj/100 g)] for 1 wk. After this adaptation period, 48 rats were randomly assigned to six dietary treatment groups, 8 rats in each group. Each rat was fed diet 13 g/d from the 1st wk, which was then increased by 2 g/d per week. Control group was fed on a standard chow, and the other groups were fed on a high-fat diet (HFD)(DongChuang Lab Animal Ltd, HuNan, China, 16.52% (wt/wt) protein, 25.17% (wt/wt) fat, and 56.66% (wt/wt) carbohydrate, calories (1810 kj/100 g). Each interventional group was administered with *B. L66-5*, *B. L75-4*, *B. M13-4* and *B. FS31-12*, respectively. Model and control groups were given equivalently 0.9% saline. All rats had free access to water and were supplied with bacteria liquid or 0.9% saline by intragastric gavage at a fixed time every day. The assigned diets were given to the rats for 6 wk. BW was measured weekly and caloric intake was accounted finally. All the rats were sacrificed by ether for further studies. The care and use of animals followed our institutional and national guidelines and all experimental procedures involving animals were approved by the ethics committee of the Central South University.

Oral glucose tolerance test

At the 6th weekend after dietary treatment, the rats were deprived of diet for 12 h, then given glucose solution (5 g/kg) by intragastric gavage. Blood samples were drawn from tails to do the oral glucose tolerance test (OGTT) test. Serum glucose was measured at 0, 30, 60, 90 and 120 min by fast blood glucose meter (OneTouch-Ⅱ, Johnson, America).

Assay for weight gain and fat index

After the OGTT test, the rats were fasted for 12 h and euthanized with ether. Liver, retroperitoneal (RET) and

epididymal (EPI) white adipose tissues were immediately removed and weighed.

Measurement: (1) Lee's index: (bodyweight)$^{1/3}$ × 10^3/ stem length (length from nasal tip to anus); (2) boby fat index: viscera fat/body weight ratio (viscera fat includes RET and EPI white adipose tissues); and (3) liver index: liver/body weight ratio.

Assay for serum triglycerides, total cholesterol and insulin

Blood samples were collected immediately in sterile tubes by heart puncture. Serum was collected by centrifugation at 2000 × *g* for 15 min at 4℃. The serum samples were analyzed for triglycerides (TG), total cholesterol (TCH) and insulin according to protocols of triglycerides fluid monoreagent (GPO-PAP) and cholesterol oxidase peroxidase-amidopyrine (CHOD-PAP) analysis (CHOD-PAP) and radioimmunoassay (Dongou Bio-Tech Ltd, Wenzhou and 3v Bio-Tech Ltd, Weifang, China).

Assay for liver lipid

Liver tissues (100 mg) were pulverized in liquid nitrogen to prepare 10% tissue homogenate. These homogenates were extracted under 4℃ with chloroform: methanol (2:1) for 48 h, then centrifuged at 12 000 × *g*/min for 15 min at 4℃. The concentrations of TG and TCH in supernatant were determined according to the protocols.

Liver histopathology

Liver tissues were routinely fixed in paraffin-embedded sections, and stained with hematoxylin and eosin (HE). To detect lipid droplets, sections were stained with Sudan Ⅳ and counterstained with hematoxylin. Each histologic section was observed for 5 fields of high power field. The classification and degree of fatty deposition are as follows: mild fatty degeneration (+): fatty hepatocytes occupying 30%-50% of the hepatic parenchyma, moderate fatty degeneration (++): 50%-75%, and severe fatty degeneration (+++): > 75%.

Statistical analysis

All data were presented as the mean ± SD. Univariate analysis of variance test was applied to determine the statistical significance of the difference among the groups, using the General Linear Models procedure of SPSS15.0 (SPSS Inc., Chicago, IL, USA). Some rank/frequency data were analyzed by nonparametric test, with the significance level set at $P < 0.05$.

RESULTS

Caloric intake, BW increment and obesity indexes in high-fat diet treated rats administered with different strains of Bifidobacteria

No rat died throughout the study. Caloric intake in control group (standard chow) was significantly lower than that of model group (HFD) (9055.68 ± 1246.62 kj *vs* 14562.32 ± 541.55 kj, $P < 0.05$). However, among model group and the four *Bifidobacteria* groups which were fed on high-fat diets, caloric intake showed no significant difference ($P > 0.05$). These indicate that the different groups of HFD rats had a similar caloric consumption (Figure 1A). The weight of all groups was increased every week, especially the model group and group *B. M13-4*. At the end of the 3rd wk, the BW increment in group *B. L66-5* was significantly less than in model group (90.26 ± 27.06 g *vs* 115.75 ± 15.13 g, $P < 0.05$), and further increased (156.05 ± 33.19 g *vs* 186.98 ± 16.25 g, $P < 0.05$) at the end of 5th wk. At the 6th week-end, weight increment in group *B. L66-5* was much less than the model group (175.19 ± 31.24 g *vs* 212.55 ± 18.54 g, $P < 0.05$). However, the BW increment was significantly higher in group *B. M13-4* than in the model group (264.27 ± 26.91 g *vs* 212.55 ± 18.54 g, $P < 0.05$). No significant change of BW was found in group *B. L75-4* and group *B. FS31-12* compared with the model group (Figure 1B).

Both the Lee's and liver indexes were decreased in all interventional groups. The liver index was obviously lower in control, *B. L75-4*, *B. M13-4* and *B. FS31-12* groups than in model group (4.04% ± 0.36%, 4.27% ± 0.50%, 4.27% ± 0.47%, 4.04% ± 0.36% *vs* 4.98% ± 0.72%, $P < 0.05$). The body lipid index showed no significant differences among the groups ($P > 0.05$) (Figure 1C). The Lee's index was significantly lower in groups *B. L66-5* and *B. FS31-12* than in model group (312.65 ± 20.18, 311.22 ± 17.52 *vs* 327.98 ± 8.90, $P < 0.05$, Figure 1D).

Effects of Bifidobacteria strains on glucose and lipid metabolism in high-fat diet treated rats

Effect of four *Bifidobacteria* strains on serum glucose: In control group, the peak of serum glucose appeared at 30 min and returned to normal at 120 min when glucose was significantly lower than in model group (5.32 ± 0.98 *vs* 7.17 ± 1.08, $P < 0.05$). In high-fat diet fed groups, the peak was postponed to 60-120 min and lasted longer than that in control group. In these groups, the serum glucose did not return to normal until 120 min. The serum glucose at 5 time points showed no significant differences among model group and interventional groups ($P > 0.05$) (Figure 2A).

Changes of serum insulin, TG and TCH and liver TG and TCH: The serum insulin (INS) showed no significant differences among the groups ($P > 0.05$) (Figure 2B). The TG levels in serum decreased significantly in all intervention groups compared with the model group (1.59 ± 0.73, 1.54 ± 0.30, 1.23 ± 0.65, 1.47 ± 0.70 *vs* 2.23 ± 0.76, $P < 0.05$), so did the TG levels in supernatant of liver homogenate in those groups (0.13 ± 0.02, 0.31 ± 0.16, 0.34 ± 0.08, 0.29 ± 0.10 *vs* 0.56 ± 0.04, $P < 0.05$) (Figure 2C). In groups *B. L66-5* and *B. FS31-12*, the TCH level in serum and liver were obviously lower than in model group (1.11 ± 0.18, 1.37 ± 0.26 *vs* 1.72 ± 0.38, $P < 0.05$ and 1.27 ± 0.08, 0.62 ± 0.6 *vs* 1.47 ± 0.05, $P < 0.05$, Figure 2D), while the other strains also showed a downward trend ($P > 0.05$).

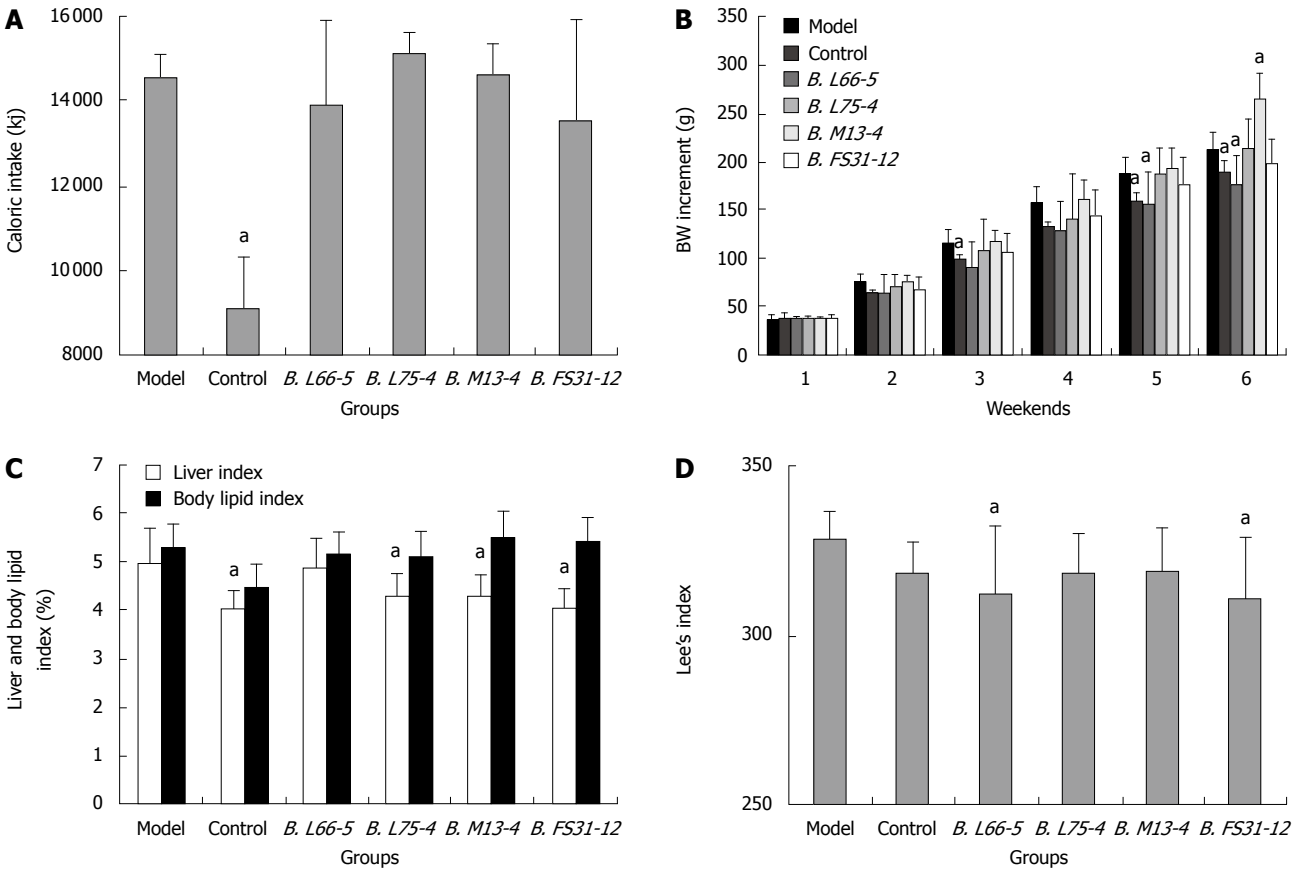

Figure 1 Caloric intake, body weight increment and obesity indexes in high-fat diet treated rats with different strains of *Bifidobacteria*. A: Sum of caloric intake in all groups at the 6th weekend; B: Body weight (BW) increment in all groups for 6 wk; C: Liver index and body lipid index in all groups; D: Lee's index in all groups. Results are shown as mean ± SD (n = 8). $^{a}P < 0.05$ *vs* model group.

Hepatic lipid deposition in high-fat diet induced rats treated with different strains of Bifidobacteria

Moderate degree of microvesicular steatosis was observed in model group. No fatty vacuolization was found in groups *B. L66-5* and *B. FS31-12*. Hepatocyte steatosis was obviously alleviated in groups *B. L75-4* and *B. M13-4* compared with model group (χ^2 = 30.754, P = 0.000) (Table 1 and Figure 3). Sudan Ⅳ staining showed that a plenty of scarlet lipid droplets deposited in the livers of model group, which confirmed the results shown in HE staining. Expectedly, lipid droplets were obviously decreased in all intervention groups compared with the model group, especially in groups *B. L66-5* and *B. FS31-12* (Figure 4).

DISCUSSION

To demonstrate the relationship between the administration of different strains of *Bifidobacteria* and the status of glucose and lipid metabolism, we established a murine obese model based on a 6-wk administration of high-fat diet (HFD), characterized by a significant increase of BW gain, fat mass, TG and TCH in serum and liver, and obesity indexes. Our results demonstrated that administration of the four *Bifidobacteria* (*Bifidobacteria L66-5*, *L75-4*, *M13-4* and *FS3-1-1-2*) played a role in reducing serum and liver TG and TCH, as well as liver lipid deposition. Furthermore, to our surprise, among the four strains of *Bifidobacteria*, two contrary results were yielded in BW changes: *B. M13-4* showed a significant increase in BW while *B. L66-5* showed a decrease in BW based on a similar caloric consumption. *Bifidobacteria L75-4* and *FS3-1-1-2* strains had no significant effect in BW change. However, all the four strains showed no significant influence on serum INS and glucose level. Based on our results, different *Bifidobacteria* strains lead to different responses of energy and fat metabolism in rat models.

Recent researches illustrated that gut microbiome should be considered as a set of genetic factors that, together with host genotype and life style (energy intake and expenditure), contribute to the pathophysiology of obesity. Turnbaugh *et al*[13] observed microbiota samples of obese mice, after transferring the microbiota to germ-free lean mice, significant fat gain was obtained and calorie extraction improved. BW increase has also been observed after administration of *Bifidobacterium*. In a preterm infant study in 1997, the authors added *Bifidobacterium breve* (about 0.5 × 10^9 live bacteria) to very low-birth weight infant formula, and their BW gain became significantly greater than in control group after administration for 4 wk[14]. In other cultures, *Lactobacillus rhamnosus GG* (1 × 10^7 cfu/g) also led to a weight growth in term infants after being supplemented to formulas for 4 mo[15]. However, *Bifidobacterium longum*[16], *Bifidobacterium lactis*[17,18], and *Bifidobacteria* combined with several species of *lactobacilli*

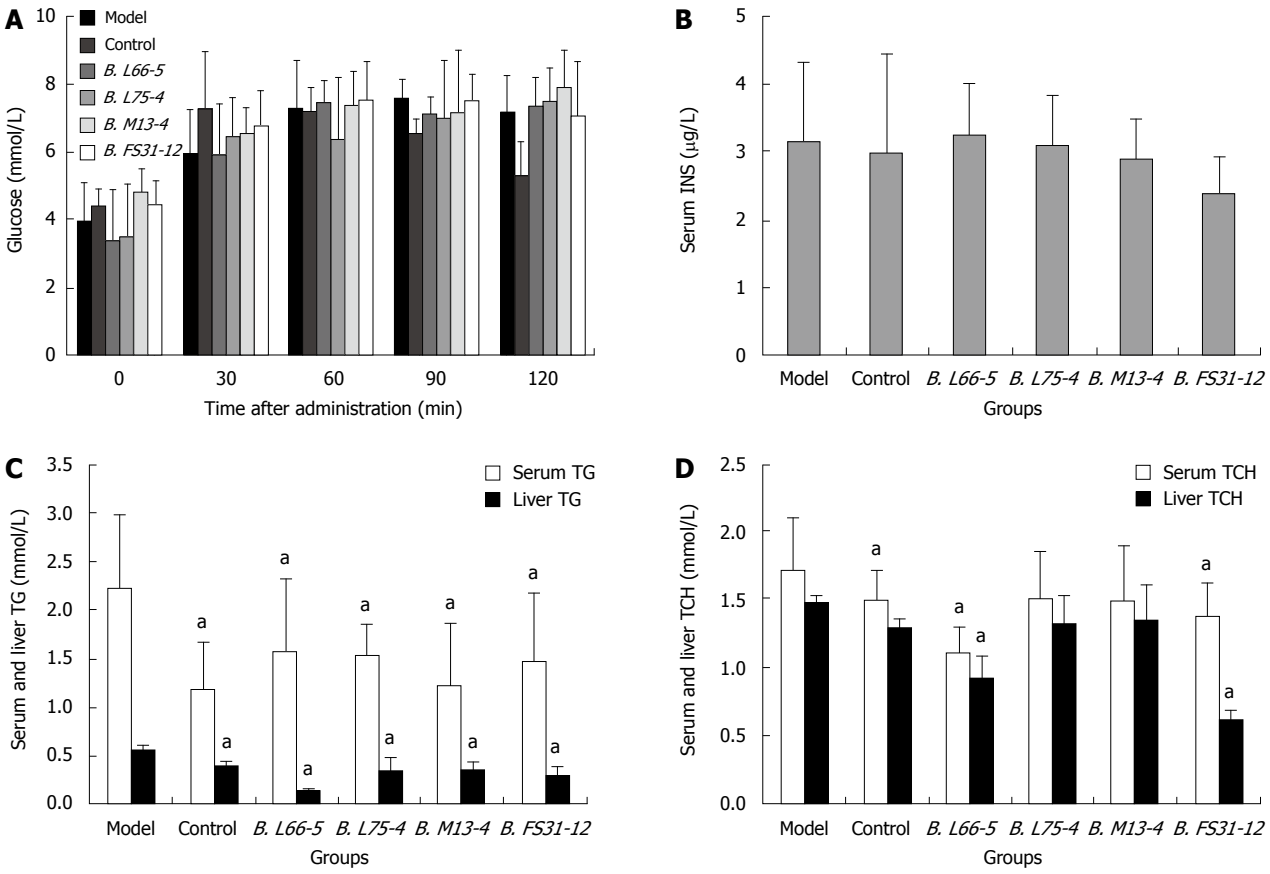

Figure 2 Effects of *Bifidobacteria* strains on glucose and lipid metabolism in high-fat diet treated rats. A: Effect of four *Bifidobacteria* strains on serum glucose; B: Serum insulin (INS) concentration in all groups; C: Serum and liver triglycerides (TG) in all groups; D: Serum and liver total cholesterol (TCH) in all groups. Results are shown as mean ± SD (n = 8), $^aP < 0.05$ *vs* model group.

Table 1 Degrees of liver lipid deposition in all groups

Groups	*n*	Degree of fatty deposition[1]			
		-	+	++	+++
Model	8	0	7	1	0
Control	8	8	0	0	0
B. L66-5	8	8	0	0	0
B. L75-4	8	6	2	0	0
B. M13-4	8	5	3	0	0
B. FS31-12	8	8	0	0	0

[1]The standard of classification and score for the degree of lipid deposition in liver was referred to the liver histopathology mentioned in MATERIALS AND METHODS.

plus *fructooligosaccharides*[19] have not shown any effects. Their similar concentrations and intervention periods indicated different effects *in vivo*. In this study, *B. M13-4* strain can decrease serum and liver TG, TCH and liver index, while no apparent changes were found in body lipid index and Lee's index with an obvious BW gain. It suggests that *B. M13-4* alleviated lipid deposition in liver although more fat was accumulated in the body. The mechanisms may be complex: (1) Intestinal microbiota can help the host to digest polysaccharides and absorb monosaccharides and short-chain fatty acids, which finally converse to lipids[20]; (2) Gut microbiota can modulate some signal pathways associated with energy balance in the gut epithelium: Gpr41, a short-chain fatty-acid binding G protein-coupled receptor, and peptide tyrosine tyrosine[21]; (3) *Bifidobacteria* can also help the host to eradicate *Campylobacter*[22] or *Eandida* and *Enterococcus*[23] to stabilize their intestinal flora; and (4) The amount of visceral fat is positively correlated with the insulin sensitivity[24,25]; the possible effect in improving insulin sensitivity to alleviating visceral adiposity of probiotics is limited in our study and worth further studies. Host colonized by *B. M13-4* absorbed more fat and transmitted them into body fat. This may contribute to the patients with fat/energy malabsorption. We should also reappraise the probiotics use in healthy and obese people for their potential effects such as fat/energy over-absorptions and weight over-growth.

To our surprise, rats colonized by *B. L66-5* showed a weight loss. The opposite outcomes in *B. M13-4* and *B. L66-5*, and strains showing no effect in BW, including *B. L75-4* and *B. FS3-1-1-2*, and other strains, including *Lactobacillus acidophilus ATCC 43121*[4], *Lactobacillus gasseri SBT2055*[5] and *Bifidobacterium longum*[6], *VSL#3*[12], *Lactobacillus reuteri*[26], may result from different interactions between strains and intestinal microbia, inappropriate dosage, variability in end-points, and subjects. Anyhow, *B. L66-5*

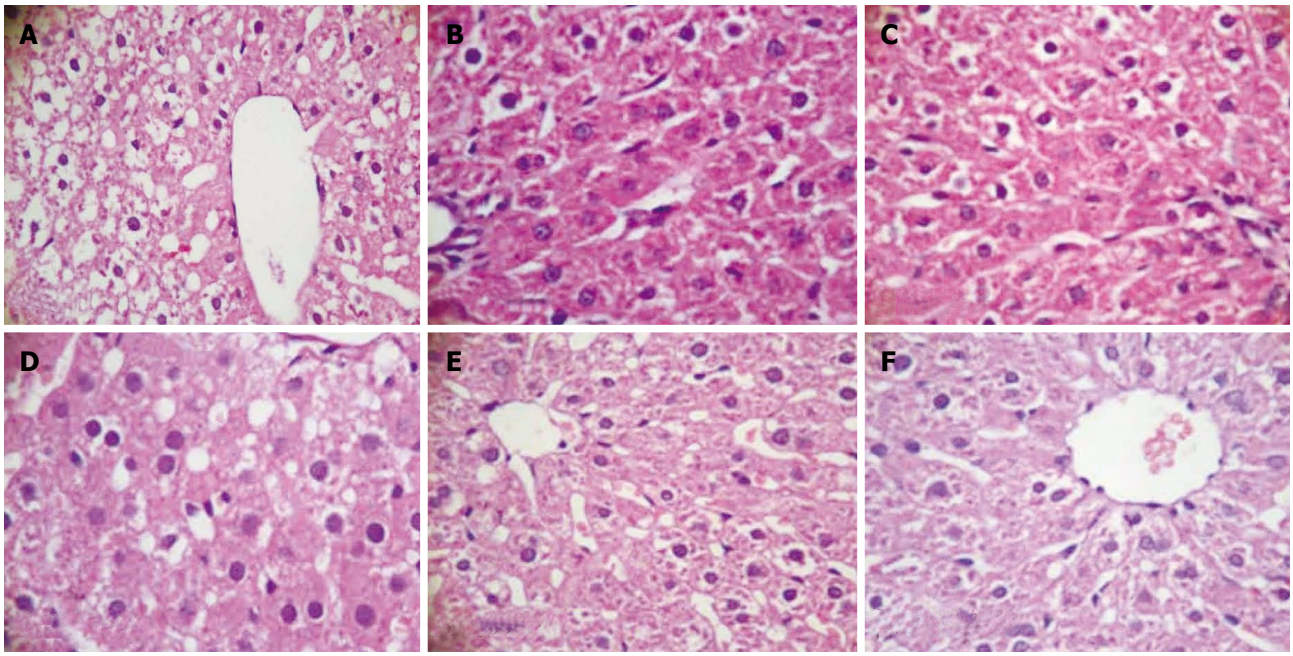

Figure 3 Hepatic tissue sections of each group in HE staining (HE, light microscope, × 400). A: Model; B: Control; C: *B. L66-5*; D: *B. L75-4*; E: *B. M13-4*; F: *B. FS31-12*.

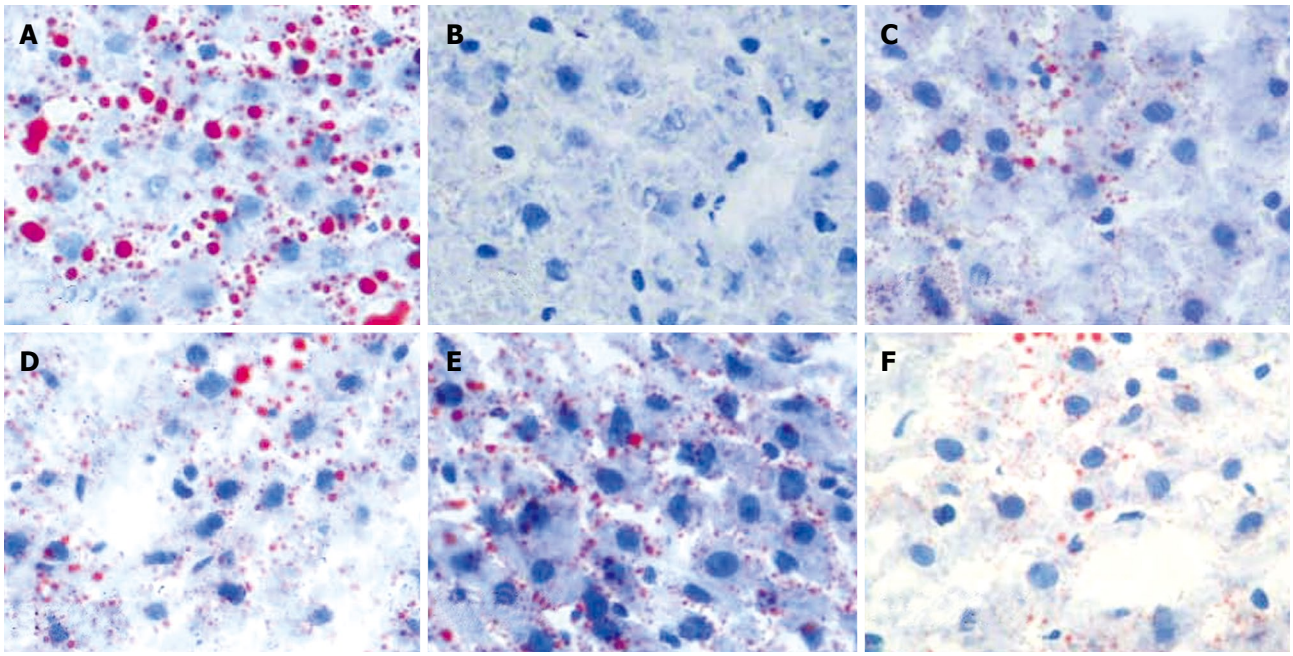

Figure 4 Hepatic tissue sections of each group in Sudan Ⅳ-staining (Sudan Ⅳ, light microscope, × 400), and lipid droplets are scarlet. A: Model; B: Control; C: *B. L66-5*; D: *B. L75-4*; E: *B. M13-4*; F: *B. FS31-12*.

was specific in regulating fat harvest and utilization, and may be a new therapeutic candidate for weight control.

All the four strains showed their effects in reducing serum TG and TCH, especially the strain *B. L66-5*. Administration of *B. L66-5* decreased the serum and liver TG and TCH and BW, and alleviated liver lipid deposition. Recent studies showed that probiotics had hypocholesteremia effects in both rat and human, including *Bifidobacterium longum*[6], *Lactobacillus acidophilus ATCC* 43121[4], *Lactobacillus plantarum MA2*[9], *Lactobacillus gasseri*[27], *Lactobacillus reuteri*[26,28], *Bacillus polyfermenticus SCD*[29], *etc*. Some specific probiotics strains could reduce serum TCH and TG, and increase the ratio of high-density lipoprotein/low-density lipoprotein (HDL/LDL). The mechanisms involved may be as follows: (1) assimilation of cholesterol by bacterial cells; (2) deconjugation of bile acids by bacterial acid hydrolyses (reduces cholesterol reabsorption, increases cholesterol excretion of deconjugated bile salts, and increases cholesterol uptake by low-density lipoprotein receptor pathway in the liver

as a compensatory response); (3) cholesterol binding to bacterial cell walls; and (4) inhibition of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver through the action of short-chain fatty acids, the end products of carbohydrate fermentation in the gut[30]. Furthermore, our results showed an individual amelioration effect in hepatic steatosis. The degree of fatty deposition in liver was obviously alleviated in all intervention groups as shown by liver histopathology. Probiotics VSL#3 also showed an effect in improving high-fat-diet induced hepatic steatosis in rats through lowering liver inflammatory signaling, increasing the expression of peroxisome proliferators-activated receptor α and hepatic natural killer T cell numbers[31,32]. However, the signaling capabilities of the four *Bifidobacteria* need further studies.

The four strains showed no significant differences in serum INS and glucose level. Similar negative results were also documented by Esposito *et al*[32] and Sato *et al*[5]. In our study, we fed the rats with high-fat diet for 6 wk, and none had a significant change of INS level, while in other HFD induced SD rats, insulin resistance did not occur until 8 mo or 36 wk[33,34]. So the next experimental period may prolong to 8 mo or longer.

In conclusion, we established a murine obese model based on a 6-wk administration of high-fat diet, which partially resembles the disorder of energy metabolism in human. The response of glucose and lipid metabolism to several strains of *Bifidobacteria* was evaluated. It was indicated that administration of the four strains of *Bifidobacteria* resulted in decreased serum/liver TG, serum/liver TCH, and hepatic steatosis, with no significant response to glucose and INS level. To our surprise, the data we presented demonstrated that administration of strain *B. L66-5* led to BW loss, decreased serum TG/TCH and decreased hepatic adiposity, while administration of strain *B. M13-4* resulted in significant increase of BW gain with alleviated hepatic adipose and serum/liver TG in rats. Thus, it is concluded that the response of energy metabolism to administration of *Bifidobacteria* is strain dependent. Different strains of *Bifidobacteria* might drive different directions of fat distribution. *B. M13-4* action may generate a new conception: certain probiotics may promote BW gain by more effective fat absorption, and a cautious assessment is needed before probiotics therapy is given, especially in obese people. *B. L66-5* might act as a new therapeutic probiotic candidate in controlling BW gain. Further studies should focus on evaluating how the administration of these *Bifidobacteria* modifies gut microbiota of obese rats.

COMMENTS

Background

Obesity is becoming a global epidemic. Recent researches have demonstrated that obesity may lead to the composition shift of gut microbiota in both mice and humans. The intentional manipulation of community structure of gut microbiota may be a novel strategy to treat obesity.

Research frontiers

Bifidobacterium is one of the most numerous "probiotic" in mammalian gut among commensal bacteria, and exhibited a significant effect in lowering serum total cholesterol. Specific strains of *Bifidobacteria* for energy metabolism may be helpful in management of obesity.

Innovations and breakthroughs

This study evaluates the effects of the administration of four strains of *Bifibofacteria* in obese rats. It demonstrated an interesting action of these strains on harvest energy from nutrients and regulation of lipid storage.

Applications

The manuscript gives new and interesting information about the key role of gut microbiota in the harvest energy from nutrients and regulation of lipid storage and metabolism.

Terminology

Probiotic bacteria are defined as living microorganisms that have beneficial effects in human health.

Peer review

This study evaluates the effects of the administration of four strains of *Bifibofacteria* on obese rats. It demonstrated an interesting action of these strains on harvest energy from nutrients and regulation of lipid storage. It is an interesting work that gives new information about the role of gut microbiota in host metabolism.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3402

World J Gastroenterol 2010 July 21; 16(27): 3402-3405
ISSN 1007-9327 (print)

BRIEF ARTICLE

Sessile serrated adenomas: Demographic, endoscopic and pathological characteristics

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Received: February 6, 2010 Revised: April 6, 2010
Accepted: April 13, 2010
Published online: July 21, 2010

Abstract

AIM: To study the demographic and endoscopic characteristics of patients with sessile serrated adenoma (SSA) in a single center.

METHODS: Patients with SSA were identified by review of the pathology database of Mayo Clinic Arizona from 2005 to 2007. A retrospective chart review was performed to extract data on demographics, polyp characteristics, presence of synchronous adenomatous polyps or cancer, polypectomy methods, and related complications.

RESULTS: One hundred and seventy-one (2.9%) of all patients undergoing colonoscopy had a total of 226 SSAs. The mean (SE) size of the SSAs was 8.1 (0.4) mm; 42% of SSAs were ≤ 5 mm, and 69% were ≤ 9 mm. Fifty-one per cent of SSAs were located in the cecum or ascending colon. Approximately half of the patients had synchronous polyps of other histological types, including hyperplastic and adenomatous polyps. Synchronous adenocarcinoma was present in seven (4%) cases. Ninety-seven percent of polyps were removed by colonoscopy.

CONCLUSION: Among patients with colon polyps, 2.9% were found to have SSAs. Most of the SSAs were located in the right side and were safely managed by colonoscopy.

Key words: Sessile serrated polyp; Sessile serrated adenoma; Colonoscopy

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Gurudu SR, Heigh RI, De Petris G, Heigh EG, Leighton JA, Pasha SF, Malagon IB, Das A. Sessile serrated adenomas: Demographic, endoscopic and pathological characteristics. *World J Gastroenterol* 2010; 16(27): 3402-3405 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3402.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3402

INTRODUCTION

Colorectal carcinoma (CRC) follows the adenoma-carcinoma sequence[1] in the majority of patients. CRC development secondary to DNA microsatellite instability (MSI-high) with a deficiency of DNA mismatch repair occurs only in a minority of patients[2,3]. MSI-high carcinoma usually progresses along the "serrated pathway"[4-6]. Historically, hyperplastic polyps (HPs) have usually been regarded as non-neoplastic lesions with no malignant

potential. However, some lesions previously diagnosed as HP but with malignant potential, which are now known as "sessile serrated adenomas" (SSAs), are increasingly being recognized[7,8]. SSAs may develop MSI as they progress toward carcinoma, and are potential precursors of sporadic microsatellite unstable CRC. Clinical and demographic characteristics including age, sex, racial distribution, endoscopic characteristics (location, gross appearance), recurrence rate, and the rate of progression of these polyps to carcinoma are not well known. There are no published consensus practice guidelines, and the optimal surveillance colonoscopy interval of these polyps is unknown, due to the paucity of clinical information regarding these patients.

The aim of our study was to identify demographic and endoscopic characteristics of patients with SSA in a single center and report the clinical experience with SSA in a large group practice.

MATERIALS AND METHODS

Patients with SSA were identified by review of an institutional pathology database from the years 2005-2007. Patient charts were reviewed for data on demographics and colonoscopy details. Colonoscopy reports were reviewed to obtain the number, size, and location of polyps, as well as the morphological appearance and the polypectomy methods. In addition, data were collected on synchronous adenomatous polyps or cancer, and the number of colonoscopies needed to eradicate the polyps, the need for surgical therapy, and colonoscopy-related complications. All colonoscopies were done with standard white light examination technique with standard definition colonoscopes. SSA was defined in practice and in this report as polypoid lesions that lacked typical or conventional dysplasia, but that showed architectural features of disordered growth[9,10]. These features are: basal crypt dilatation, horizontal orientation of deep crypts, prominent serration extending deep into the crypts, irregular crypt branching, and inverted crypts. Other criteria consistent with abnormal proliferation were also useful in the diagnosis of SSA; these were nuclear atypia and/or oval nuclei in mid/upper crypts, prominent nucleoli in middle/superficial crypts, dystrophic goblet cells, irregular distribution of goblet cells, mitoses in mid/upper crypts, and excessive crypt or luminal mucin (Figure 1). We classified the polyps as SSA even if the diagnostic changes were only identified focally.

Descriptive analysis was performed using statistical software (SPSS version 17; SPSS Inc., Chicago, IL, USA) to study the demographic and clinical characteristics of these patients.

RESULTS

Among patients undergoing colonoscopy from 2005 to 2007 (21 238 colonoscopies), a total of 5991 patients were found to have polyps. Of these, 171 (2.9%) patients had a total of 226 SSAs. The majority of patients with SSAs in our study were Caucasian (164, 96%) with a mean (SE) age of 65.9 (0.8) years and a mean body mass index of 29.6 (0.4). Ninety-one patients (53%) were male. The SSA characteristics are summarized in Table 1. The mean size of the SSAs was 8.1 (0.4) mm (range 2-40 mm); 42% were ⩽ 5 mm and 69% were ⩽ 9 mm in size. The majority of SSAs were located in the right side of the colon (Figure 2). All SSAs were described as sessile or flat in appearance (Figure 3). The mean number of SSAs per patient was three (range 1-24); 58 (34%) patients had one SSA, 43 (25%) had two and 70 (41%) had three or more. Approximately half (87, 51%) of the patients had synchronous polyps of other histological appearance. The most common type of associated pathology in these synchronous polyps was tubular adenoma in 62 (49%) polyps, tubulovillous adenoma (TVA) in 13 (10%) and hyperplastic pathology in 50 (40%). One polyp was a small carcinoid. Synchronous adenocarcinoma was present in seven (4%) cases and all of these cancers were present in the cecum or ascending colon.

Thirty-one percent of SSAs were removed by cold biopsy/cold snare, 4% by hot biopsy, 63% by snare cautery, and 2.7% required surgical excision due to size or associated malignancy. Polyp removal techniques depended on the judgment and preference of the endoscopist. All polyps ⩾ 2 cm in size were removed by saline-assisted polypectomy. There were no complications associated with the endoscopic resection of the SSAs. Although up to nine pathologists interpreted the histopathology of these polyps, the majority (75%) were diagnosed as SSA by two

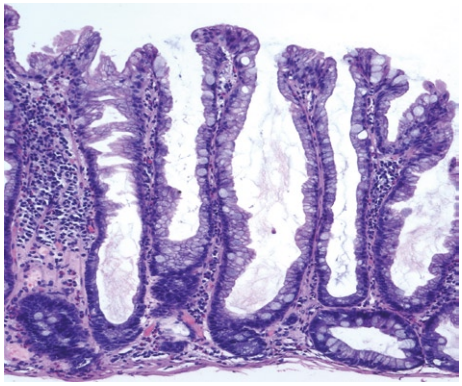

Figure 1 Sessile serrated adenoma. Flask-shaped glands with dilated and irregular architecture of the gland bases with abundant luminal mucin.

Table 1 Sessile serrated adenoma characteristics

Mean size of SSA	8.1 mm (range 2-40 mm) ⩽ 5 mm (42%), ⩽ 9 mm (69%)
Mean No. of SSAs per patient	3 (range 1-24)
Location of SSAs	51%: Cecum, ascending colon 49%: Remaining colon
Appearance	Flat or sessile (all polyps)
Synchronous polyps of other histology	Present in 87 (51%) patients TA: 62 (49%) TVA: 13 (10%) HP: 50 (40%)
Synchronous adenocarcinoma	7 (4%), all in right side of the colon

SSA: Sessile serrated adenoma; TA: Tubular adenoma; TVA: Tubulo-villous adenoma; HP: Hyperplastic polyp.

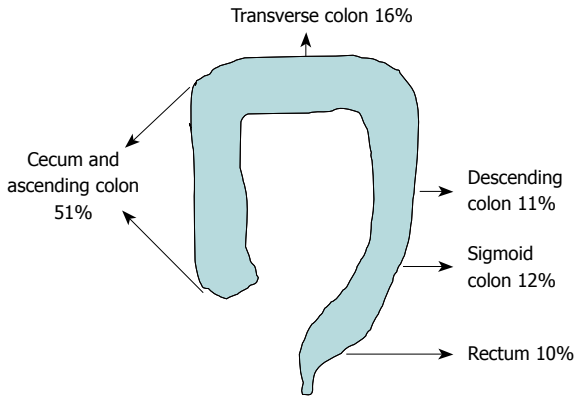

Figure 2 Distribution of sessile serrated adenomas in patient population.

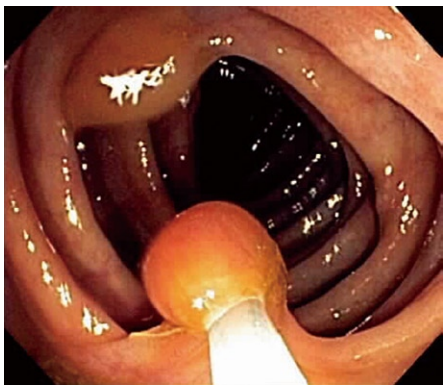

Figure 3 Colonoscopic image of sessile serrated adenoma (sessile, flat with yellow appearance).

highly experienced gastrointestinal pathologists. In the clinical practice of pathologists interpreting specimens, in 12.6% cases, the diagnosis of SSA was confirmed in consultation by a second pathologist within the department.

DISCUSSION

Colorectal serrated polyps are a group of morphologically related lesions that include aberrant crypt foci, conventional HPs, advanced serrated adenomas (ASAs)[11] and SSAs. These lesions share some histological features, however, they differ significantly at the molecular level, and are biologically distinct[12]. In 1990, Longacre and Fenoglio-Preiser[13] first used the term serrated adenoma. While many patients probably had serrated lesions described as hyperplastic in the past, some have mixed hyperplastic and adenomatous features. Unfortunately, often there is significant disagreement among pathologists in the diagnosis of these polyps. In a study of 185 serrated polyps, five gastrointestinal pathologists had only moderate overall agreement ($\kappa = 0.58$), with near perfect agreement on traditional serrated adenoma, also called ASA, but varying most in the SSA and hyperplastic categories[14]. In our study, the majority of these polyps (75%) were diagnosed as SSA by two experienced gastrointestinal pathologists, however, up to nine pathologists interpreted the histopathology of polyps. In 12.6% of our cases, a second pathologist's opinion was obtained by intradepartmental consultation for diagnosing SSA, which suggests difficulty in day to day clinical interpretation using standard published criteria. Simple standardized diagnostic criteria and terminology could improve interobserver agreement among pathologists, and represent a limitation in the interpretation of this study, and an opportunity for improvement in overall clinical practice.

In the present study, approximately 3% of all polyps were SSAs, which is similar to previous studies[15,16]. However, a prospective study using magnifying chromoendoscopy has reported a 9% prevalence of SSAs[17]. All examinations in our study were performed with standard white light colonoscopes, and the diagnosis of SSAs was made in real time clinical practice between 2005 and 2007. Polyps in the database were not re-examined, which could have changed the actual prevalence. Endoscopically, SSAs commonly appear flat or sessile, have a soft, smooth surface, and are often covered with mucus, which gives an initial yellow appearance[9]. Small, flat, right-sided lesions potentially can be missed by white light colonoscopy. Newer techniques such as high definition colonoscopy, narrow band imaging or chromoendoscopy could help to distinguish these polyps from normal mucosa.

The majority of patients with SSA in our study had multiple SSAs (mean = 3, range = 1-24). Approximately half of these patients also had synchronous polyps of other histological type, such as tubular adenoma, TVA and HPs. Synchronous right-sided adenocarcinoma was seen in 4% of cases.

As noted in other studies[15,18,19], the majority (67%) of SSAs in our study were located proximal to the splenic flexure. Forty-two percent of all SSA were $\leqslant$ 5 mm, and 69% were $\leqslant$ 9 mm in size. In a study of 13 992 colonoscopies from the Clinical Outcomes Research Initiative (CORI) repository[20], when pathology results were available, 1.7% of polyps that measured 5 mm ($n = 3744$) had advanced neoplasia [one cancer, one high-grade dysplasia (HGD), 44 TVA]. In polyps 6-9 mm in size ($n = 1198$), 6.6% had advanced neoplasia (two cancers, nine HGD, and 53 TVA), which suggests that not all small polyps are innocent. The natural history of SSAs is not well understood. Although carcinoma associated with advanced serrated adenoma has been reported to be a distinct type of neoplasm, which accounts for 5.8%-7.5% of all colorectal carcinomas and up to 17.5% of proximal colon cancers[21,22], the overall impact of SSAs on cancer risk is not known because of a lack of data on their true prevalence. There have been reports of rapid progression of SSA to invasive cancer as early as within 8 mo [23]. The current surveillance interval recommendation[24,25] for small SSAs is mainly based on indirect evidence, and is similar to that for small adenoma (5 years). Surveillance of large SSAs or more than three SSAs is similar to large or advanced adenoma (3 years).

The retrospective design and lack of long-term longitudinal follow-up data are major limitations of our study. Demographically, our patient population was predominantly Caucasian and above ideal body weight. These factors could limit the applicability of our results to other populations. Future large prospective studies are needed to understand the natural history of SSAs and to establish

clinical practice guidelines for optimal cost-effective management and surveillance of patients with SSA.

COMMENTS

Background

Colorectal carcinoma (CRC) usually follows a sequence of development from adenomatous polyp to carcinoma. In a minority of patients, CRC development occurs due to alterations of small areas of DNA known as microsatellite instability (MSI). These patients have a defect in their DNA repair mechanism. Carcinomas that develop by this mechanism often have a special serrated appearance. One type of polyp with a serrated appearance, hyperplastic polyps (HPs), have usually been regarded as non-neoplastic lesions with no malignant potential. However, some lesions previously diagnosed as HPs, but with malignant potential, are now known as sessile serrated adenomas (SSAs), and are increasingly being recognized. SSAs can develop MSI as they progress toward carcinoma, and are potential precursors of sporadic microsatellite unstable CRC.

Research frontiers

SSA are uncommon colon polyps. The clinical and demographic characteristics of patients with these polyps, including age, sex, racial distribution, endoscopic characteristics (location, gross appearance), recurrence rate, and rate of progression of these polyps to carcinoma are not well known, and were evaluated in this study.

Innovations and breakthroughs

Between 2005 and 2007, 171 (2.9%) patients undergoing colonoscopy had a total of 226 SSAs. The average size of the SSAs was 8.1 mm. Forty-two percent of SSAs were ⩽ 5 mm and 69% were ⩽ 9 mm. Fifty-one per cent of SSAs were located in the cecum or ascending colon. Approximately half of the patients had coexisting colon polyps of other histological types, including hyperplastic and adenomatous polyps. Coexisting adenocarcinoma was present in seven (4%) cases. Ninety-seven percent of polyps were removed by colonoscopy.

Applications

Most of the SSAs were located in the right side of the colon and were safely managed by colonoscopy. Concurrent lesions including adenomas and right-sided colon cancers were not uncommon. The natural history of SSAs is being learned, and greater concern about small polyps might exist in the future, as some can be aggressive or associated with other lesions.

Peer review

This is an extensive study of a very important topic. The disadvantage of the study is its retrospective nature; especially concerning the endoscopic findings and follow-up.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3406

World J Gastroenterol 2010 July 21; 16(27): 3406-3410
ISSN 1007-9327 (print)

BRIEF ARTICLE

Can chronic gastritis cause an increase in fecal calprotectin concentrations?

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Received: March 3, 2010 Revised: March 30, 2010
Accepted: April 6, 2010
Published online: July 21, 2010

Abstract

AIM: To evaluate fecal calprotectin concentrations (FCCs) in subjects with chronic gastritis and the correlation between FCCs and gastritis activity score.

METHODS: FCCs were measured in 61 patients with histological diagnosis of gastritis and in 74 healthy volunteers. Histological grading of gastritis was performed according to the updated Sydney gastritis classification. Patients were subdivided into 2 groups according to the presence/absence of an active gastritis. Patients with chronic active gastritis were divided into 3 subgroups on the basis of the activity score (mild, moderate, marked). FFCs in relation to *Helicobacter pylori* (*H. pylori*) infection and proton pump inhibitor (PPI) use were also evaluated.

RESULTS: FCCs in patients with chronic active gastritis were not significantly different to FCCs either in subjects with non active gastritis or in healthy controls. Among patients with chronic active gastritis (even marked), FCCs did not significantly differ according to activity score. No significant differences in FCCs were found when considering *H. pylori*, as well as when considering PPI chronic use.

CONCLUSION: FCCs were not significantly increased in subjects with chronic gastritis, even in those patients with a marked neutrophil infiltration.

Key words: Chronic gastritis; Fecal calprotectin; Intestinal inflammation; Neutrophils

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Montalto M, Gallo A, Ianiro G, Santoro L, D'Onofrio F, Ricci R, Cammarota G, Covino M, Vastola M, Gasbarrini A, Gasbarrini G. Can chronic gastritis cause an increase in fecal calprotectin concentrations? *World J Gastroenterol* 2010; 16(27): 3406-3410 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3406.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3406

INTRODUCTION

Calprotectin is a calcium and zinc binding protein, mainly contained in neutrophils where it accounts for more than 60% of cytosolic proteins. It has well-known antimicrobial activity, both bacterial and fungicidal[1]. Elevated concentrations of calprotectin can be measured in plasma, synovial fluid, urine, liquor, saliva and feces when an

inflammation process with recruitment of neutrophils is ongoing[2,3]. In particular, the presence of calprotectin in feces quantitatively relates to neutrophil migration towards the gastrointestinal tract[4]. Its levels are closely correlated with the fecal excretion of ^{111}In-labelled leukocytes[5]. Therefore, it is considered a useful marker of intestinal inflammation[6]. Several recent studies reported a significant increase in fecal calprotectin concentrations (FCCs) in intestinal conditions characterized by a conspicuous neutrophil infiltrate, such as inflammatory bowel diseases (IBDs) and non-steroidal antiinflammatory drug (NSAID) enteropathy[7-9]. It may accurately distinguish IBD from non-IBD conditions (such as irritable bowel syndrome)[10,11]. It has also been proposed as a reliable marker able to predict clinical relapse in IBD patients[12,13]. Diagnostic accuracy of FCCs in colorectal neoplasia has not been univocally established yet[14].

Chronic gastritis represents a common and heterogeneous inflammatory process. It can be morphologically characterized by a variable inflammatory infiltrate in the lamina propria, within the epithelium and within the foveolar lumen[15]. According to the updated Sydney System, the presence of a neutrophil infiltrate characterizes the "activity" of gastritis[15].

The aim of our study was to evaluate FCCs in subjects with chronic gastritis and the possible correlation between FCCs and the activity score, according to the updated Sydney System gastritis classification.

MATERIALS AND METHODS

Patients

Between May 2008 and December 2008, subjects who were referred to the Endoscopy Center of "Gemelli Hospital" for upper gastrointestinal endoscopy, were invited to enter the study. In those subjects who agreed to participate in the study, the extraction of at least 5 biopsy samples (2 from the antrum, 2 from the corpus and one from the incisura angularis) had been undertaken to correctly characterize an eventual gastritis process, in accordance with Sydney's recommendations[15]. However, when esophageal lesions, gastric ulcers, gastric polyps or duodenal lesions were found during the endoscopy, the necessary biopsy specimens were taken, and these subjects were not included in the study. In addition, subjects with IBDs or family history of IBDs, colorectal cancer, chronic use of NSAIDs, history of gastric resection, coexisting and severe cardiopulmonary, hepatic, renal, neurologic, psychiatric, endocrine and rheumatologic diseases, malignancy, pregnancy, alcohol abuse, other intestinal disorders characterized by increased mucosal permeability and inflammatory changes, were not considered for the study.

All the eligible subjects were asked to provide a stool sample for measurement of calprotectin levels, within 2 d of endoscopic examination, before starting specific therapy. Stools were also examined to exclude infectious intestinal diseases. All subjects were asked if they were taking proton pump inhibitor (PPI) therapy for at least since 1 mo before the endoscopy.

According to the updated Sydney System, depending on the presence/absence of a neutrophil infiltrate, patients with chronic gastritis were divided into 2 groups: group A which consisted of patients with active gastritis and group B which consisted of patients with non active gastritis. Furthermore, adult healthy volunteers participated as a further control group, providing their own stool sample.

Procedures were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Each subject gave written informed consent for the study. The study was approved by the Institutional Board of Department of Internal Medicine, Catholic University of Rome.

Histological evaluation

The biopsy samples were fixed in 4% buffered formalin, processed in the usual manner, and paraffin embedded. The sections were stained with hematoxylin and eosin for histological evaluation; Giemsa stain was also used to evaluate the presence of *Helicobacter pylori* (*H. pylori*). The sections were evaluated by 2 separate expert gastrointestinal pathologists working blind. The degree of activity of inflammation was assessed using a semiquantitative 3-tiered scale (mild, moderate, marked) according to the updated Sydney System[15]. The infiltration of neutrophil granulocytes was defined as "mild" when isolated cells of this type were identified in the lamina propria only with difficulty, after a thorough search; it was defined as "moderate" if neutrophils were either easily detectable in the lamina propria or were found within the epithelium, provided they were not crowded; finally, the infiltration was defined as "marked" when a dense neutrophil infiltrate, usually involving both lamina propria and epithelium, was strikingly evident at low power magnification. When activity differed among antrum, corpus and incisura angularis, the activity grade in the most severely affected compartment was considered; when activity grade changed among different biopsies of the same gastric compartment, the predominant grade was considered, according to the updated Sydney classification[16]. *H. pylori* status was evaluated as present/absent in all the examined biopsy samples. *H. pylori* density score was graded as mild, moderate and marked, according to the updated Sydney classification[16].

Fecal calprotectin measurement

Each subject was instructed to collect and return a single stool sample within 48 h of defecation. Upon receipt, the stools were frozen and stored at -20℃ for subsequent biomarker determination.

The stool samples were prepared and analyzed according to the manufacturer's instructions (Calprest; Eurospital SpA, Trieste, Italy). A portion of each sample (40-120 mg) was measured and an extraction buffer containing citrate and urea was added in a weight per volume ratio of 1:50. The samples were mixed for 30 s by a vortex method and homogenized for 25 min. One

Table 1 Demographic data and mean fecal calprotectin concentrations of the different study groups (mean ± SD)

Groups	*n*	Sex (M/F)	Age (yr)	FCCs (μg/g)
Patients	61	28/33	49.64 ± 13.80	28.25 ± 23.43
Active gastritis	35	15/20	49.66 ± 14.15	29.70 ± 21.26
Mild	15	6/9	48.07 ± 14.56	31.44 ± 22.55
Moderate	10	7/3	47.60 ± 13.72	31.08 ± 23.68
Marked	10	2/8	53.30 ± 14.64	26.57 ± 17.66
Non active gastritis	26	13/13	49.90 ± 13.61	25.97 ± 22.55
Healthy controls	74	32/42	45.93 ± 12.42	31.20 ± 19.18

FCCs: Fecal calprotectin concentrations.

milliliter of the homogenate was transferred to a tube and centrifuged for 20 min. Finally, the supernatant was collected and frozen at -20℃. In most cases, time from sampling to preparation and freezing was estimated to be 1-3 d, except for a few samples that took 4-6 d before handling. The supernatants were thawed and analyzed later with Calprest, a quantitative calprotectin ELISA, for determination of calprotectin in stools. The within-assay coefficient of variation was 1.5%. Calprotectin was expressed as μg/g of feces.

Statistical analysis

Statistical comparison of age and sex among patients with chronic active gastritis, non active gastritis and healthy controls was performed by the *t*-test for unpaired data and χ^2 test. FCCs among subjects with active gastritis, non active gastritis and healthy controls groups were compared by the *t*-test for unpaired data.

In subjects with chronic active gastritis, FCC differences among the subgroups identified by density of neutrophil infiltration (activity score) were analyzed by means of one-way analysis of variance (ANOVA). The *post hoc* effect was assessed by Bonferroni *t*-test. Comparison between FCCs and *H. pylori* status, FCCs and PPI use, was performed by means of the *t*-test for unpaired data. FCC differences among the subgroups identified by density of *H. pylori* infection were analyzed by ANOVA. The *post hoc* effect was assessed by the Bonferroni *t*-test. The statistical analysis of categorical parameters was performed by the χ^2 test. All values were assessed as mean ± SD. A *P*-value of 0.05 or less was regarded as significant.

RESULTS

During the study period, 929 subjects had an upper intestinal endoscopy; 696 were ruled out on the basis of the above-mentioned exclusion criteria. Of the 247 eligible patients, 61 Caucasians (28 male, 33 female, mean age 49.64 ± 13.80 years) gave their consent to participate in the study. Seventy four adult healthy volunteers (32 male, 42 female, mean age 45.93 ± 12.42 years) entered the study as controls. The demographic data of the different study groups are summarized in Table 1. There were no significant differences between the groups regarding age and sex.

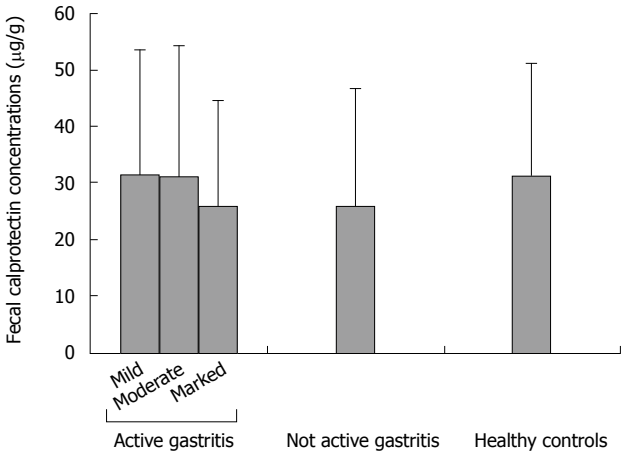

Figure 1 Mean fecal calprotectin concentrations ± SD in the different study groups.

According to the updated Sydney System classification, 35 patients showed chronic active gastritis (group A); in particular, 15 showed mild activity, 10 moderate activity and 10 marked activity. When separately analyzed by antrum gastritis activity (AGA) and corpus gastritis activity (CGA), 21 patients showed predominant antral activity (4 mild AGA without CGA; 7 moderate AGA, 6 with mild CGA and 1 without CGA; 10 marked AGA, 7 with mild CGA and 3 with moderate CGA), 2 showed predominant corpus activity (both with mild CGA without AGA), and the other 12 patients showed a concordant activity between antrum and corpus (9 mild and 3 moderate in both AGA and CGA).

Of the 26 patients with non active chronic gastritis (group B), 15 showed a predominant chronic mononuclear infiltrate, 4 intestinal metaplasia and 7 glandular atrophy.

Mean FCCs were not significantly different between group A and group B, and they both did not differ significantly from FCCs in healthy volunteers (*P* = NS for all comparisons).

When considering only patients with chronic active gastritis, mean FCCs were not significantly different among the 3 subgroups identified by the different degree of neutrophil infiltrate (Table 1 and Figure 1). Also, when separately considering antrum and corpus gastritis, mean FCCs did not correlate with the degree of activity in either subgroup.

When considering the presence of *H. pylori* infection in the whole study group, 24 patients were *H. pylori* positive (7 with mild infection, 8 moderate and 9 marked), while 37 patients were *H. pylori* negative; mean FCCs neither significantly differed between the 2 subgroups (27.35 ± 22.64 *vs* 28.84 ± 24.21, *P* = NS), nor correlated with degree of *H. pylori* infection (*P* = NS for all comparisons). On the other hand, both the presence and density of *H. pylori* significantly correlated with neutrophilic infiltration. In particular, in subjects with chronic active gastritis, 5/15 (33%) with a mild active gastritis, 8/10 (80%) with a moderate active gastritis, and 10/10 (100%) with a severe active gastritis, were *H. pylori* positive, whereas in the group

with non active gastritis, only 1/26 (3.8%) was *H. pylori* positive ($P < 0.001$). In addition, when considering *H. pylori* density, of the 7 patients with a mild *H. pylori* density score, 4 showed mild active gastritis and 3 moderate active gastritis; of the 8 patients with moderate *H. pylori* density, 3 showed moderate active gastritis and 4 showed marked active gastritis, while one had non active gastritis; of the 9 patients with marked *H. pylori* density, one showed mild active gastritis, 2 moderate active gastritis and 6 marked active gastritis ($P < 0.05$).

Finally, when considering PPI use, 22 patients were on PPI therapy and 39 patients were not; mean FCCs were not significantly different between the 2 groups (32.88 ± 25.90 *vs* 25.64 ± 25.83, P = NS).

DISCUSSION

Our study showed no significant differences in FCCs between patients with chronic active gastritis and non active chronic gastritis controls, regardless of the degree of neutrophil infiltration. In addition, FCCs in both groups did not significantly differ with regard to that in healthy controls.

Fecal calprotectin has recently emerged as a reliable marker of intestinal inflammation[14]. Different studies regarding fecal calprotectin have been carried out in bowel diseases, mainly IBDs[8-11]. Up to now, no specific studies have been designed to evaluate FCCs in upper gastrointestinal tract diseases. The few available data on this topic can only be gathered from studies evaluating FCCs in different conditions throughout the gastrointestinal tract. In this regard, only Summerton *et al*[17], in 2002, performed a study evaluating FCCs in different gastrointestinal inflammatory and cancer conditions. In particular, 26 patients showed upper gastrointestinal inflammation because of gastritis and duodenitis. FCCs were in the normal range in all these subjects. Nevertheless, a correlation between FCCs and histological severity of inflammation was not performed in this study.

Chronic gastritis is a very common clinical condition. The updated Sydney System provided the term "activity" as an expression of the presence of neutrophils on a background of chronic inflammation[15].

As expected, we found that patients with non active chronic gastritis did not show increased FCCs, since a neutrophil infiltrate is lacking in these conditions. Nevertheless, we also found that FCCs were not significantly increased in active chronic gastritis, even in subjects with a marked activity score (and so a high grade of neutrophil infiltration). This result could be explained by the consideration that the inflammatory process, and in particular the neutrophil recruitment occurring in gastritis, is far less severe than in other intestinal conditions, mainly IBDs. Furthermore, our findings can be also explained by the smaller extent of inflamed tissue found in gastritis with respect to that in IBDs. In this regard, Sipponenn *et al*[18] reported that subjects with ileal Crohn's disease showed lower fecal markers (calprotectin and lactoferrin) compared to subjects with colonic involvement. They supposed that this finding might be explained by the limited extent of ileal disease, even in the presence of endoscopic and histological inflammation. In addition, in 39 children with IBDs, it has been shown that FCCs were closely related not only to disease severity, but also to disease extent[19].

In our study, we did not consider all those subjects with endoscopic findings involving the esophagus, duodenum or with gastric polyps and ulcers, because our aim was to evaluate FCCs only in chronic gastritis, relating these levels to the neutrophil infiltrate classified according to a validated histological score. Further studies, specifically aimed at this purpose, should clarify if FCCs might be increased in other upper gastrointestinal diseases different from chronic gastritis.

It has been reported that a neutrophil infiltrate is almost always present in *H. pylori* gastritis and usually disappears within a few days of antibiotic therapy[20]. In agreement with data in the literature, we found that the gastritis activity score was closely correlated with the degree of *H. pylori* infection. In particular, only one patient with an absent neutrophil infiltrate was *H. pylori* positive, while all 10 patients with marked active gastritis showed *H. pylori* infection. However, no significant differences were found when FCCs was compared between *H. pylori*-positive and *H. pylori*-negative subjects, regardless of *H. pylori* density score.

Concerning the relationship between PPI therapy and FCCs, Poullis *et al*[21], in a letter, reported that patients using PPIs had significantly higher FFCs compared to those not on PPIs. Nevertheless, data on their population were lacking; they did not undergo endoscopy, and other causes of increased FCCs, such as IBDs and NSAID use were not excluded. On the other hand, it has been reported that PPIs may also inhibit proton pumps present on membranes of phagolysosomes of neutrophils, interfering with neutrophil release of reactive oxygen species, commonly mediated by lysosomal acidification[22,23]. We found that FCCs were not significantly different between subjects taking PPIs and subjects who did not, thus suggesting that gastric pH is unlikely to be responsible for the low levels of FCC we found. On the other hand, it is not possible to exclude that gastric acidity could interfere with FCCs. Until now, no data have been available on this topic and further studies should be encouraged.

In conclusion, we showed that in subjects with chronic active gastritis, even marked, FCCs were not significantly increased when compared with FCCs either in subjects with non active gastritis or in healthy controls. Thus we recommend that in subjects with high FCCs, causes of gut inflammation other than chronic gastritis should be checked.

COMMENTS

Background

Fecal calprotectin is a valid marker of intestinal inflammation, being quantitatively related to neutrophil migration towards the gastrointestinal tract. Fecal calprotectin concentrations (FCCs) are significantly increased in intestinal diseases characterized by a conspicuous neutrophil infiltration, mainly inflam-

matory bowel diseases. Chronic gastritis morphologically shows a variable neutrophil infiltrate, which characterizes the gastritis activity, according to the updated Sydney System classification.

Research frontiers

No systematic study has ever been performed to evaluate FCCs in subjects with chronic gastritis and the possible correlation between FCCs and gastritis activity score.

Innovations and breakthroughs

The authors found no significant difference between FCCs in patients with chronic active gastritis and FCCs either in subjects with non active gastritis or in healthy controls. Among patients with chronic active gastritis (even marked), FCCs did not correlate with the activity score.

Applications

The authors recommend that in subject with high FCCs, causes of gut inflammation other than chronic gastritis should be checked.

Terminology

Calprotectin: A calcium and zinc binding protein, mainly contained in neutrophils where it accounts for more than 60% of cytosolic proteins. It has well-known antimicrobial activity, both bacterial and fungicidal. Elevated concentrations of calprotectin can be measured in plasma, synovial fluid, urine, liquor, saliva and feces when an inflammation process with recruitment of neutrophils is ongoing. The presence of calprotectin in feces quantitatively relates to neutrophil migration towards the gastrointestinal tract. Active gastritis: according to the updated Sydney System, the presence of a neutrophil infiltrate characterizes the "activity" of gastritis.

Peer review

The study is set up correctly. The material studied is big enough to allow conclusions to be drawn. The paper is written sufficiently well, the Introduction gives a good overview of the study background and the authors clearly raised the hypothesis of the study. The description of the method and material studied is accurate. The aim of the study is fulfilled. The Results are presented clearly and have been discussed sufficiently well.

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3411

World J Gastroenterol 2010 July 21; 16(27): 3411-3417
ISSN 1007-9327 (print)

BRIEF ARTICLE

Pancreatic and pulmonary mast cells activation during experimental acute pancreatitis

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Supported by The Project SAF2006-08449 and a grant from Fundación para la Investigación del Hospital General Universitario de Alicante; Inmaculada Lopez-Font has a Juan de la Cierva contract supported by the Spanish Ministry of Science and Innovation
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Received: January 13, 2010 Revised: March 25, 2010
Accepted: April 1, 2010
Published online: July 21, 2010

Abstract

AIM: To study the activation of pancreatic and pulmonary mast cells and the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages during acute pancreatitis.

METHODS: Pancreatitis was induced by intraductal infusion of 5% sodium taurodeoxycholate in rats. The mast cell inhibitor cromolyn was administered intraperitoneally (i.p.) 30 min before pancreatitis induction. The pancreatic and pulmonary tissue damage was evaluated histologically and mast cells and their state of activation were evaluated. Peritoneal and alveolar macrophages were obtained and the expression of tumor necrosis factor α was determined. Myeloperoxidase activity was measured to evaluate the effect of mast cell inhibition on the progression of the inflammatory process. Finally, the effect of plasma on cultured mast cells or macrophages was evaluated *in vitro*.

RESULTS: The mast cell stabilizer significantly reduced inflammation in the pancreas and lung and the activation of alveolar macrophages but had no effect on peritoneal macrophages. Mast cell degranulation was observed in the pancreas during pancreatitis but no changes were observed in the lung. Plasma from rats with pancreatitis could activate alveolar macrophages but did not induce degranulation of mast cells *in vitro*.

CONCLUSION: Pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the inflammatory response related to pancreatic damage.

Key words: Cytokines; Inflammation; Macrophages; Mast cells; Pancreatitis

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Lopez-Font I, Gea-Sorlí S, de-Madaria E, Gutiérrez LM, Pérez-Mateo M, Closa D. Pancreatic and pulmonary mast cells activation during experimental acute pancreatitis. *World J Gastroenterol* 2010; 16(27): 3411-3417 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3411.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3411

INTRODUCTION

Acute pancreatitis represents a substantial clinical problem with increasing incidence and it is associated with high morbidity and mortality[1]. The most important predictor of mortality is the development of persistent or multiple organ failure and the commonest affected organ is the lung[2,3]. In these cases, acute lung injury is frequently related to early deaths in the first week of the disease[4]. The mechanisms involved in triggering distant organ inflammation are unclear, however, in addition to the release of activated hydrolytic enzymes, different pathways have been reported, including cytokines[5], oxygen-derived free radicals[6] or activated complement[7].

Among these mechanisms, mast cells have been reported to contribute to several aspects of pancreatitis-associated lung injury. These cells release a number of mediators, including histamine, tumor necrosis factor (TNF)α or monocyte chemotactic protein-1 (MCP-1) which could have a strong effect on pulmonary endothelial cells, thus potentiating the progression of inflammation[8,9]. The expression of different adhesion molecules increases early after pancreatitis induction, and in some of these molecules this increase could be prevented by administering mast cell degranulation inhibitors such as sodium cromolyn[10,11]. These observations suggest that masts cells are responding to mediators released during pancreatitis and, when activated, play a role in the induction of endothelial lung dysfunction and in the progression of the local and systemic inflammatory process.

Mast cells are usually located close to endothelial cells and this explains their effect on endothelial dysfunction when activated during pancreatitis. However, tissue-related variability on the number, phenotype and distribution of mast cell populations have been reported, resulting in different activation during inflammatory processes[12]. In the case of acute pancreatitis, the use of mast cell stabilizers prevented changes in systemic inflammation and in endothelial permeability in different organs[10], however, the involvement of the particular mast cell populations remains unclear.

In this work we have evaluated the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages in an experimental model of acute pancreatitis.

MATERIALS AND METHODS

Animal model of acute pancreatitis

Male Wistar rats (250-300 g b/w) (n = 6 each group) were anaesthetized with 10% urethane (1 mL/100 g, i.p.). The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Severe acute pancreatitis was induced by retrograde infusion into the biliopancreatic duct of 5% sodium taurocholate (Sigma Chemical, St. Louis, MO, USA) in a volume of 0.1 mL/100 g b/w using a *Harvard* '22' infusion pump (Harvard Instruments, Edenbridge, UK)[13]. Control animals received an intraductal infusion of saline solution (0.9% NaCl). In a group of animals, cromolyn (Sigma, St Louis, MO, USA) (5 mg/kg b/w) was administered i.p. 30 min before pancreatitis induction. Three hours after induction, tissue samples of pancreas and lung were obtained, immediately frozen and maintained at -80℃ until processed. This time point was selected because we previously reported that, in this model, a significant systemic inflammation is initiated three hours after the induction of pancreatitis[14]. Plasma samples were pelleted and the supernatant was stored at -40℃ until use. Pancreas and lung samples were also obtained and stored for histological analysis.

Histological analysis

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraplast, sectioned in 5 μm slices and stained with toluidine blue (0.1%). The dye was allowed to dry on the slide for a few seconds; the slides were then rinsed in xylene for 5-10 min. and rinsed twice with acetone. Finally, the slides were cleared in xylene and mounted in diphenylphthalein xylene. Sections were evaluated by light microscopic examination.

Cell culture

Peritoneal macrophages were harvested by 5 peritoneal washes with 10 mL of phosphate buffered saline (PBS) containing 3 units/mL heparin. The obtained cell suspension was centrifuged (300 × g, 7 min). Cells were suspended in the RPMI1640 culture medium containing 10% fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). Aliquots of about 3×10^6 cells were plated in 6 well plates and cultured at 37℃ under a gas phase of air/CO_2 (95:5). After an attachment period of 4 h, the non-adhered cells were removed by shaking. The resulting adherent population consisted of > 92% peritoneal macrophages.

Alveolar macrophages were obtained by bronchoalveolar wash. After exsanguinations, lung and trachea were excised *en bloc* and washed 5 times with 10 mL cold (4℃) saline solution. The supernatant was centrifuged at 300 × g for 7 min and the cells were resuspended in RPMI1640 culture medium containing 10% fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). Aliquots of about 3×10^6 cells were plated in 6 well plates and cultured at 37℃ un-

der a gas phase of air/CO_2 (95:5). After an attachment period of 4 h, the non-adhered cells were removed by shaking. The resulting adherent population consisted of > 95% alveolar macrophages.

The rat mast cell line RBL-2H3 was maintained as a monolayer culture in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an incubator with 5% CO_2 at 37℃.

In vitro effect of plasma

The effects of circulating mediators on mast cells or macrophages were evaluated by incubating alveolar macrophages or the mast cell line RBL-2H3 with plasma obtained from controls, animals with pancreatitis or with cromolyn treated pancreatitis. Cells were cultured in 12 well plates in the presence of 20% plasma in the culture media. One hour after culture at 37℃, the levels of histamine were measured in RBL-2H3 supernatants. For macrophages, RNA was obtained and the expression of TNFα was evaluated by quantitative real-time polymerase chain reaction (RT-PCR).

RNA isolation and RT-PCR

Total RNA from cells was extracted using the TRizol® reagent (Invitrogen, Carlsbad, CA, USA). The RNA was quantified by measurement of the absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), and reverse transcription was then performed on 1 μg RNA sample by adding iScript reagents. The reaction was incubated at 25℃ for 5 min, 42℃ for 30 min, and 85℃ for 5 min, and then stored at -80℃.

Subsequent PCR amplification was performed in a DNA Engine, Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA) using IQTM SYBR Green Super mix and the correspondent rat primers: TNFα forward: 5'-AACTCCCAGAAAAGCAAGCA-3' reverse: 5'-CGAGCAGGAATGAGAAGAGG-3'; GAPDH forward: 5'-CTGTGTCTTTCCGCTGTTTTC-3', reverse: 5'-TGTGCTGTGCTTATGGTCTCA-3'.

Initial denaturation was followed by 40 cycles of DNA amplification with fluorescence detection at the end of the elongation step (SYBR Green format). Reactions were performed in duplicate and threshold cycle values were normalized to GAPDH gene expression. The specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to GAPDH was calculated by using the ΔC(t) formula.

Lipase

Plasma lipase was determined using commercial turbidimetric assay kits from Randox (Antrim, UK), according to the supplier's specifications.

Histamine analysis

Histamine levels in plasma and in cell culture supernatants were evaluated using a commercial ELISA assay from Labor Diagnostika Nort (Nordhorn, Germany) according to the supplier's specifications.

TNFα

TNFα concentration in the cell culture medium was measured using a commercial kit for rat TNFα from BLK International (Badalona, Spain), according to the supplier's specifications.

Myeloperoxidase

Neutrophilic infiltration was assessed by measuring myeloperoxidase (MPO) activity. MPO was determined photometrically with 3,3',5,5'-tetramethylbenzidine as substrate. Tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer at pH 6.0. Homogenates were disrupted for 30 s using a Labsonic sonicator (Braun Biotech, Inc., Allentown, PA, USA) at 20% power and submitted to three cycles of snap freezing in dry ice and thawing before a final 30 s sonication. Samples were incubated at 60℃ for 2 h and then spun down at 4000 × *g* for 12 min. The supernatants were collected for MPO assay. Enzyme activity was assessed photometrically using 630 nm wavelength. The assay mixture consisted of 20 μL supernatant, 10 μL tetramethylbenzidine (final concentration 1.6 mmol/L) dissolved in DMSO, and 70 μL H_2O_2 (final concentration 3.0 mmol/L) diluted in 80 mmol/L phosphate buffer, pH 5.4. The results are expressed as units (U) MPO activity per g protein.

Protein measurement: Total protein concentration in homogenates was determined using a commercial kit from BioRad (Munich, Germany).

Statistical analysis

Data are expressed as mean ± SE. Means of different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed to evaluate significant differences between groups. Differences were assumed to be significant when $P < 0.05$.

RESULTS

Mast cell activation during pancreatitis

Histological analysis revealed the presence of mast cells in the interlobular areas of control pancreas (Figure 1A) and a general pancreatic mast cell degranulation after induction of pancreatitis (Figure 1B). This degranulation was prevented by cromolyn treatment (Figure 1C). In lungs, mast cells could also be observed (Figure 1D), however, no apparent degranulation was detected in histological samples of the lung 3 h after induction of pancreatitis (Figure 1E). Cromolyn treatment had no effect on lung mast cells (Figure 1F).

Effects of mast cell inhibition

Pancreatitis resulted in increased levels of circulating lipase and histamine in plasma as well as enhanced MPO activity in both pancreas and lung (Figure 2). The inhibition of mast cell degranulation with cromolyn did not

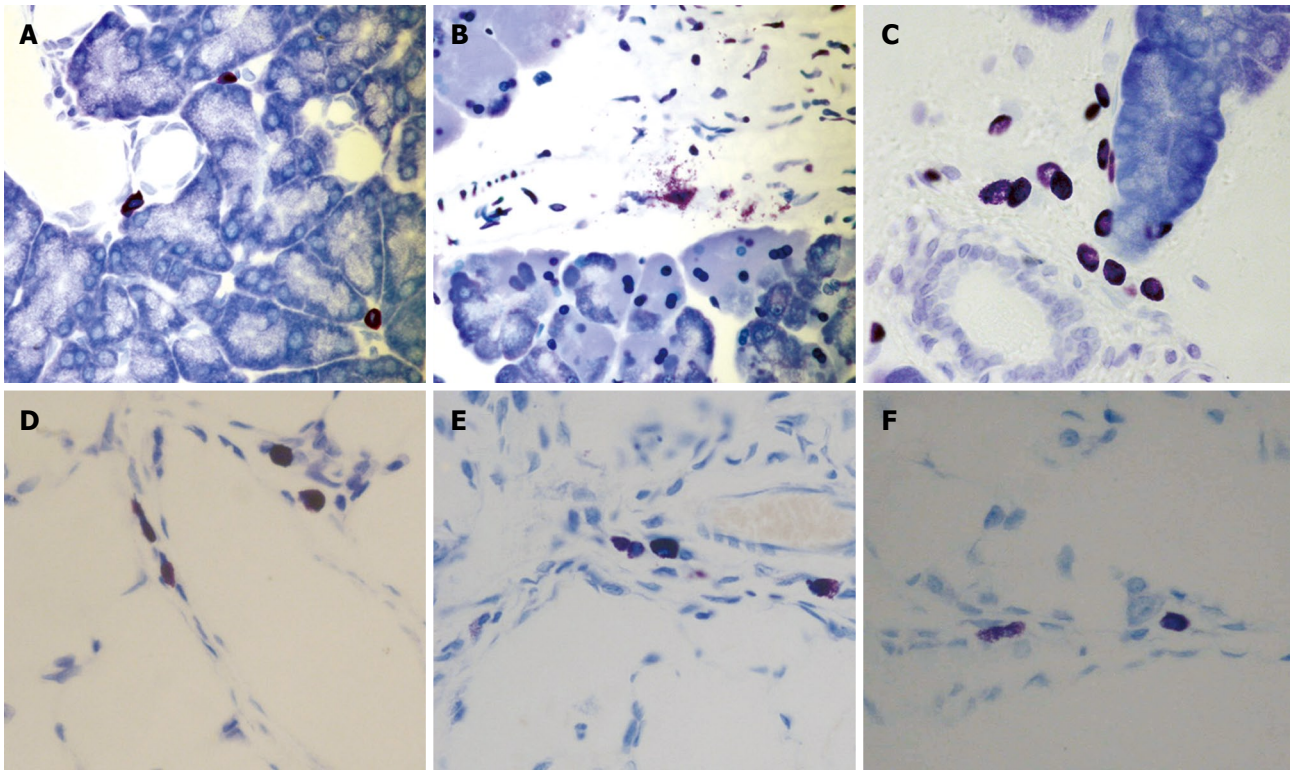

Figure 1 Presence of mast cells in pancreas (A-C) and lung (D-F), in control (A and D). Three hours after induction of pancreatitis (B and E) and under cromolyn treatment (C and F). Degranulating mast cells were observed in the pancreas after pancreatitis induction (B). Cromolyn treatment prevented mast cell degranulation (C). In contrast, no evident degranulation was observed in lung after induction of pancreatitis. Toluidine blue, × 40.

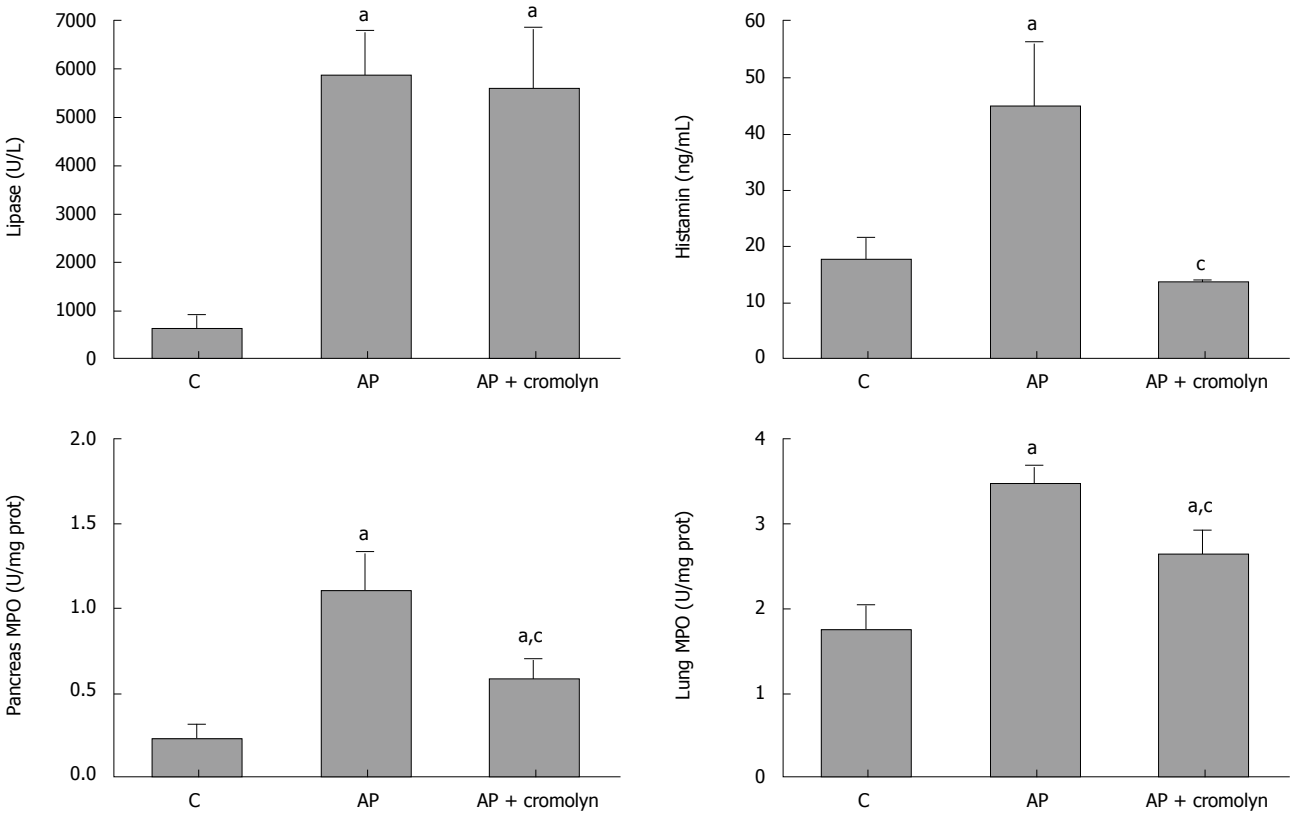

Figure 2 Effect of mast cell inhibitor, cromolyn. Three hours after pancreatitis induction, increased levels of lipase and histamine were detected in plasma. Cromolyn treatment had no effect on lipase, which is related to acinar cell damage, but prevented the increase in histamine. In tissue, leukocyte infiltration was evaluated by measuring myeloperoxidase (MPO) activity. Pancreatitis resulted in increased MPO activity in both pancreas and lung. Cromolyn treatment partially prevented these increases. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

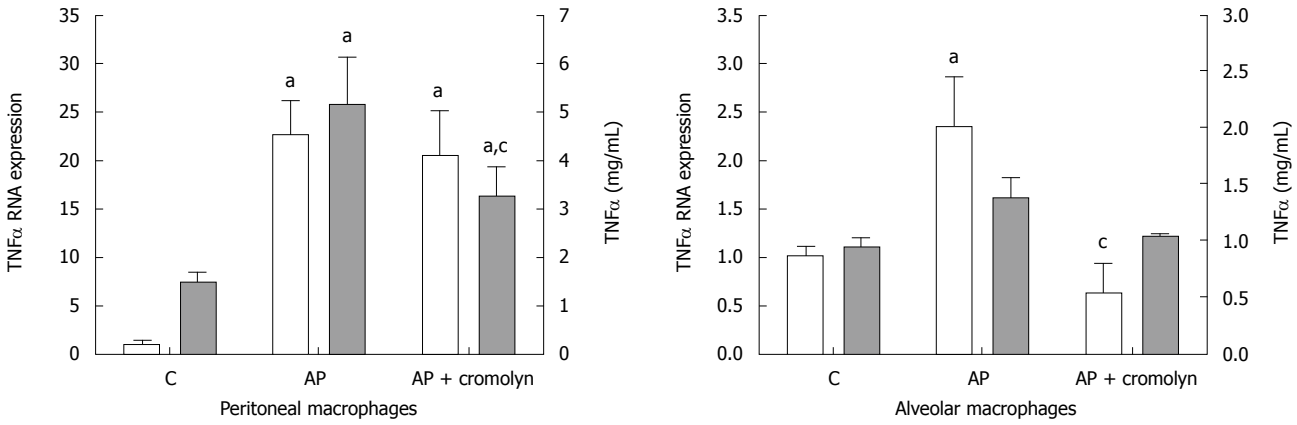

Figure 3 Both peritoneal and alveolar macrophages were activated after induction of pancreatitis, but the expression of tumor necrosis factor α mRNA in peritoneal macrophages was one order of magnitude higher than that observed in alveolar macrophages. Tumor necrosis factor (TNF)α release was induced in peritoneal cells, while in alveolar cells the observed increase was not statistically significant. Cromolyn treatment completely prevented the activation of alveolar macrophages. In contrast, peritoneal macrophages remained activated under cromolyn treatment. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

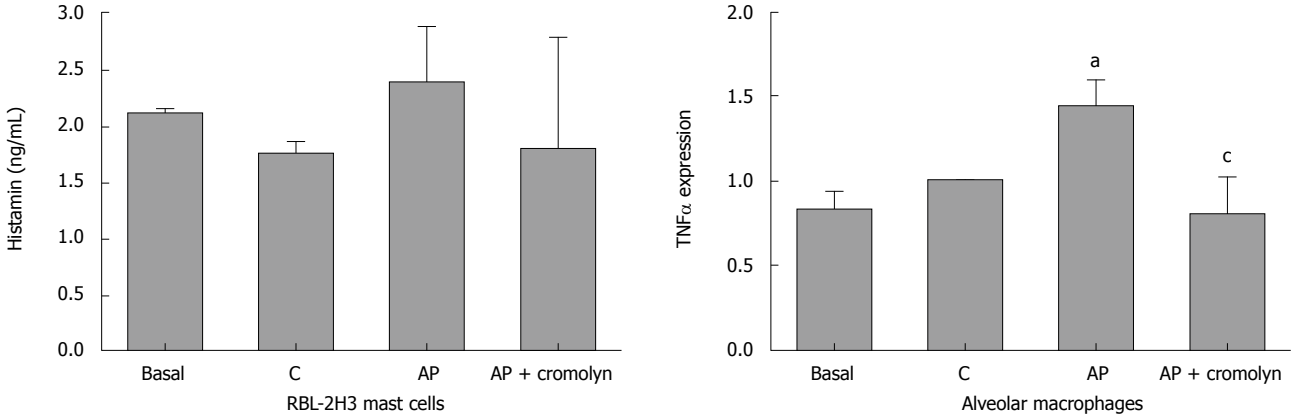

Figure 4 The effect of plasma on cultured mast cell line RBL-2H3 and alveolar macrophages. Mast cells were not activated by plasma from animals with pancreatitis. In contrast, the expression of tumor necrosis factor (TNF)α in alveolar macrophages was induced by plasma from animals with pancreatitis. This induction was not observed when animals were treated with cromolyn. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

modify the lipase levels, but resulted in a reduction in pancreatic MPO activity, indicating that this treatment had no effect on acinar cell damage but reduced pancreatic inflammation. Inhibition of the inflammatory process in lung was also observed. Nevertheless, these inhibitions did not achieve the control values and MPO activity remained significantly increased with respect to the control group in both pancreas and lung.

Changes in peritoneal and alveolar macrophages

Pancreatitis resulted in the induction of TNFα expression in both peritoneal and alveolar macrophages (Figure 3). However, at this time point the activation observed in peritoneal macrophages was one order of magnitude greater than that observed in alveolar macrophages. This was reflected in the release of TNFα which only showed a significant increase in peritoneal macrophages after pancreatitis induction, while the increase observed in alveolar macrophages was not statistically significant (Figure 3). Inhibition of mast cell degranulation had no effect on peritoneal macrophage activation, but completely prevented the increase observed in TNFα expression in alveolar macrophages.

Effect of plasma on cultured mast cells and macrophages

Incubation of the RBL-2H3 cell line with plasma obtained from animals with pancreatitis did not result in increased histamine release (Figure 4). In contrast, this plasma was able to induce the activation of alveolar macrophages, reflected in an increase in the expression of TNFα. This induction was not observed when plasma was obtained from animals treated with cromolyn (Figure 4).

DISCUSSION

The development of systemic inflammation during the progression of severe acute pancreatitis involves multiple pathways and cell systems. Among them, mast cells appear to play a pivotal role between the hydrolytic enzymes released by damaged pancreatic acinar cells and the stress-induced response mediated by free radicals and inflammatory mediators.

In this sense, mast cells seem to play a role amplifying the acute inflammatory response in different organs early after the onset of pancreatitis. Several mediators known to be released by mast cells, including platelet activating factor (PAF), histamine or prostaglandin D_2 (PGD_2), have been shown to be increased a few minutes after the induction of pancreatitis in experimental models[15,16]. In addition, the administration of mast cell inhibitors results in a reduction of the local and systemic inflammatory response and, in particular, prevents changes in endothelial cells and vascular permeability[10].

However, it is important to evaluate the particular role of the different mast cell populations in this process, in order to design therapeutic strategies centered on these cells. Due to the rapid activation of pancreatic mast cells in the onset of pancreatitis, the obvious therapeutic target may be pulmonary mast cells that are suspected of being activated in the later stages of the disease.

In the present study, we evaluated the degranulation of mast cells in pancreas and lung and found a different response during pancreatitis. Histological evaluation showed a clear and extensive degranulation of mast cells located in pancreatic tissue (Figure 1B). As expected, this degranulation was prevented by cromolyn administration (Figure 1C). In contrast, no clear evidence of mast cell degranulation was observed in lung tissue (Figure 1E).

This was a surprising result, taking into account that cromolyn administration resulted in a clear reduction in lung inflammation revealed by MPO activity and by a lower activation of alveolar macrophages (Figure 3). In addition, these results are in line with other authors who reported on the critical role of mast cells in the inflammatory response in lung during pancreatitis.

An explanation for this apparent contradictory result is an indirect effect of pancreatic mast-cell derived mediators on distant organs. Activation of mast cells results in the immediate release of mediators that play a role in the activation of circulating leukocytes as demonstrated by Zhao *et al*[11]. On the other hand, the progression of inflammation in pancreatic tissue is modified by cromolyn treatment (Figures 1 and 2). Consequently, it is suspected that the profile of pro-inflammatory mediators released to the bloodstream by pancreatic tissue and their ability to induce lung endothelial dysfunction could be modified by pancreatic mast cell inhibition.

To evaluate this possibility we treated alveolar macrophages as well as the mast cell line RBL-2H3 *in vitro* with plasma obtain from the different experimental groups. Our results indicate that plasma from the pancreatitis-induced group did not stimulate the production of significant amounts of histamine in culture (Figure 4). This result suggests that while pancreatic damage could enhance the activation and degranulation of mast cells in the pancreas a few minutes after pancreatitis induction[8], mediators present in plasma are not sufficient to activate these cells in distant organs.

However, plasma from pancreatitis animals was able to induce the activation of macrophages *in vitro*, reflected in the increased expression of TNFα. This effect was clearly reduced when animals were treated with cromolyn (Figure 4). Together, these results indicate that pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the inflammatory response related to pancreatic damage. The early activation reported in pancreatic mast cells may make the use of these cells as a pharmacological target difficult due to the short therapeutic window.

COMMENTS

Background

Mast cells have been reported to contribute to several aspects of pancreatitis associated lung injury. However, the involvement of particular mast cell populations remains unclear.

Research frontiers

Using an experimental model of acute pancreatitis in rats, the authors evaluated the activation of mast cells from pancreas and lung as well as the effect of mast cell inhibitors on progression of the inflammatory reaction.

Innovations and breakthroughs

Pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the systemic inflammatory response related to pancreatic damage.

Applications

The identification of active mast cells in the early stages of pancreatitis may improve our understanding of their role in this disease and the possible therapeutic strategies focussed on these cells.

Terminology

Cromolyn is a mast cell stabilizer that prevents the release of inflammatory mediators, such as histamine, from these cells.

Peer review

In this work, the authors have evaluated the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages in an experimental model of acute pancreatitis. The manuscript portrays a good effort by its authors.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3418

World J Gastroenterol 2010 July 21; 16(27): 3418-3422
ISSN 1007-9327 (print)

BRIEF ARTICLE

Double balloon enteroscopy examinations in general anesthesia

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Received: March 26, 2010 Revised: April 26, 2010
Accepted: May 3, 2010
Published online: July 21, 2010

Abstract

AIM: To demonstrate that the double balloon enteroscopy (DBE) can be safely performed in general anesthesia with intubation.

METHODS: We performed a retrospective examination between August 2005 and November 2008 among patients receiving intubation narcosis due to DBE examination. The patients were grouped based on sex, age and physical status. Anesthesia records included duration of anesthesia, quantity of medication used and anesthesia-related complications. We determined the frequency of complications in the different groups and their relation with the quantity of medication used and the duration of anesthesia.

RESULTS: We compiled data for 108 cases of general anesthesia with intubation. We did not observe any permanent anesthesia-related complications; the most frequent side effects of anesthesia were hypotension (30.55%), desaturation (21.29%), and apnea (17.59%). These complications were significantly more frequent among patients with multiple additional diseases [hypotension (23.1% *vs* 76.9%, $P = 0.005$), desaturation (12.3% *vs* 69.2%, $P < 0.001$) and apnea (7.7% *vs* 53.8%, $P = 0.001$)], however, their incidence was not proportional to the quantity of medication used or the duration of anesthesia.

CONCLUSION: General anesthesia with intubation is definitely a viable option among DBE methods. It is highly recommended in patients with multiple additional diseases.

Key words: Double balloon enteroscopy; General anesthesia; Intubation; Sedation; Patient autonomy

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Zubek L, Szabo L, Lakatos PL, Papp J, Gal J, Elo G. Double balloon enteroscopy examinations in general anesthesia. *World J Gastroenterol* 2010; 16(27): 3418-3422 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3418.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3418

INTRODUCTION

Although gastroenterological endoscopic examinations are performed with some form of sedation or anesthesia at increasing rates worldwide, gastroscopy and colonoscopy are still often performed without any sedation, even today[1]. A reason for the widespread use of anesthesia is that patients receiving sedation are more satisfied, because they recall less pain and discomfort related to the intervention. Also, gastroenterology specialists

can examine patients that are otherwise not suitable for examination because of psychological reasons or strong abdominal pain.

However, the dissemination of gastroenterology sedation has limitations[2]. This is partly because the intervention costs significantly more because of personnel and infrastructure requirements, and also because anesthesia itself may also have moderate or severe side effects[3]. According to the literature, over 50% of the complications are heart- or lung-related (aspiration, airway obstruction, low ventilation frequency, vaso-vagal episode, oversedation). So far, there is no consensus on whether sedation should be performed by gastroenterology specialists, anesthetist physicians or assistants, or the patient (patient-controlled anesthesia); or whether the medication should be administered as a bolus, as a continuous infusion, or automatically provided based on pre-calculated plasma level (target controlled infusion)[4].

The emergence of double balloon enteroscopy (DBE) among endoscopic examinations also means a shift of paradigm for internal medicine specialists, because its safe and efficient completion requires an advanced level of anesthesia. As the method has only been widely used for a couple of years, little data are available on the respective anesthetic procedures. According to the literature, three methods of sedation are used with significant geographical preferences, including conscious sedation, deep sedation (propofol anesthesia) and general anesthesia.

The goal of our research was to assess the suitability and advantages of general anesthesia with intubation for DBE.

MATERIALS AND METHODS

Patients

We retrospectively analyzed the data from 108 patients that had not been pre-selected, in whom DBE was carried out under general anesthesia with intubation. The interventions were carried out in the 1st Department of Internal Medicine of Semmelweis University, Budapest, Hungary between August 2005 and November 2008. Patients were classified into groups based on sex, age, physical status (ASA Physical Status Classification System) and DBE indication[5]. Anesthesia records included the duration of the intervention, anesthesia protocol, quantity of medication used, and complications.

Following recovery from anesthesia, the patients were asked to recall memories of the intervention and describe any possible complaint.

Method of anesthesia

Electrocardiography and transdermal oxygen saturation were constantly monitored during the intervention, and non-invasive blood pressure measurements were also performed every 5 min. Based on the literature, the definitions were as follows: hypotension, systolic blood pressure < 90 mmHg; desaturation, transdermal oxygen saturation < 90%; and apnea, > 30 s pause in respiration.

During intervention, proper anesthesia was provided by the combined administration of benzodiazepine, opioids and propofol in all cases; the mentioned medications were selected based on availability because these are all readily available in every endoscopy laboratory. We supposed that complete anesthesia was reached with their combined usage, and we also wished to adapt continuously the degree of anesthesia to the requirements of the intervention.

First, peripheral venous access was provided, and then infusion was administered (500-1000 mL). All patients received 0.5 mg atropine prior to the intervention, followed by gradual intravenous midazolam injection (3-10 mg) to reach a consciousness level equivalent to conscious sedation. All patients received 1-1.5 μg/kg fentanyl, and induction of narcosis was achieved by 1 mg/kg propofol as a bolus. For the maintenance of narcosis, further doses of propofol were used. We used two anesthesia protocols. According to these, propofol was either provided as continuous infusion or given in discrete fractions. In the case of continuous use, the infusion rate was set at 200 mg/h; for fractioned use, 25 mg fractions were given as a bolus following induction and intubation, until the end of intervention. If the degree of anesthesia was insufficient (patient motion, changes in vegetative reactions), we increased the speed of propofol infusion, or another fraction was administered. Fentanyl was repeatedly provided every 30 min at 0.5-1 μg/kg.

Statistical analysis

Arithmetic mean and SD values were used for continuous parameters, whereas frequency percentages were calculated for discrete parameters. Statsoft version 8.0 software (www.statsoft.com) was used for statistical analysis. The quantity of medication was compared using non-parametric variance analysis (Kruskal-Wallis analysis of variance), and the frequency of the observed complications was compared with Fisher's exact test among the various groups. $P < 0.05$ was considered statistically significant.

RESULTS

The indications for intervention in the 108 patients enrolled in the study are presented in Table 1. In patients with obscure gastrointestinal bleeding (OGIB), abnormal small-bowel findings were seen in 41 patients (65.1%). Most of them were classified as probable (angiodysplasia, erosion), and others as definitive (e.g. small ulcers) causes of bleeding. Other definitive causes were malignant disease, found in five patients, including polypoid gastrointestinal stromal tumor (GIST) in three patients, non-Hodgkin lymphoma (NHL) in one, and melanoma in one. In suspected inflammatory bowel disease (IBD), enteroscopy confirmed the diagnosis in five out of 12 cases. In patients with suspected neoplasia/stenosis, malignant disease was proven in three cases. In patients with known polyposis syndromes [familial adenomatous polyposis (FAP) or Peutz-Jeghers syndrome], small-bowel polyps were removed in eight patients. The average insertion length was 209 cm (50-460 cm, SD: 113 cm). Using the oral route (n = 95), a larger proportion of the

Table 1 Indications for double balloon enteroscopy

	n (%)
Suspected malignancy/stenosis	8 (7.4)
OGIB	63 (58.3)
Peutz-Jeghers syndrome	5 (4.6)
Polyposis	6 (5.6)
Angiodysplasia	6 (5.6)
IBD	12 (11.1)
Chronic cramping pain	6 (5.6)
Unknown fever or loss of weight	1 (0.9)
Irritable bowel syndrome	1 (0.9)

DBE: Double balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; IBD: Inflammatory bowel disease.

Table 2 Demographic and clinical data of patient groups

	P1 (SD)	P2 (SD)	P3 (SD)	Sum (SD)
n	65	30	13	108
Age (yr)	45.88 (15.93)	60.42 (13.36)	70.69 (19.47)	52.53 (18.44)
Duration (min)	91.85 (24.79)	79.17 (19.17)	65.77 (10.77)	85.18 (23.72)
Propofol (mg)	464.31 (91.84)	410.33 (66.82)	346.92 (61.29)	435.18 (91.16)
Midazolam (mg)	7.17 (1.29)	5.50 (1.04)	4.08 (0.76)	6.31 (1.60)
Fentanyl (mg)	0.1307 (0.0350)	0.1217 (0.0284)	0.0731 (0.0259)	0.1213 (0.0369)

small intestine was accessible for examination (226 cm, SD: 107 cm) compared with procedures that started with anal endoscope insertion and colonoscopy (n = 13, 98 cm, SD: 58 cm, $P < 0.01$).

Fifty-five patients were male (50.92%) and 53 were female (49.08%), with the average age being 52.53 years (SD: 18.44 years). The patients were classified into three groups based on the ASA Physical status classification system (ASA P1-P3). P1 included 65 patients (average age: 45.87 years, SD: 15.93 years), P2 included 30 patients (average age: 60.42 years, SD: 13.36 years), and P3 included 13 patients (average age: 70.69 years, SD: 19.47 years). The three groups were compared based on the duration of the intervention, the quantity of medication used, and the observed complications. Demographic and clinical data of the three groups are shown in Table 2.

The average length of the intervention was 85.18 min (SD: 23.72 min); with 91.85 min in P1 (SD: 24.79 min), 79.17 min (SD: 19.17 min) in P2, and 65.77 min (SD: 10.77 min) in P3. Although the time of intervention gradually decreased with deteriorating physical status, a significant difference was only found between P1 and P3 ($P < 0.001$).

The average amount of propofol used during the intervention was 435.18 mg (SD: 91.16 mg) per patient, with 464.31 mg (SD: 91.84 mg) in P1, 410.33 mg (SD: 66.82 mg) in P2, and 346.92 mg (SD: 61.29 mg) in P3. The dose of propofol decreased in patients with deteriorating physical status and significant differences were found between groups P1 and P2 ($P = 0.027$) and P1 and P3 ($P < 0.001$).

Table 3 Number and ratio of frequent complications *n* (%)

	P1	P2	P3	Sum
n	65	30	13	108
Hypotension	15 (23.1)	8 (12.3)	10 (76.9)	33 (30.6)
Desaturation	8 (12.3)	6 (20.0)	9 (69.2)	23 (21.3)
Apnea	5 (7.7)	7 (23.3)	7 (53.8)	19 (17.6)

The average quantity of midazolam used per patient was 6.31 mg (SD: 1.60 mg); with 7.14 mg (SD: 1.29 mg) in P1, 5.5 mg (SD: 1.04 mg) in P2, and 4.08 mg (SD: 0.76 mg) in P3. Significant differences were found between the groups P1 and P2 ($P < 0.001$) and P1 and P3 ($P < 0.001$), as well as P2 and P3 ($P = 0.045$). The average amount of fentanyl used per patient was 0.1213 mg (SD: 0.0369 mg); with 0.1307 mg (SD: 0.0350 mg) in P1, 0.1217 mg (SD: 0.0284 mg) in P2, and 0.0731 mg (SD: 0.0259 mg) in P3. Significant differences were found between groups P1 and P3 ($P < 0.001$) and P2 and P3 ($P = 0.001$).

Among anesthesia-related complications recorded during the intervention, hypotension, desaturation and apnea occurred frequently. Table 3 presents the number of complications and their comparison between the groups. We analyzed statistically the correlation between the occurrence of the above complications (hypotension, desaturation and apnea) and patients' physical status, the duration of the intervention, and the quantity of medication (propofol, midazolam, fentanyl).

Only the physical-status-based classification and the occurrence of the recorded complications showed a significant positive correlation. ASA P stage significantly influenced the frequency of hypotension ($P = 0.005$), de-saturation ($P < 0.001$) and apnea ($P < 0.001$). These complications were more frequently observed among patients classified into group P3 than would have been expected based on random incidence.

A significant positive correlation was not found between the quantity of medication and complications. There was a significant negative correlation between propofol dosage and the development of hypotension ($P = 0.002$). We found a significant negative correlation between midazolam dosage and the three most frequent complications (hypotension, $P = 0.001$; de-saturation, $P = 0.004$; apnea, $P = 0.001$). There was a significant negative correlation between fentanyl dosage and desaturation ($P = 0.003$), but not with the other complications. There was a significant negative correlation between the duration of the intervention and the frequency of desaturation ($P = 0.018$) and apnea ($P = 0.040$).

Among the 108 DBE anesthesia cases, three imminent anesthesia-related problems had to be resolved. In one case, peripheral venous access could not be provided due to the physical status of the patient, but instead, central venous access was established without any complication. In another case, intubation could not be completed and hence enteroscopy had to be delayed. One week later, both the intubation and intervention were completed with

the application of a depolarizing muscle relaxant. For a third patient, who had obesity and chronic obstructive pulmonary disease, continuous respiration assistance and oxygen supply had to be provided, and extubation could only be performed in the seated position due to breathing difficulty.

Anesthesia was not related to any permanent or severe complication (aspiration, malignant dysrhythmia, resuscitation, malignant hyperthermia) in any case. More than 98% of the patients had amnesia concerning events during anesthesia. Frequent complaints included discomfort at the site of peripheral venous access, sore throat or dysphagia, and abdominal distension.

DISCUSSION

Thanks to international recommendations based on the accumulating amount of data published about sedation techniques related to endoscopic interventions performed in the gastrointestinal tract, these interventions have become extremely safe[6-8]. Severe complications are generally rare and deadly fatal complications mostly affect patients in a severely impaired or terminal physical state[9]. Data concerning recently introduced enteroscopic examinations and the related sedation techniques are scarce, and randomized, multicenter comparative studies with large numbers of patients have been lacking.

The goal of our study was to examine the utility of general anesthesia with intubation as a method of choice for DBE. The enrolled patients were divided into three groups according to the ASA Physical Status Classification System.

We first examined whether the 108 enteroscopy cases corresponded with published data in terms of intervention indications and duration. Among the indications, OGIB (58.33%), IBD (11.11%) and tumor (7.41%) were the most frequent in our practice, as in the literature (OGIB: 59%-62.8%; IBD: 2.9%-6.4%; tumor: 8.3%-10.2%)[10-12]. The average duration of the intervention in our study (85.18 min) was also found to be similar to that in the literature (53-113 min)[13,14]. The detailed outcome of the endoscopic procedures has been published in a separate paper[15].

However, we need to highlight some differences in sedation complications. Anesthesia-related complications occurred at much higher frequencies in our practice than during conscious sedation described in the literature, but these have either quickly resolved without any or with minor medical intervention. Hypotension was found to be the most frequent complication in our study (30.55%), which occurred at much lower frequencies during conscious sedation (1.8%-23.08%)[10,13,16]. If hypotension was observed, we increased intravenous fluid therapy, although the positive effects of the procedure are not obvious[17], and we also decreased the administration of propofol and fentanyl. Hypertensive drugs were not used in any case, and hypotension resolved within minutes with the above procedures.

The frequency of desaturation was 21.29% in our study, which is similar to the frequencies reported in the literature (0%-30.78%)[13,18]. In cases of hypoxia, transient or continuous oxygen inhalation was necessary, depending on the patient's requirements, and if oxygen levels normalized, we discontinued oxygen administration. Apnea was observed in 17.59% of the cases in which respiratory assistance was initiated, which was discontinued as soon as spontaneous respiration was restored. However, some patients required continuous respiratory assistance during the intervention. The severe complication of aspiration did not occur during intubation, and probably this is the most prominent difference between intubation and conscious sedation (0% *vs* 1.2%-2.77%).

We found a significant positive correlation between the number of complications and poor physical status. Poor physical status and senior age both predict occurrence of hypotension, desaturation and apnea. With the increase in ASA physical status level, the duration of the intervention and the quantity of medication decreased.

We consider it important to highlight that side effects observed during anesthesia are not related to medication use, because there was no significant positive correlation found between the frequency of complications and dose of propofol, midazolam or fentanyl. For some complications, the opposite was true, as patients with poor health status (P3), who experienced the most complications, received much less anesthetic.

The amount of medication used for narcosis in our study was higher than that required for examinations performed under conscious sedation[13]. The reason for this is that a greater amount of medication is required for deeper sedation (general anesthesia). Also, patients receiving orotracheal intubation require more medication to tolerate the procedure.

The ratio of complete amnesia observed among patients examined in intubation narcosis was much higher (98%) than among those receiving venous sedation (24%-56%)[14].

Therefore, who is advised to undergo general anesthesia as an alternative sedation method performed by an anesthetist? Every patient who belongs to a sedation-related risk group. Such risk factors include emergency interventions; senescence; cardiac, lung, renal or liver diseases possibly resulting in organ failure; pregnancy; drug or alcohol abuse; disorientation; post-prandial or non-cooperative patients, and alleged airway obstruction[4]. In our study, patients classified as P3 or higher also belonged to the high-risk group, therefore, ASA physical status helps us to choose the right method of anesthesia.

General anesthesia with intubation was a viable option when performing DBE in all three patient groups. With the deterioration of physical status (increasing ASA P status), the advantage of intubation narcosis increases compared to other sedation methods. In the case of poor physical status, the number of complications significantly increases, however, these are readily treatable due to pre-existing intubation. The occurrence of hypoxia, apnea or aspiration can result in an emergency situation in patients sedated without intubation, which can lead to deterioration of the patient's physical status and halt the course of

the examination, thus significantly increasing the number of complications and healthcare costs. General anesthesia can also be used safely in ASA groups P1-2 because severe or permanent anesthesia-related complications have not been observed in any case. Alternative anesthetic methods that suit the patient's needs will be justified in the future if, in the institutions performing enteroscopy, venous sedation (conscious or deep) and general anesthesia are provided. Self autonomy of patients with good health status, who are suitable for ambulatory intervention (ASA P1-2), should be emphasized, and after providing sufficient information, choices of alternative anesthesia methods should be offered.

Our study had some limitations, because the study was retrospective and patients were not randomized. Patient numbers were not equal between the groups, with especially few patients in group P3. Another limitation was that we performed only general anesthesia with intubation; the other sedation methods that were used for comparison were based on published data only. We compared the average frequency of side effects observed in our patients with those reported in the literature.

COMMENTS

Background

Three methods of sedation are used for double balloon enteroscopy (DBE), with significant geographical preferences, including conscious sedation, deep sedation (propofol anesthesia) and general anesthesia. The goal of this research was to assess the suitability and advantages of general anesthesia with intubation for DBE.

Research frontiers

The best anesthetic method for DBE is not clear at present, because all sedation methods have many different advantages and disadvantages.

Innovations and breakthroughs

This research shows that the ASA physical status classification system helps us to choose the right anesthetic method. With the deterioration of physical status (increasing ASA P status), the advantage of intubation narcosis increases compared to other sedation methods. The authors found a significant positive correlation between the number of complications and poor physical status. They consider that the side effects observed during anesthesia are not related to medication use.

Applications

Based on the results of this clinical study, general anesthesia with intubation was a viable option for DBE in all three patient groups. Alternative anesthetic methods that suit the patient's needs will be justified in the future in institutions that perform enteroscopy.

Peer review

The study demonstrates that the double balloon enteroscopy examination can also be safely performed in general anaesthesia with intubation.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3423

World J Gastroenterol 2010 July 21; 16(27): 3423-3426
ISSN 1007-9327 (print)

BRIEF ARTICLE

Does bilioenteric anastomosis impair results of liver resection in primary intrahepatic lithiasis?

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Received: January 14, 2010 Revised: March 25, 2010
Accepted: April 2, 2010
Published online: July 21, 2010

Abstract

AIM: To evaluate the long-term results of liver resection for the treatment of primary intrahepatic lithiasis. Prognostic factors, especially the impact of bilioenteric anastomosis on recurrence of symptoms were assessed.

METHODS: Forty one patients with intrahepatic stones and parenchyma fibrosis/atrophy and/or biliary stenosis were submitted to liver resection. Resection was associated with a Roux-en-Y hepaticojejunostomy in all patients with bilateral stones and in those with unilateral disease and dilation of the extrahepatic biliary duct (> 2 cm). Late results and risk factors for recurrence of symptoms or stones were evaluated.

RESULTS: There was no operative mortality. After a mean follow-up of 50.3 mo, good late results were observed in 82.9% of patients; all patients submitted to liver resection alone and 58.8% of those submitted to liver resection and hepaticojejunostomy were free of symptoms (P = 0.0006). Patients with unilateral and bilateral disease showed good late results in 94.1% and 28.6%, respectively (P < 0.001).

CONCLUSION: Recurrence of symptoms in patients with hepaticojejunostomy showed that this may not be the ideal solution. Further studies are needed to establish the best treatment for patients with bilateral stones or unilateral disease and a dilated extrahepatic duct.

Key words: Biliary lithiasis; Bilioenteric anastomosis; Cholangitis; Intrahepatic lithiasis; Liver resection

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Herman P, Perini MV, Pugliese V, Pereira JC, Machado MAC, Saad WA, D'Albuquerque LAC, Cecconello I. Does bilioenteric anastomosis impair results of liver resection in primary intrahepatic lithiasis? *World J Gastroenterol* 2010; 16(27): 3423-3426 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3423.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3423

INTRODUCTION

Primary intrahepatic lithiasis or hepatolithiasis, is a prevalent disease in Southeastern Asia but is rare in the Western world; it is a challenging condition due to its varied forms of presentation and complex treatment. In some Western countries, it has been increasingly diagnosed and a relative incidence of 2.1% from all cases of biliary stone disease has been reported[1,2]. The goals of treatment are to promote stone clearance, control bile infection, decompress the biliary tree, and prevent progressive hepatic dysfunc-

tion. Since each patient has a distinctive stone distribution within the biliary tree, treatment has to be individualized accordingly. Liver resection has been reported to promote excellent long-term results, since stones and biliary strictures can be simultaneously removed reducing the risk of recurrence. In patients with unilateral stones, liver resection is considered a potentially curative treatment[3-8]. For bilateral stones, the ideal treatment has not yet been established; bilioenteric anastomosis or a percutaneous approach associated or not with liver resection have been employed with good long-term results in up to two thirds of cases.

Although resection can lead to a cure in patients with bilateral disease, the recurrence of symptoms is not rare. Moreover, it has been shown that patients submitted to liver resection associated with a bilioenteric anastomosis, had higher rates of recurrent cholangitis when compared to those submitted to resection only[4,8].

The purpose of this study is to report our experience with patients submitted to liver resection for the treatment of non-oriental hepatolithiasis, and to evaluate the influence of different prognostic factors, especially bilioenteric anastomosis, on late results.

MATERIALS AND METHODS

Ninety eight patients with symptomatic primary intrahepatic lithiasis were treated at our institution between 1990 and 2006.

According to our treatment protocol, liver resection was indicated in patients with irreversible hepatic lesions such as unilateral or segmental liver fibrosis/atrophy or the presence of intrahepatic biliary stenosis. A complementary Roux-en-Y hepaticojejunostomy was performed in patients with unilateral liver disease who presented with common bile duct stones with a duct diameter larger than 2 cm, and in all patients with bilateral stones[2].

Forty one patients (41.8%) underwent liver resection; data regarding gender, age, history of cholangitis and previous biliary surgery, intrahepatic stone location, liver function tests, intraoperative findings, type of surgery performed and postoperative outcome are presented in Table 1.

There were 16 men (39.9%) and 25 women (60.1%), with a mean age of 41.3 years (range 18 to 67 years). A history of right upper quadrant pain was present in all cases, jaundice in 31 patients (75.6%), cholangitis in 25 (61%) and nineteen (46.3%) had previously undergone biliary tract surgery: cholecystectomy in 13, hepaticojejunostomy in 3 and cholecystectomy plus common bile duct exploration in 3. None of the patients showed any sign of liver failure at physical examination.

Preoperative diagnosis was based on ultrasonography, helicoidal three-phase tomography, endoscopic or percutaneous cholangiography that in the last 5 years were replaced by magnetic resonance cholangiography. A complementary operative cholangiography was performed in all cases.

Indications for liver resection were: parenchymal atrophy in 27 patients, intrahepatic biliary stenosis in 8 and unilobular severe liver fibrosis in 6. Two patients were submitted to liver resection in a septic condition, due to cholangitis.

Table 1 Analysis of the effect of each variable on late results

Variable (*n*)	Late complications (poor results) *n* (%)	Statistical analysis
Gender		
Female (25)	6 (24.0)	$P = 0.1406$
Male (16)	1 (6.3)	
Previous biliary surgery		
No (22)	3 (13.6)	$P = 0.5291$
Yes (19)	4 (21.1)	
History of cholangitis		
Yes (25)	7 (28.0)	$P = 0.0608$
No (16)	0 (0)	
Preoperative serum bilirubin		
Normal (32)	6 (18.8)	$P = 0.5905$
Raised (9)	1 (11.1)	
Preoperative white blood cells		
Normal (37)	6 (16.2)	$P = 0.6574$
Raised (4)	1 (25.0)	
Stone location		
Unilateral (34)	2 (5.9)	$P < 0.0001$
Bilateral (7)	5 (71.4)	
Type of surgery		
Liver resection (24)	0 (0)	$P = 0.0006$
Liver resection + HJ (17)	7 (41.2)	
Major liver resection (more than 3 segments)		
Yes (14)	1 (7.1)	$P = 0.2237$
No (27)	6 (22.2)	

HJ: Hepaticojejunostomy.

Mean follow-up was 50.3 mo, ranging from 18 to 198 mo. Long-term results were considered good when no recurrence of symptoms or complications of the disease such as cholangitis or liver abscess during the follow-up period were observed.

Independent variables and their impact on late prognosis were compared using Student's t and Pearson's χ^2 tests. Statistical significance was set at $P < 0.05$.

RESULTS

Forty one patients were submitted to liver resection, 34 (82.9%) had unilateral disease and the left lobe was more frequently affected (28 cases). Bilirubin, alkaline phosphatase and gamma glutamyl transpeptidase serum levels were raised in 21.9%, 61% and 53.7% of patients, respectively.

Five patients underwent right hepatectomy (12.2%), nine left hepatectomy (22%), twenty six bisegmentectomy 2-3 (63.4%) and one patient underwent a segment 5 resection. A Roux-en-Y hepaticojejunostomy was associated with liver resection in 14 patients as follows: seven with bilateral and seven (21.8%) with unilateral disease and common bile duct dilation larger than 2 cm in diameter. Another three patients with unilateral stones who had previously been submitted to hepaticojejunostomy were submitted to liver resection and the anastomosis was maintained. All patients had a drain placed at the site of resection.

There was no operative mortality. Two patients submitted to liver resection (right hepatectomy and bisetorectomy 2-3) in a septic condition had an uneventful outcome. Four patients (9.8%) had a postoperative biliary fistula and were conservatively managed with an uneventful outcome; one patient (2.4%) developed a right subphrenic abscess which was percutaneously drained with good outcome.

Thirty two patients with unilateral and two with bilateral disease (82.9%) had good long-term results. Seven patients (17.1%), 2 with unilateral and 5 with bilateral stones, had late complications of the disease: cholangitis associated with recurrent stones in three (bilateral disease); cholangitis in two (unilateral disease); liver abscess associated with recurrent stones in one and liver abscess in one (all with bilateral stones).

One of these patients had caudate lobe recurrent stones and an abscess 93 mo after resection of segments 2 and 3, and died 28 d after drainage of the abscess due to sepsis; one had a liver abscess percutaneously drained with good outcome; three patients with cholangitis and stone recurrence, received antibiotic therapy and percutaneous stone removal and have remained well; two patients with cholangitis were treated with systemic antibiotics with good outcome. The long-term mortality rate was 2.4%.

The overall rate of good long-term results was 82.9% and was 94.1% and 28.6%, respectively for unilateral and bilateral disease. Comparing the data of good results between patients with unilateral and bilateral disease, statistical analysis showed a significant difference ($P < 0.001$) (Table 1).

All patients submitted to liver resection only, showed good long-term results (100%), while seven of seventeen patients (41.2%) who underwent liver resection associated with hepaticojejunostomy had late postoperative complications. A comparison between liver resection alone and resection associated with hepaticojejunostomy showed a statistically significant difference ($P = 0.0006$) (Table 1).

Twenty seven out of 34 patients with unilateral disease were submitted to liver resection alone and all had a good outcome. Of the remaining seven patients with unilateral disease who were submitted to liver resection associated with a bilioenteric anastomosis, two had recurrence of symptoms (2/7, 28.5%). A comparison between liver resection alone and resection associated with hepaticojejunostomy for patients with unilateral disease, showed a statistically significant difference ($P = 0.0498$).

DISCUSSION

Primary intrahepatic lithiasis is a rare disease in Western countries but, the high number of cases diagnosed in our institution, led to a treatment protocol based on presentation of the disease[2,9], where 41 out of 98 patients with symptomatic hepatolithiasis underwent liver resection.

The aim of treatment was the removal of intrahepatic and extrahepatic stones as well as duct strictures and to promote adequate drainage of the remaining segments of the biliary tree. Liver resection is the only treatment that can achieve these goals, thus reducing the risk of recurrence[4,5,7,8,10-14]. In this series, liver resection was indicated in patients with irreversible lesions such as biliary strictures or severe parenchymal fibrosis or atrophy, criteria initially proposed by Choi and Wong[6] and employed by many others[4,7,15,16].

Hepatic resection for the treatment of hepatolithiasis can lead to low rates of cholangitis or stone recurrence and good long-term results ranging from 80% to 98%[3-5,7,10-13,16]. In this series, good late results were observed in 100% of the patients submitted to liver resection only, showing that in some situations cure of the disease is possible.

With regard to the long-term results, seven patients (17.1%), 2 with unilateral and 5 with bilateral stones, all submitted to liver resection and bilioenteric anastomosis, had complications: five had cholangitis and two had liver abscesses. One of these patients died and the other 6 were treated successfully.

Patients with unilateral disease had significantly better results compared to those with bilateral stones, 94.1% and 28.6% had good late results, respectively. These data are comparable to other reports from the Far East and to our own previous experience, where good results were achieved in 80% to 100% of patients with unilateral stones and in 50% to 80% of those with bilateral disease[3-7,9-11]. These results can be explained by the fact that in patients with unilateral disease, all the compromised liver parenchyma is removed, potentially leading to cure of the disease, while the same is not always possible in those with bilateral disease. Indeed, if one looks at our data, good late results were achieved in all patients with unilateral stones who did not present with extrahepatic biliary disease. However, if stones were present in the remnant parenchyma or there was a dilation of the extrahepatic biliary tree and a biliary drainage procedure and hepaticojejunostomy was required, the rate of good results fell significantly to 58.8%. This was probably due to two factors: (1) Associated extrahepatic biliary disease (persistence of a possible cause for stone formation and/or inadequate biliary or stone drainage); and (2) Bilateral disease (persistence of affected liver tissue).

Most authors emphasize that at long-term follow-up, patients submitted to liver resection associated with a bilioenteric anastomosis, have a worse prognosis when compared to those submitted to resection only[4,8]. In recent years, reports have shown higher rates of postoperative cholangitis in patients submitted to hepaticojejunostomy[17-19].

Although patients submitted to hepaticojejunostomy had a higher incidence of poor late results, it is difficult to state whether cholangitis in these cases was due to recurrent stones in the remnant liver or to the presence of a bilioenteric anastomosis. Indeed, Roux-en-Y hepaticojejunostomy is the procedure of choice because the long jejunal loop is employed to avoid bacterial reflux into the liver. In an attempt to solve this question, we compared only patients with unilateral disease, with and without hepaticojejunostomy and, despite a small number of patients; there was a significant difference between the groups

showing a direct effect of the bilioenteric anastomosis on patient outcome.

Although the majority of groups perform a Roux-en-Y hepaticojejunostomy in patients with bilateral stones, the real benefits of this procedure have not yet been proven. Indeed, Li *et al*[19] showed that stones located in the lateral and posterior segments of the liver, do not drain easily through the biliary anastomosis. Moreover, Chen *et al*[20] showed excellent results employing percutaneous treatment without any surgical treatment in patients with bilateral stones. According to this data and reinforced by the poor results in our patients with hepaticojejunostomy, a biliary anastomosis may not be the ideal solution for these patients. Further studies are needed to establish the best treatment for bilateral hepatolithiasis and for those with unilateral disease and a dilated extrahepatic duct.

This study with the largest non-oriental series of primary intrahepatic lithiasis showed that liver resection can lead to the cure of unilateral hepatolithiasis. However, in patients with bilateral disease and in those with extrahepatic biliary duct dilation, where a hepaticojejunostomy was performed, more than 30% of patients had symptom recurrence and a rigorous follow-up is necessary. For the late group of patients, other treatment modalities such as resection associated with percutaneous treatment instead of hepaticojejunostomy should be considered.

COMMENTS

Background

Surgical treatment of primary intrahepatic lithiasis in a Western country was evaluated. The paper reports the largest non-oriental series of liver resection for hepatolithiasis. Prognostic factors were evaluated and bilateral disease treated with a bilioenteric anastomosis had a negative impact on outcome.

Research frontiers

It may be necessary for surgeons who deal with this challenging disease to reevaluate the benefit of hepaticojejunostomy.

Innovations and breakthroughs

Evaluation of prognostic factors in patients submitted to surgical treatment of primary intrahepatic lithiasis.

Applications

The benefit of other treatment modalities such as resection associated with percutaneous treatment instead of hepaticojejunostomy in patients with extrahepatic biliary duct dilation and for those with bilateral intrahepatic stones.

Peer review

This is an interesting manuscript on a challenging group of patients.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3427

World J Gastroenterol 2010 July 21; 16(27): 3427-3431
ISSN 1007-9327 (print)

BRIEF ARTICLE

Prevalence of type 2 diabetes in Algerian patients with hepatitis C virus infection

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Received: April 6, 2010 Revised: April 27, 2010
Accepted: May 4, 2010
Published online: July 21, 2010

Abstract

AIM: To investigate the prevalence of, and risk factors for, diabetes mellitus (DM) in Algerian patients with chronic hepatitis C virus (HCV) infection and in a control group.

METHODS: A cross-sectional study was undertaken. A total of 416 consecutive patients with viral chronic hepatitis attending the Internal Medicine Department of the University Hospital Center Touhami Benflis in Batna [290 HCV-infected and 126 hepatitis B virus (HBV)-infected patients] were prospectively recruited.

RESULTS: The prevalence of DM was higher in HCV-infected patients in comparison with HBV-infected patients (39.1% *vs* 5%, $P < 0.0001$). Among patients without cirrhosis, diabetes was more prevalent in HCV-infected patients than in HBV-infected patients (33.5% *vs* 4.3%, $P < 0.0001$). Among patients with cirrhosis, diabetes was more prevalent in HCV-infected patients, but the difference was not significant (67.4% *vs* 20%, $P = 0.058$). The logistic regression analysis showed that HCV infection [odds ratio (OR) 4.73, 95% CI: 1.7-13.2], metabolic syndrome (OR 12.35, 95% CI: 6.18-24.67), family history of diabetes (OR 3.2, 95% CI: 1.67-6.13) and increased hepatic enzymes (OR 2.22, 95% CI: 1.1-4.5) were independently related to DM in these patients.

CONCLUSION: The high prevalence of diabetes in HCV-infected patients, and its occurrence at early stages of hepatic disease, suggest that screening for glucose abnormalities should be indicated in these patients.

Key words: Prevalence; Hepatitis C virus; Hepatitis B virus; Diabetes mellitus; Algeria

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Rouabhia S, Malek R, Bounecer H, Dekaken A, Bendali Amor F, Sadelaoud M, Benouar A. Prevalence of type 2 diabetes in Algerian patients with hepatitis C virus infection. *World J Gastroenterol* 2010; 16(27): 3427-3431 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3427.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3427

INTRODUCTION

Hepatitis C virus (HCV) infection is a common worldwide medical problem; it is one of the major causes of chronic liver disease. According to recent World Health Organization estimates the worldwide prevalence of HCV infection is 2.2%, affecting approximately 130 million people globally[1]. Patients with chronic HCV infection may develop various extrahepatic manifestations, including cryoglobulinemia, the presence of serum antibodies, glomerulonephritis, sialoadenitis and porphyria cutaneous tarda[2]. Several studies from different parts of the world have reported that HCV infection may also contribute to the development of diabetes mellitus (DM), and higher prevalence of type 2 DM has been observed in patients with HCV infection than in those with other forms of chronic hepatitis[3-5]. However, the prevalence of type 2 DM in patients with HCV infection has not been reported in Algeria, as far as we know.

Thus, in order to examine the prevalence of DM in patients with chronic HCV infection in Batna (Algeria), we conducted a cross-sectional study assessing the prevalence of this metabolic disorder in patients with HCV infection in comparison with the prevalence in those with hepatitis B virus (HBV) infection. In addition, risk factors associated with DM development such as age, body mass index (BMI), diabetic familial history and metabolic syndrome were also evaluated to clarify the possible role of chronic HCV infection in association with development of diabetes.

In this study, the prevalence of diabetes in patients with chronic HCV has not been compared to that in the general population, because patients with chronic liver disease, regardless of etiology, have a higher prevalence of diabetes mellitus[6]. We have chosen patients with chronic hepatitis B as a control group, because HBV infection is the second leading cause of chronic hepatitis after HCV in Algeria[7].

MATERIALS AND METHODS

Patients

From September 2004 to September 2007, we conducted a cross-sectional study by enrolling patients with chronic viral hepatitis admitted to the University Hospital Center Touhami Benflis in Batna, Algeria. The diagnosis of HCV infection was made if patients were positive for anti-HCV antibody and HCV RNA. The presence of anti-HCV antibody was assessed using the third generation microparticle enzyme immunoassay test. The presence of HCV RNA was confirmed by Cobas Ampliprep/Roche Taq Man (Pasteur Institute, Algiers and Sadelaoud Laboratory, Batna, Algeria). HBV infection was diagnosed if patients had evidence of hepatitis B surface antigen. Patients with concomitant HCV and HBV infection were excluded. There was no serologic evidence of co-infection with other hepatotropic viruses or with human immunodeficiency virus. Patients having other causes of liver disease, in particular those known to be involved in the pathogenesis of diabetes, such as hemochromatosis or alcoholic liver disease, were excluded. None of the study patients had received corticosteroids during the previous 6 mo before the study. Patients with a history of, or evidence of, pancreatitis, pancreatic tumor, hepatic tumor or cirrhosis with Child-Pugh category C were excluded from the study. None of the study patients had previously received anti-viral treatment. No woman was pregnant in this study. Patients who were infected with HCV or HBV after being diagnosed with diabetes were also excluded from the analysis.

According to the American Diabetes Association criteria[8], patients were assigned a diagnosis of DM if they were using oral hypoglycemic medication or insulin, or if they showed fasting glucose greater than 126 mg/dL on two occasions, or glucose greater than 200 mg/dL, 2 h after an oral glucose tolerance test, performed in patients with impaired fasting glucose (fasting glucose concentration $\geqslant$ 110 mg/dL and $<$ 126 mg/dL).

A diagnosis of cirrhosis was established either by histology or by presumptive diagnosis made when patients had ascites, hematologic evidence of hypersplenism, esophageal varices or relevant ultrasonographic findings. Liver biopsy was performed in patients with increased alanine aminotransferase (ALT) and who gave their informed consent beforehand. Liver biopsy specimens were analyzed by a single experienced pathologist, who was informed of clinical and biologic data. Fibrosis was assessed using the METAVIR score[9]. Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis), and F4 (cirrhosis).

The BMI and family history of DM were recorded for each patient during enrollment. The BMI was expressed as the body weight divided by the square of the body length (kg/m^2). Overweight was defined as a BMI 25-29.9 kg/m^2 and obesity as a BMI $\geqslant$ 30 kg/m^2. The family history of diabetes was obtained from the patients themselves and was recorded as positive if their first-degree relatives had DM. The metabolic syndrome was diagnosed according to the National Cholesterol Education Program's Adult Treatment Panel Ⅲ definition[10].

Statistical analysis

All data values were expressed as mean ± standard deviation. Results were compared between HCV and HBV patients using the χ^2 test for categorical variables and Student *t*-test for continuous variables. Stepwise multivariate logistic regression was performed to evaluate the predictive variables associated with the presence of diabetes in the study patients. All statistical analyses were performed using Epi info 2000 (Statistics Program for Public Health. CDC, Atlanta, USA), and a P value < 0.05 was considered significant.

RESULTS

In total, 416 patients (290 HCV-infected patients and 126 HBV-infected patients) were enrolled in this study. Considerable differences could be noted when the demographic characteristics of the two groups were compared (Table 1).

Table 1 Characteristics of all patients in the chronic viral hepatitis cohort *n* (%)

Clinical features	Virological diagnosis		*P*
	HBV (*n* = 126)	HCV (*n* = 290)	
Age (yr)			
< 40	57 (45.2)	13 (4.5)	< 0.001
40-59	60 (47.6)	193 (66.6)	< 0.001
⩾ 60	9 (7.1)	84 (29)	< 0.001
Male sex	79 (62.7)	78 (26.9)	< 0.001
Family history of diabetes	38 (30.2)	103 (35.5)	0.28
BMI (kg/m^2), mean ± SD	25.22 ± 4.48	26.27 ± 4.51	0.05
BMI ⩾ 25 kg/m^2	68 (54)	161 (55.5)	0.42
Metabolic syndrome	20 (15.9)	122 (42.1)	< 0.0001
Increased ALT	15 (11.9)	177 (61)	< 0.0001
Cirrhosis	5 (4)	51 (17.6)	0.00018

HBV: Hepatitis B virus; HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase.

Table 2 Analysis of variables associated with diabetes in chronic viral hepatitis *n* (%)

Variables	Virological diagnosis		*P*
	HBV (*n* = 121)	HCV (*n* = 261)	
Diabetic	6 (5)	102 (39.1)	< 0.0001
Age (yr)			
< 40	0/56 (0)	1/12 (8.3)	
40-59	4/58 (6.9)	61/174 (35.1)	< 0.0001
⩾ 60	2/7 (28.6)	40/75 (52.6)	0.56
Family history of diabetes	1/37 (2.7)	52/92 (56.5)	< 0.0001
BMI ⩾ 25 kg/m^2	5/66 (7.6)	70/147 (47.6)	< 0.0001
Metabolic syndrome	4/18 (22.2)	75/108 (69.4)	< 0.001
Increased ALT	2/14 (14.3)	79/155 (51)	0.01
Cirrhosis	1/5 (20)	29/43 (67.4)	0.058
No cirrhosis	5/116 (4.3)	73/218 (33.5)	< 0.0001

HBV: Hepatitis B virus; HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase.

Patients with hepatitis C were older than those with hepatitis B (55 ± 9 years *vs* 40 ± 13 years, $P < 0.0001$). Percentage of men was lower in the HCV-infected patients than in those with HBV (26.9% *vs* 62.7%, $P < 0.0001$). In addition, there were significantly more patients with metabolic syndrome among the hepatitis C patients, and more cirrhosis in the hepatitis C group.

DM was observed more often in HCV-infected patients than in HBV-infected patients (39.1% *vs* 5%, $P < 0.0001$). However, this difference is statistically significant only in patients aged between 40 and 60 years.

We compared variables associated with diabetes in patients with patent diabetes (6 HBV-infected patients and 102 infected HCV patients) and in those who were non-diabetics (115 HBV-infected patients and 159 HCV-infected patients). Patients with impaired fasting glucose (5 HBV-infected patients and 29 HCV-infected patients) were excluded from this comparison.

A family history of diabetes appeared to be matched in the present study; of the subjects infected with HCV and HBV, 35.5% and 30.2%, respectively, had a familial history of DM. Patients with a family history of diabetes were more likely to have DM compared with those without (41.08% *vs* 21.73%, $P < 0.0001$). For patients with a family history of DM, the prevalence of diabetes was significantly higher in subjects with HCV infection compared with those with HBV infection (56.5% *vs* 2.7%, $P < 0.000001$) (Table 2).

Metabolic syndrome was more frequent in HCV-infected patients (42.1% *vs* 15.9%, $P < 0.0001$). Patients with metabolic syndrome were more likely to have DM compared with those without (62.69% *vs* 11.32%, $P < 0.000001$). For patients with metabolic syndrome, DM was significantly more frequent in HCV-infected patients than in those with HBV infection (69.4% *vs* 22.2%, $P < 0.001$). In obese or overweight subjects (BMI ⩾ 25 kg/m^2), DM was more frequent in HCV-infected patients (47.6% *vs* 7.6%, $P < 0.0001$).

Liver disease appeared more severe in the HCV group. Cirrhosis was more frequent in HCV-infected patients

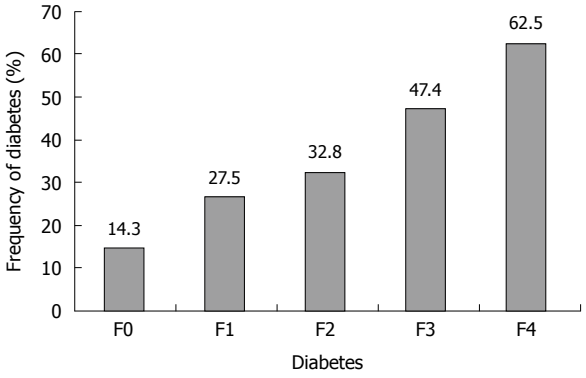

Figure 1 Frequency of diabetes by stage of fibrosis in hepatitis C virus-infected patients.

than in those with HBV infection (17.6% *vs* 4%, $P < 0.001$). In patients without cirrhosis, DM was more frequent in patients with HCV infection than in those with HBV infection (33.5% *vs* 4.3%, $P < 0.00001$). However, in patients with cirrhosis, diabetes was more prevalent in HCV-infected patients, but the difference was not significant (67.4% *vs* 20%, $P = 0.058$).

In HCV-infected patients in whom liver biopsy was performed, DM prevalence increased progressively and significantly with the fibrosis stage (Figure 1). DM was more frequent in patients with increased ALT plasma concentration (> 40 IU/L) compared with those with normal ALT plasma concentration (47.92% *vs* 12.67%, $P < 0.00001$). In patients with increased ALT plasma concentration, DM was significantly more frequent in HCV-infected patients than in those with HBV infection (51% *vs* 14.3%, $P = 0.01$).

The multiple regression analysis revealed that the major independent variables associated with type 2 diabetes were metabolic syndrome [odds ratio (OR) 12.35, $P = 0.0001$, 95% CI: 6.18-24.67], HCV infection (OR 4.73, $P = 0.0029$, 95% CI: 1.69-13.20), family history of diabetes (OR 3.2, $P = 0.0004$, 95% CI: 1.67-6.13) and increased ALT (OR 2.22, $P = 0.027$, 95% CI: 1.09-4.52) (Table 3).

Table 3 Factors associated with the development of diabetes in patients chronically infected with hepatitis virus

Variables	Odds ratio	95% CI	*P* value
Metabolic syndrome	12.35	6.18-24.67	0.00001
Hepatitis C	4.73	1.69-13.20	0.0029
Family history of diabetes	3.2	1.67-6.13	0.0004
Increased ALT	2.22	1.09-4.52	0.027

ALT: Alanine aminotransferase.

DISCUSSION

Our epidemiological and virological data suggest that HCV infection is more closely related to diabetes than HBV infection. Diabetes was observed in 39.1% of patients with HCV infection, as compared with 5% of HBV-infected subjects in our population. However, our study has some limitations, related to the small size of the control group. Indeed, chronic hepatitis B is much less common than chronic hepatitis C in our area[7]. Our findings are in concordance with similar epidemiological studies from different part of the world.

Allison *et al*[11] published, in 1994, the first article about a link between viral hepatitis C and diabetes. In their retrospective study of 100 cirrhotic patients listed for transplantation, these authors reported that the prevalence of type 2 DM was higher in patients with HCV-associated cirrhosis than in cirrhotics with other underlying liver diseases. In a cross-sectional survey including 9841 persons, Mehta *et al*[12] found that HCV-positive persons who were older than 40 years had an increased risk for type 2 diabetes mellitus, more than 3-fold when compared to persons without HCV infection. However, no difference was seen between HBV-infected subjects and the general population[12].

In a retrospective analysis of 1117 patients with chronic viral hepatitis, diabetes was present in significantly more patients with HCV compared to those with HBV infection (21% *vs* 12%)[13]. In a separate case-control trial included in the same report, the prevalence of HCV infection was significantly higher among patients with diabetes than among controls (4.2% *vs* 1.6%).

Diabetes mellitus has been more often seen in cirrhotic patients[14]. However, in a cohort of 45 non-cirrhotic patients with chronic hepatitis C the prevalence of type 2 DM was 33%, higher than in the matched control group and in a group of patients with chronic hepatitis B[15]. Furthermore, in a large retrospective study DM was present in 23.6% of patients with hepatitis C, and in 9.4% of those with hepatitis B[16].

Recently, in a Spanish study which included 525 chronic hepatitis C patients treated with peginterferon plus ribavirin, patients were followed up after treatment. The incidence of altered baseline glucose and the appearance of type 2 DM was greater in non-responders than in sustained responders, even after multivariate analysis including such confounding variables as previous type 2 DM in relatives, age older than 40 years and male sex. Thus, hepatitis C virus clearance induced a decrease in insulin resistance index during short time follow-up and decreased the incidence of type 2 DM in long-term follow-up[17].

Shintani *et al*[18], in an experimental model, observed that the HCV core antigen transgenic mouse had higher basal insulin levels than non-transgenic mice, and readily developed diabetes when fed a high-fat diet, in addition to exhibiting marked insulin resistance as demonstrated by the insulin tolerance test.

In the present study, logistic regression analysis confirmed that family history of diabetes, metabolic syndrome and increased transaminases were the major independent variables associated with DM. This finding is consistent with reports in the literature[19-21].

The mechanisms by which hepatitis C induces increased insulin resistance and the risk for development of diabetes has not been completely understood. Liver fibrosis progression has long been considered responsible for the appearance of insulin resistance and type 2 DM in patients with chronic liver diseases[22]. However, in our study, diabetes occurs in the early stages of liver disease. The mechanism through which HCV is associated with insulin resistance involves direct viral effects, proinflammatory cytokines and suppressors of cytokine signaling[23-25].

In conclusion, this study shows a higher prevalence of DM in patients with HCV infection than in those with HBV infection, and that DM occurs at an early stage of hepatic disease. However, other factors such as metabolic syndrome, family history of diabetes and increased transaminases seem also to be important risk factors for the development of diabetes in Algeria.

ACKNOWLEDGMENTS

The advice from Professor Francesco Negro (Geneva, Switzerland), Professor Dominique Valla (Clichy, France) and Christian Pagnoux, MD (Paris, France), was greatly appreciated while performing this study. The authors also thank Professor Daoud Roula (Constantine, Algeria), Rabah Ait Hamouda (Batna, Algeria) and Saadi Berkane (Algiers, Algeria), for their critical review of the manuscript. In addition, they are indebted to Professor AbdelAziz Agti and Mss Asma Bounecer (Batna, Algeria) for English grammar review. Finally, the authors thank Roche for paying the publishing fee.

COMMENTS

Background

A higher prevalence of diabetes mellitus (DM) has been observed in patients with hepatitis C virus (HCV) infection than in those with other forms of chronic hepatitis and several mechanisms have been implicated in the pathogenesis of DM. However, there is no information from Algeria regarding this issue, and few reports from Africa.

Research frontiers

Recent data link HCV infection with diabetes. However, diabetes is a multifactorial disease; other factors such as age, weight, family history of diabetes and cirrhosis contribute to the development of diabetes.

Innovations and breakthroughs

This is a cross-sectional study assessing the prevalence of diabetes in patients with HCV infection in comparison with the prevalence in those with hepatitis B virus (HBV) infection. It is the first study of its kind performed in Algeria. In addition, risk factors associated with DM development were also evaluated.

Applications

Diabetes plays a role in the initiation and progression of liver injury. The high prevalence of diabetes in HCV-infected patients, and its occurrence at early stages of hepatic disease, suggest that screening for glucose abnormalities should be indicated in these patients.

Peer review

The authors used 290 HCV-infected and 126 HBV-infected patients. It would be better if they had used equal numbers for both the groups or slightly fewer.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3432

World J Gastroenterol 2010 July 21; 16(27): 3432-3436
ISSN 1007-9327 (print)

BRIEF ARTICLE

Association of *E-cadherin* (*CDH1*) gene polymorphisms and gastric cancer risk

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Received: March 24, 2010 Revised: April 24, 2010
Accepted: May 1, 2010
Published online: July 21, 2010

Abstract

AIM: To investigate the associations between *CDH1* gene polymorphisms and gastric cancer (GC) risk predisposition.

METHODS: We analyzed four *CDH1* polymorphisms (*+54 T>C*, *-160 C>A*, *-616 G>C*, *-3159 T>C*) in an Omani population, by extraction of genomic DNA from the peripheral blood of 192 patients with GC and 170 control participants and performed *CDH1* genotyping using DNA sequencing.

RESULTS: *CDH1 -160 -AA* genotype was associated with an increased risk of GC (OR = 3.6, 95% CI: 1.1-11.8) (P = 0.03). There was no significant association between the other polymorphisms and GC risk. The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C*, *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), but did not reach statistical significance.

CONCLUSION: The current study suggests that the *-160 AA* genotype was associated with an increased risk of GC in Oman.

Key words: Gastric cancer; Polymorphism; *CDH1*

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Al-Moundhri MS, Al-Khanbashi M, Al-Kindi M, Al-Nabhani M, Burney IA, Al-Farsi A, Al-Bahrani B. Association of *E-cadherin* (*CDH1*) gene polymorphisms and gastric cancer risk. *World J Gastroenterol* 2010; 16(27): 3432-3436 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3432.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3432

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and second most common cause of cancer mortality worldwide; therefore, it remains a global health burden[1,2]. GC has been associated with *Helicobacter* infection and environmental factors such as smoking, salted fish, and low intake of fruit and vegetables[3,4]. However, while these factors might affect large proportions of some populations, only subsets of these populations develop GC, and

therefore, increased genetic susceptibility has been postulated. Possible genetic risk factors have included single nucleotide polymorphisms (SNPs) in several pathways involved in chronic inflammation of gastric mucosa and subsequent carcinogenesis. The involved SNPs affect agents such as pro-inflammatory cytokines, xenobiotic metabolizing enzymes, and growth factors[5-11]. The study of these molecular pathways has helped to identify individuals at higher risk, particularly when examined with *Helicobacter pylori* (*H. pylori*) infection and other environmental exposure[7,8].

Adhesion molecules, especially the calcium-dependent intercellular adhesion molecule E-cadherin and its *CDH1* gene (located on chromosome 16), play a central role in carcinogenesis and metastasis[10,12]. The *CDH1* gene encodes a transmembrane glycoprotein that mediates intercellular adhesion and cellular polarity. The E-cadherin protein is a tumor invasion suppressor, and loss of its function results in transition to an invasive phenotype in human epithelial cancers[10,12].

Several SNPs in the *CDH1* gene are associated with GC. The most widely studied polymorphism is *CDH1 -160C>A*, where the *A* allele decreases transcriptional activity of the *CDH1* gene and E-cadherin expression, and increases susceptibility to GC in some populations[9,13-19]. Moreover, several other SNPs, including *+54 T>C*, *-3159 T>C*, *-160 C>A*, *-2076 C>T* and *-616 G>C*, were studied in Japanese and Italian populations, which resulted in the identification of haplotypes associated with increased risk of GC[12,20].

The above studies have highlighted the ethnic variation in frequency and risk predisposition of these SNPs[15,16]. Therefore, we studied in an Omani population, four *CDH1* gene polymorphisms (*+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C*) that were previously examined in Japanese and Italian populations[12,20]. We evaluated the potential association of these SNPs and their haplotypes with GC susceptibility in a case-control design.

MATERIALS AND METHODS

Study participants

The study population consisted of a series of unrelated patients with GC who were diagnosed at two main hospitals in the Sultanate of Oman (Sultan Qaboos University Hospital and Royal Hospital). The healthy control group comprised persons of the same ethnic and geographical origin as the patients. The Medical Research and Ethics Committee of the College of Medicine of Sultan Qaboos University approved the study design. The study participants provided informed consent prior to participation, in compliance with the Declaration of Helsinki.

Genotyping method

From each participant, 10 mL blood was collected in an EDTA tube and stored frozen until the extraction of the DNA. DNA was extracted from whole blood using a commercial DNA blood kit (Gentra Puregene DNA Purification kit; Qiagen, Gaithersburg, MD, USA) and stored until processing for genotyping.

Analysis of the *CDH1* SNPs, *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C*, was performed using multiplex polymerase chain reaction (PCR) with an ABI premix. Genomic DNA from whole blood was used as a PCR template in a total reaction volume of 10 μL that contained 10 pmol designed primers: *+54 T>C (rs3743674)*: [5'-CCCCTGGTCTCATCATTTC-3' (forward) and 5'-AATTCCTCCAAGAATCCCCAG-3' (reverse)]; *160 C>A (rs16260)*: [5'-TGATCCCAGGTCTTAGTGAG-3' (forward) and 5'-GCTCCTCAGGACCCGAAC-3' (reverse)]; *-616 G>C (rs7203904)*: [5'-TTGACTGAGGCCACAGAGTG-3' (forward) and 5'-CTGCCTAAATCTGCTGAGCC-3' (reverse)]; *-3159 T>C* (*rs2010724*): [5'-GAGCTTCCCAGAGCCTTTCT-3' (forward) and 5'-ATTGGACTTGCCAAGGGTG-3' (reverse)]. PCR was performed as follows: one cycle at 94℃ for 10 min, 35 cycles at 94℃ for 30 s, 59℃ for 30 s, and 72℃ for 30 s, followed by 72℃ for 5 min. The final extension was at 72℃ for 10 min. PCR products were analyzed on a 2.5% agarose gel stained with ethidium bromide and photographed under UV light. The PCR product was subsequently sequenced in an ABI PRISM 3100 sequencer using BigDye Terminator v3.1 Cycle Sequencing method (Applied Biosystems,USA) as recommended by the manufacturer. Candidate SNP regions were detected and typed with the aid of DNA Star Software (DNASTAR, Madison, WI, USA).

Statistical analysis

The genotypic distributions of different polymorphic loci in the control samples were compared with those expected from the Hardy-Weinberg equilibrium using the χ^2 test. The differences in frequency distributions of the genotypes between the patient and control groups were also tested using the χ^2 test. Age- and sex-adjusted ORs and 95% CIs were calculated using logistic regression analysis. Haplotype frequencies, haplotype-survival analyses, and standardized disequilibrium coefficients (D) were calculated using Thesias software available at http://genecanvas.ecgene.net/. $P < 0.05$ was considered statistically significant. Analysis of data was performed using SPSS version 10.0 software (SPSS, Chicago, IL, USA).

RESULTS

One hundred and ninety-two GC patients and 170 unrelated controls were included. The age range for the participants included in the study was 19-80 years, and the mean ages for the patients and controls were 55.1 ± 12.5 and 32.8 ± 6.6 years, respectively. The percentages of male and female participants were 58.3% and 41.7% for GC patients respectively, and 56.5% and 43.5% for controls. *H. pylori* infection status was available in 116 GC patients and 90 control participants, with a positivity rate of 58% and 60% (Table 1). Most GC patients in this cohort presented at an advanced stage, with slight predominance of non-intestinal type according to Lauren's classification, as shown in Table 2.

Table 1 Demographic data, *Helicobacter* status, and smoking in gastric cancer patients and control subjects

Variable	GC patients	Control
No. of subjects	192	170
Age (yr), mean ± SD	32.8 ± 6.6	55.1 ± 12.5
Male, %	58.30	56.50
H. pylori status, *n*	116[1]	90[1]
Positive, *n* (%)	67 (58)	54 (60)

[1]The number of GC patients and control participants for whom *Helicobacter pylori* (*H. pylori*) serology was available. GC: Gastric cancer.

Table 2 Clinicopathological features of 192 gastric cancer patients

Variable	*n* (%)
Lauren's classification	
Intestinal	93 (48.5)
Mixed and diffuse	99 (51.5)
Histological grade	
G1	11 (5.8)
G2	80 (41.6)
G3	101 (52.6)
T stage	
T1 + T2	32 (16.7)
T3 + T4	160 (83.3)
Lymph node involvement	
Negative	26 (13.5)
Positive	166 (86.5)
TNM stage	
Ⅰ + Ⅱ	33 (17.2)
Ⅲ + Ⅳ	159 (82.8)

CDH1 genotypic frequencies and GC risk

The frequencies of the *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes are shown in Table 3. The SNP analysis was successful in the majority of GC patients and control subjects, however, 15-23 samples failed for GC patients and 4-13 samples for control subjects, as shown in Table 3. The allelic distributions for control subjects did not deviate significantly from those expected from the Hardy-Weinberg equilibrium. There was a significant association between the *CDH1-160 AA* genotype, with an increased risk of GC, with OR 3.6 (95% CI: 1.1-11.8, $P = 0.03$) (Table 3). There was no significant association between the other *CDH1* polymorphisms and GC risk (Table 3).

Table 3 *CDH1* genotype frequencies and their associated risk of gastric cancer predisposition

CDH1 genotype	Patients *n* (%)[1]	Control *n* (%)[1]	OR[2] (95% CI)	*P* value
+54 T>C	*n* = 174	*n* = 157		
TT	25 (14.4)	22 (14.0)	1	
TC	70 (40.2)	75 (47.8)	0.9 (0.4-2.2)	0.9
CC	79 (45.4)	60 (38.2)	0.9 (0.4-2.4)	0.8
CC + TC	149 (85.6)	135 (86.0)	0.9 (0.4-2.1)	0.8
TT + TC	95 (54.6)	97 (61.8)	1.0 (0.5-2.0)	1.0
C allele	66.0	62.0		
-160 C>A	*n* = 174	*n* = 166		
CC	93 (53.6)	93 (56.0)	1	
CA	60 (34.5)	65 (39.2)	0.6 (0.3-1.1)	0.1
AA	21 (12.0)	8 (4.8)	3.6 (1.1-11.8)	0.03
AA + CA	81 (46.5)	73 (44.0)	0.8 (0.4-1.5)	0.8
CC + CA	153 (88.1)	158 (95.2)	3.4 (1.4-13.9)	0.01
A allele	29.0	24.0		
-616 G>C	*n* = 172	*n* = 159		
GG	84 (48.8)	71 (44.7)	1	
GC	65 (37.8)	69 (43.4)	0.9 (0.6-1.8)	0.7
CC	23 (13.4)	19 (12.0)	1.8 (0.6-5.1)	0.3
GC + CC	88 (51.2)	88 (55.4)	1.0 (0.6-1.9)	0.9
GG + GC	149 (86.6)	140 (88.1)	1.8 (0.7-5.2)	0.3
C allele	32.0	34.0		
-3159 T>C	*n* = 177	*n* = 166		
TT	52 (29.7)	47 (28.3)	1	
TC	72 (41.1)	78 (47.0)	0.9 (0.4-1.7)	0.7
CC	53 (30.3)	41 (24.7)	1.0 (0.5-2.0)	0.9
CC + TC	125 (71.4)	119 (71.7)	0.9 (0.5-1.7)	0.8
TT + TC	124 (70.8)	125 (75.3)	1.1 (0.54-2.2)	0.8
C allele	50.0	48.0		

[1]The number of patients and control indicates successful single nucleotide polymorphism analysis for each polymorphism; [2]Age and sex-adjusted.

Haplotype analysis

The common haplotypes were identified, as shown in Table 4. There were significant differences in the distribution of these haplotypes between patients and controls (Table 4). The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), respectively, but did not reach statistical significance.

Table 4 Frequencies of *CDH1* haplotypes and associated risk of gastric cancer predisposition

Haplotype	Frequency (%)		OR[1] (95% CI)	*P* value
	Patient	Control		
TCGT	20.1	22.1	1	
TACG	10.4	11.0	0.99 (0.5-1.9)	1.0
CCGT	16.5	17.7	1.0 (0.5-1.8)	1.0
CCGC	7.0	5.0	1.5 (0.7-3.5)	0.3
CCCT	11.1	9.9	1.1 (0.5-2.3)	0.8
CCCC	15.1	16.6	0.9 (0.6-1.6)	0.8
CAGC	10.7	7.1	1.5 (0.8-3.0)	0.2
CACC	5.8	5.7	1.1 (0.5-2.4)	0.8

[1]Age and sex-adjusted.

DISCUSSION

Six polymorphisms of the *CDH1* gene have been studied previously in Caucasian, East Asian, and Mexican populations and included: *-616 G>C*, *-160 C>A*, *-3159 T>C*, *+54 T>C*, *2076C>T* and *347G>GA*[12-17,20]. A recent meta-analysis has highlighted the role of ethnic differences by showing that the associations between these polymorphisms and GC among Asian and Caucasian populations are in opposite directions[15,18]. Therefore, we investigated the association between GC and the *CDH1 +54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* polymorphisms in an Omani population, an ethnic group in which the association between GC and these polymorphisms has not been studied previously.

The most widely studied *CDH1* polymorphism in various cancers is *CDH1 -160 C>A*[13-19]. In the present study, we found that this polymorphism affected the risk of developing GC. The carriage of the *CDH1 -160 AA* genotype increased the risk of GC (OR: 3.6, 95% CI: 1.1-11.8) (P = 0.03). Two meta-analyses have suggested that the association of *CDH1 -160 AA* with GC risk is ethnicity-dependent, whereby the OR estimates for *CDH1 -160 AA* carriers are less than 1.0 for Asians but significantly greater than 1.0 for Caucasians[15,18]. Thus, our results are consistent with the findings in Caucasian populations. The explanation for this observation remains unclear, however, the A variant decreases transcription efficiency by 68% compared with the C allele *in vitro*[9]. The altered expression of adhesion molecule E-cadherin results in tumor development and carcinogenesis. Possible explanations for the discrepancy between ethnic groups include the frequency of the polymorphism in the population studied or linkage disequilibrium with other, perhaps undiscovered, functional SNPs in the *CDH1* gene. The present study shows that there is no association between the *CDH1 +54 T>C* and *-616 G>C* SNPs and GC development. Although a study by Zhang *et al*[13] has found an association between *+54 T>C* and esophageal and gastric cancer, other studies were negative[15].

It has been suggested that haplotype analysis might be more useful than single SNP analysis in identifying cancer risk[12,20]. In particular, the combined analysis of *CDH1 -160 C>A*, *-2076C>T* and *+54 T>C* has suggested that a haplotype ATT increases susceptibility to GC, whereas the CTT haplotype has a protective effect[12,20]. Yamada *et al* have studied the *+54 T>C*, *-160 C>A*, *-616 G>C*, *-2076 T>C* and *3159 T>C* polymorphisms and have found that the TCGTT haplotype is the most common haplotype and has a protective effect, whereas the TAGTC haplotype increases susceptibility to GC[12,20]. The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), respectively, but did not reach statistical significance. The reason for the difference can be attributed to differences in polymorphisms studied, genetic background and local environmental factors, and highlights the need for comparative studies between different ethnic groups.

In conclusion, the current study confirms the ethnic variations in the association between *CDH1 -160 C>A* polymorphisms and GC susceptibility. We demonstrated that the *-160 AA* genotype was associated with an increased risk of GC. This finding could allow the identification of higher-risk groups who might benefit from intensive prevention strategies (aimed at infections or environmental factors). A better understanding of the functional aspects of these polymorphisms in tumor tissue could lead to a better understanding of tumor biology and behavior, and elucidate the discrepancies observed between and within studies.

COMMENTS

Background

E-cadherin plays a central role in carcinogenesis and metastasis. *E-cadherin* (*CDH1*) gene polymorphisms at various loci and their significance for predisposition to gastric cancer (GC) risk have been studied previously with different results that have suggested ethnic variation. The authors investigated the associations between *CDH1* gene polymorphisms and GC risk predisposition.

Research frontiers

A better understanding of *CDH1* gene polymorphisms in GC could lead to a better understanding of tumor biology and behavior.

Innovations and breakthroughs

The current study confirms the ethnic variations in the association between *CDH1 -160 C>A* polymorphisms and GC susceptibility. The authors demonstrated that the *-160 AA* genotype was associated with an increased risk of GC.

Applications

These findings could allow the identification of higher-risk groups who might benefit from intensive prevention strategies (aimed at infections or environment factors).

Terminology

CDH1 gene encodes E-cadherin protein, which is an important adhesion molecule. Single nucleotide polymorphisms are DNA sequence variations that occur when a single nucleotide is altered.

Peer review

This study provides some useful epidemiological information about genetic predisposition and the risk of GC.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3437

World J Gastroenterol 2010 July 21; 16(27): 3437-3444
ISSN 1007-9327 (print)

BRIEF ARTICLE

Transcatheter arterial chemoembolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma

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Received: January 21, 2010 Revised: March 10, 2010
Accepted: March 17, 2010
Published online: July 21, 2010

Abstract

AIM: To evaluate the efficacy of transcatheter arterial chemoembolization (TACE) using a suspension of a fine-powder formulation of cisplatin (DDPH) for hepatocellular carcinoma (HCC).

METHODS: The study population was comprised of 164 patients who were treated by TACE alone. Of these patients, 76 underwent TACE using a suspension of DDPH in lipiodol (LPD) (DDPH group), and the remaining 88 underwent TACE with an emulsion of doxorubicin (ADM) with LPD (ADM group). We compared the DDPH group with the ADM group in terms of the objective early response rate, progression free survival (PFS) and overall survival (OS).

RESULTS: The objective early response rate in the DDPH group was significantly higher than that in the ADM group (54% *vs* 24%, $P < 0.001$). The PFS rate in the DDPH group was also significantly higher than that in the ADM group ($P < 0.001$). Moreover, the OS in the DDPH group was significantly longer than that in the ADM group ($P = 0.002$). Although the incidence rate of nausea or vomiting in the DDPH group was higher than that in the ADM group, the ADM group showed a higher incidence rate of the adverse events of hepatic arterial damage and leucopenia. No other serious complications were observed in either group.

CONCLUSION: We conclude that TACE using a suspension of DDPH in LPD could be a useful treatment for HCC.

Key words: Hepatocellular carcinoma; DDPH; Transcatheter arterial chemoembolization; Cisplatin; Doxorubicin

Peer reviewers: Satoshi Mamori, MD, PhD, Department of Gastroenterology and Hepatology, Shinko Hospital, 1-4-47 Wakihama-cho, Chuo-ku, Kobe, Hyogo 651-0072, Japan; Susumu Ohwada, Associate Professor, Department of Surgery, Gunma University Graduate School of Medicine, 3-39-15 Shoma-Machi, Maebashi 371-8511, Japan

Kasai K, Ushio A, Sawara K, Miyamoto Y, Kasai Y, Oikawa K, Kuroda H, Takikawa Y, Suzuki K. Transcatheter arterial chemoembolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(27): 3437-3444 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3437.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3437

INTRODUCTION

Hepatocellular carcinoma (HCC) is the cancer with the sixth highest incidence in the world[1]. The number of deaths from HCC is also increasing throughout the world[2-5]. Development of new treatments for HCC has

helped improve the patient prognosis[6,7]. Local ablating therapies such as percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) have been effective in cases of limited tumor spread and are increasingly used[7,8]. However, the majority of patients are not eligible for these modalities because of large tumor size or diffuse tumor growth. In these patients regional transcatheter arterial chemoembolization (TACE) has been widely used as a palliative treatment[9,10]. Two randomized trials from Europe and Asia recently confirmed a survival benefit after TACE using gelfoam and iodized oil (lipiodol) compared to conservative treatment[11,12]. In recent years, TACE using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been employed commonly for HCC treatment[13,14]. However, the tumors have been demonstrated to show a high frequency of recurrence after TACE[10,15,16]. Cisplatin (CDDP), a platinum compound, is an effective anticancer agent used in the treatment of various malignancies[17]. Researchers have recently reported that TACE using a suspension of CDDP powder in LPD may be more effective against unresectable HCC as compared with TACE using ADM-LPD emulsion[18,19]. However, only limited institutions have used this for TACE because it is laborious to refine the CDDP powder. Since 2004, a fine-powder formulation of CDDP (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has also been available as a therapeutic agent for intra-arterial infusion in Japan. As a result, TACE using DDPH has become widespread in Japanese institutions. Nevertheless, the efficacy of TACE using DDPH-LPD suspension has not yet been reported.

In this article, we compared the effectiveness with regard to the response rate (RR), progression free survival (PFS) and overall survival (OS) between TACE using a suspension of DDPH in LPD (DDPH-LPD suspension) and ADM-LPD emulsion. Moreover, we analyzed the prognostic factors for clinical outcome of patients treated with TACE.

MATERIALS AND METHODS

Patients

Between January 2006 and July 2009, 164 HCC patients who showed no indication for surgical resection or local ablation therapy such as RFA and PEI therapy were enrolled in the study. HCC was diagnosed by the distinctive findings on ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) and angiography, and the serum levels of des-γ-carboxy prothrombin (DCP) and α-fetoprotein (AFP). Histologic examination was not always carried out. Liver function was evaluated according to the Child-Pugh classification[20]. Tumor stage was judged by the TNM classification established by the International Union Against Cancer[21]. The extent of portal vein invasion was classified as follows: Vp 0, no invasion of the portal vein; Vp 1, invasion of the third or more distal branch of the left or right portal vein; Vp 2, invasion of the second branch of the portal vein; Vp3, invasion of the first branch of the portal vein; and Vp4, invasion of the trunk of the portal vein. After being presented with the clinical results of previous studies of TACE using DDPH-LPD suspension or TACE using ADM-LPD emulsion, all 164 patients themselves selected the therapeutic option on the basis of informed consent. All of the enrolled patients met the eligibility criteria for inclusion in the analysis described in the next paragraph. The patients were divided into two groups: one group consisting of 76 patients who underwent TACE using DDPH-LPD suspension (DDPH group), and another group consisting of 88 patients who underwent TACE using ADM-LPD emulsion (ADM group). They were all treated by TACE alone.

Informed consent was obtained from all of the patients. The study protocol was approved by the Ethics Committee of Iwate Medical University and the study was conducted in accordance with the Declaration of Helsinki 1975.

Eligibility criteria

The eligibility criteria of the patients for this study were as follows: (1) No indication for surgical resection or local ablation therapy such as RFA and PEI therapy; (2) No evidence of extra-hepatic metastasis; (3) No tumor thrombus in the main trunk of portal vein; (4) No evidence of active heart or renal diseases meeting the contraindications for ADM and CDDP therapy, respectively; (5) Eastern Cooperative Oncology Group (ECOG) performance status (PS)[22] level 0-2; (6) Hypervascular tumors showing enhancement during angiography; (7) Bidimensionally measurable hepatic lesions; (8) No uncontrolled ascites or pleural effusion; and (9) Total serum bilirubin (T-Bil) less than 3 mg/dL.

The presence of underlying liver diseases such as hepatitis or cirrhosis was confirmed by laboratory, radiological examinations and pathological examinations. We classified the chronic hepatitis patients into Child-Pugh class A, because chronic hepatitis is a known precirrhotic condition.

Preparation of the agents for TACE

We used DDPH or ADM (Adriacin; Kyowa Hakko Kogyo, Tokyo, Japan) mixed with LPD (iodized oil; Andre Guerget, Aulnay-sous-Bois, France).

The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 3-10 mL of LPD.

The ADM-LPD emulsion was prepared by the following procedure: 10-30 mg of ADM was dissolved in 1-2 mL of a contrast medium (Iomeron; Eisai Co., Ltd., Tokyo, Japan) and then mixed with 3-10 mL LPD.

The dosage of LPD and the anticancer drugs was adjusted depending on the tumor size, number of tumors, degree of liver impairment and renal function, however, the maximum dose of LPD was not allowed to exceed 10 mL.

Treatments

Hepatic arteriography, superior mesenteric arterial porto-

venography, CT during arteriography and CT during arterio-portography were performed to define the size and locations of tumor nodules and to exclude tumor thrombus in the main trunk of the portal vein. Following hepatic angiography, a catheter was selectively inserted into the hepatic artery supplying the target tumor and the DDPH-LPD suspension or the ADM-LPD emulsion was injected. In patients with several tumors in the liver, superselective catheterization was performed for each lesion. If superselective catheterization was not possible, the DDPH-LPD suspension or the ADM-LPD emulsion was injected into the right and left main hepatic artery distal to the origin of the cystic artery. After the injection, arterioembolization was performed used gelatin sponge particles (Gelpart; Nippon Kayaku, Tokyo, Japan) mixed with contrast medium.

All the patients were followed up with US, CT and/or MRI after 1 mo and every 3 mo thereafter. TACE was undertaken again when relapse of the treated lesions and/or new hepatic lesions were detected. These patients received additional TACE using the same agent during the follow-up period. The TACE was repeated until complete regression of the tumor was obtained, or until the patient could no longer be treated.

Post treatment assessment

Early tumor response was assessed by US, CT and/or MRI, conducted 1 mo after the initial treatment. We regarded LPD accumulation in the tumor as representing a necrotic area, based on previous reports of such LPD retention areas corresponding to the necrotic areas on CT[23-26]. By measurement of the two largest perpendicular diameters of the tumor, we classified the tumor response into four categories using the following criteria: complete response (CR), complete disappearance or 100% necrosis of all tumors; partial response (PR), reduction and/or necrosis, with at least 50% decrease of all the measurable lesions; progressive disease (PD), an increase of the tumor size exceeding 25% of all the measurable lesions or appearance of a new lesion; stable disease (SD), disease not qualifying for classification as CR, PR, PD.

Toxicity was evaluated by the National Cancer Institute-Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

Statistical analysis

The differences in the background clinical characteristics of the patients between the DDPH group and ADM group were assessed by Mann-Whitney's *U* test, logistic regression test, or the χ^2 test, as appropriate.

PFS and OS were calculated from the date of start of the therapy to the date on which tumor progression was documented and the date of death of the patient, respectively. Both were assessed by the Kaplan-Meier life-table method, and the differences between the two treatment groups were evaluated by the log rank test. Univariate analysis to identify the predictors of survival in the patients was conducted by the Kaplan-Meier life-table method, and the differences between the two groups were evaluated by the log rank test. Multivariate analysis to identify the predictors of survival was conducted using the Cox proportional hazards model. Statistical significance was defined as a *P* value of less than 0.05. All of the above analyses were performed using the SPSS software (version 11, SPSS, Chicago, IL, USA).

Table 1 Patient characteristics

Characteristics	DDPH group	ADM group	*P* value
No. of patients	76	88	
Age (yr) [median, (range)]	67 (32-87)	69 (21-90)	0.093
Gender (male/female)	57/19	52/36	0.031
Etiology (HBV/HCV/NBNC)	11/50/15	8/64/16	0.508
Child-Pugh classification (A/B/C)	47/26/3	45/36/7	0.303
TNM classification (Ⅰ-Ⅱ/Ⅲ-Ⅳ)	10/66	24/64	0.026
Tumor size (≤ 3.0/> 3.0 cm)	21/55	30/58	0.373
Number of tumors (1-3/≥ 4)	35/41	46/42	0.427
PVTT (Vp0-2 /Vp3)	62/14	80/8	0.080
Total bilirubin (≤ 1.5/> 1.5 mg/dL)	66/10	75/13	0.906
Albumin (≤ 3.5/> 3.5 g/dL)	38/38	45/42	0.822
AFP (≤ 1000/> 1000 ng/mL)	68/8	79/8	0.776
DCP (≤ 1000/> 1000 mAU/mL)	59/14	73/14	0.609

Data are expressed as median with range values, or the number of patients. The stages of HCC by TNM classification are clustered into two groups (Ⅰ-Ⅱ and Ⅲ-Ⅳ). The tumor characteristics and other parameters are classified as follows: tumor size: ≤ 3.0, > 3.0 cm; tumor number: 1-3, > 4; extent of PVTT: Vp 0-2, and Vp 3; serum bilirubin: ≤ 1.5, > 1.5 mg/dL; serum albumin: ≤ 3.5, > 3.5 g/dL; serum AFP levels: ≤ 1000, > 1000 ng/mL. Serum DCP levels: ≤ 1000, > 1000 mAU/mL. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin; DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD.

RESULTS

Patient profile

The characteristics of the 164 patients of both groups are summarized in Table 1. There were 109 male and 55 female patients, ranging in age from 21 to 90 years old (mean, 68 years old).

Regarding the assessment of differences in the characteristics of the patients, there were significant differences in the gender distribution and in the TNM classification between the two groups, i.e. there was a higher proportion of males ($P = 0.031$) and more subjects with advanced TNM classification ($P = 0.026$) in the DPHH group. There were no significant differences in any of the other characteristics between the two groups.

Treatments and early tumor response

The median follow-up period was 13.1 mo (range: 1-40 mo). We performed 392 TACE procedures (157 sessions in the DDPH group, 235 sessions in the ADM group) in 164 patients. The median number of TACE

Table 2 Early tumor response *n* (%)

	DDPH (*n* = 76)	ADM (*n* = 88)	*P* value
CR	2 (3)	5 (6)	
PR	39 (51)	16 (18)	
SD	23 (30)	5 (6)	
PD	12 (16)	62 (70)	
CR + PR	41 (54)	21 (24)	< 0.001

Data are expressed as number of patients and percentages. DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 3 Univariate analysis for identifying the predictors of survival

Variable	Hazard ratio	95% CI	*P* value
Treatment regimen (ADM *vs* DDPH)	0.580	0.325-1.035	0.065
Age (≤ 65 yr *vs* > 65 yr)	1.286	0.741-2.231	0.372
Gender (female *vs* male)	1.651	0.944-2.888	0.079
Etiology (NBNC *vs* HBV/HCV)	0.734	0.432-1.246	0.252
Child Pugh classification (A *vs* B/C)	1.142	0.689-1.891	0.607
TNM classification (Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ)	2.765	1.252-6.106	0.012
Tumor size (≤ 3.0 cm *vs* > 3.0 cm)	2.094	1.161-3.776	0.014
Number of tumors (1-3 *vs* ≥ 4)	2.612	1.535-4.444	0.001
PVTT (Vp0-2 *vs* Vp3)	4.714	2.520-8.819	< 0.001
Total bilirubin (≤ 1.5 mg/dL *vs* > 1.5 mg/dL)	1.730	0.874-3.422	0.116
Albumin (≤ 3.5 g/dL *vs* > 3.5 g/dL)	0.996	0.603-1.647	0.989
AFP (≤ 1000 ng/mL *vs* > 1000 ng/mL)	1.323	0.528-3.315	0.551
DCP (≤ 1000 mAU/mL *vs* > 1000 mAU/mL)	2.396	1.288-4.459	0.005

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin.

procedures was 2 sessions (range: 1-5 sessions) in the DDPH group and 3 sessions (range: 1-6 sessions) in the ADM group. The median interval to the re-treatment with TACE was 9.4 mo in the DDPH group and 3.8 mo in the ADM group. One hundred and ten sessions (70.1%) in the DDPH group and 170 sessions (72.3%) in the ADM group were treated by superselectivity of TACE. There was no significant difference in the incidence of superselectivity of TACE between the two groups.

In the DDPH group, 2 (3%), 39 (51%), 23 (30%) and 12 (16%) patients showed CR, PR, SD and PD, respectively. In the ADM group, 5 (6%), 16 (18%), 5 (6%) and 62 (70%) patients showed CR, PR, SD and PD, respectively. Therefore, the objective early response rate of the DDPH group (54%) was significantly higher than that in the ADM group (24%). The difference in the rate between the two groups was statistically significant ($P < 0.001$) (Table 2).

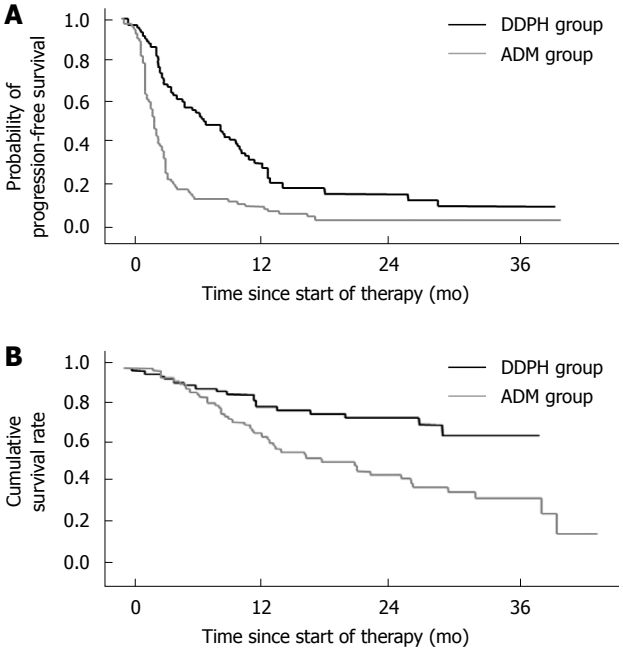

Figure 1 Comparison of the progression-free survival rates (A) and overall survival (B) between the DDPH and ADM groups. A: The progression-free survival rate was significantly higher in the DDPH group than in the ADM group (log-rank test: $P < 0.001$); B: The overall survival was significantly longer in the DDPH group than in the ADM group (log-rank test: $P = 0.002$). DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD.

PFS

The median PFS was 8.6 mo in the DDPH group and 3.0 mo in the ADM group. The PFS rates at 6, 12, 24 and 36 mo were 58%, 32%, 18% and 11%, respectively, in the DDPH group. In contrast, the corresponding values were 18%, 10%, 5% and 5%, respectively, in the ADM group. The PFS rates in the DDPH group were significantly higher than those in the ADM group ($P < 0.001$) (Figure 1A).

Survival

The median survival time (MST) in the DDPH and ADM groups was "not reached" and 20.8 mo, respectively. The OS values at 6, 12, 24, and 36 mo were 92%, 81%, 76% and 67%, respectively, in the DDPH group. The corresponding values in the ADM group were 87%, 68%, 46% and 37%, respectively. The OS in the DDPH group was significantly longer than that in the ADM group ($P = 0.002$) (Figure 1B).

Univariate analysis to identify the predictors of survival indicated five possible factors affecting the survival: TNM classification; tumor size; number of tumors; portal vein tumor thrombosis (PVTT) and serum DCP level. The treatment regimen was close to being statistically significant ($P = 0.065$) for survival (Table 3). Multivariate analysis performed using factors that were considered

Table 4 Multivariate analysis for identifying predictors of survival

Variable	Hazard ratio	95% CI	*P* value
Treatment regimen (ADM *vs* DDPH)	0.329	0.149-0.726	0.006
Gender (female *vs* male)	2.291	1.174-4.470	0.015
Number of tumors (1-3 *vs* ≥ 4)	6.541	3.201-13.363	< 0.001
PVTT (Vp0-2 *vs* Vp3)	6.704	2.581-17.418	< 0.001
Albumin (≤ 3.5 g/dL *vs* > 3.5 g/dL)	0.311	0.157-0.612	0.001

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; PVTT: Portal vein tumor thrombosis.

significant ($P < 0.1$) on univariate analysis identified the treatment regimen, gender, number of tumors, PVTT, and serum albumin as independent factors affecting the survival (Table 4).

Adverse effects

Table 5 shows a summary of the adverse effects in the two groups. The incidence rate of nausea/vomiting in the DDPH group was significantly higher than that in the ADM group ($P < 0.001$). In addition, the incidence rates of hepatic arterial damage (HAD) after TACE and leucopenia in the ADM group were significantly higher than those in the DDPH group ($P < 0.001$ and $P = 0.002$, respectively). We observed HAD in 17 patients. Although one patient in the DDPH group was observed to have slight wall irregularity of the hepatic artery (HA), HAD associated with TACE did not interfere with catheterization at the next TACE session. On the other hand, in the ADM group, we observed slight wall irregularity of HA in six patients, overt stenosis of HA in four patients and occlusion of HA in six patients. In six patients who were observed as having occlusion of HA, it became impossible to treat with repeated TACE.

No other serious complications or treatment-related deaths were observed in either group.

Table 5 Adverse events *n* (%)

Adverse effect	Treatment group (%)		*P* value
	DDPH group (*n* = 76)	ADM group (*n* = 88)	
Nausea/vomiting	64 (84)	48 (55)	< 0.001
Fever	61 (80)	73 (83)	0.571
Abdominal pain	53 (69)	63 (72)	0.958
Elevation of transaminase levels	55 (72)	62 (71)	0.993
Liver abscess	1 (1)	2 (2)	0.765
Hepatic arterial damage	1 (1)	16 (18)	< 0.001
Renal or liver failure	0 (0)	2 (2)	0.229
Leucopenia	3 (4)	12 (14)	0.002
Thrombocytopenia	4 (5)	6 (7)	0.650
Fatigue	21(28)	27 (31)	0.839

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; Data are expressed as number of patients, with the percentages indicated in parentheses.

DISCUSSION

TACE has been widely used for the treatment of unresectable HCC[9,10]. The most commonly used agent used in TACE for HCC treatment is ADM-LPD emulsion, followed by embolization with a gelatin sponge[13,14]; however, the tumors frequently recur[10,15,16] or residual tumors are observed at a high incidence. CDDP is an effective anticancer agent used in the treatment of various malignancies[17]. It has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. The antitumor activity of CDDP is closely associated with the serum concentration of the drug[27]. Therefore, the antitumor activity can be enhanced by increase of the dose. LPD acts as a selective carrier of anticancer agents and as an embolic material[23]; the anticancer agent is gradually released from the iodized oil. Although the mechanism of topical accumulation of LPD in the tumor is not yet precisely understood, it is used nonetheless to achieve a targeting drug delivery system with long-lasting accumulation in the tumor and gradual drug release. Consequently, augmented antitumor efficacy and milder side-effects have come to be expected with the use of this substance for TACE. In fact, Morimoto *et al*[28] investigated the pharmacological advantages of TACE using DDPH for hypervascular hepatic tumors in animal experiments. They reported that the tumor concentration of the platinum agent in the DDPH-LPD-TACE group was about 14 times higher than that in the DDPH-hepatic arterial infusion (HAI) group. In addition, they reported that the plasma concentrations of the platinum agent at 5 and 10 min from start of the infusion were lower in the DDPH-LPD-TACE group than those in the DDPH-HAI group. Recently, Ono *et al*[18] reported that TACE using a suspension of CDDP powder in LPD was more effective than that using ADM-LPD emulsion against unresectable HCC. Other investigators have also frequently reported favorable results obtained with TACE using a suspension of CDDP powder in LPD in HCC patients[19,29]. However, the CDDP powder for this therapy is difficult to produce because of the characteristics of the drug formulation. Therefore, CDDP powder had to be a custom-made formulation in individual institutions[30]. Consequently, when an institution was able to dispense CDDP powder in its own pharmacy department, TACE using a suspension of CDDP powder in LPD was undertaken.

A fine-powder formulation of CDDP, namely "DDPH", for intra-arterial infusion has been available for HCC treatment since 2004 in Japan. Dispensing of CDDP powder improved with the development of DDPH, and DDPH has now come to replace CDDP powder. Using DDPH-LPD suspension for TACE in HCC patients was expected to yield better therapeutic outcomes; therefore, TACE using DDPH became widespread in Japanese institutions. Nevertheless, the efficacy of TACE using DDPH-

LPD suspension has not yet been reported. Therefore, we compared the outcomes of TACE using DDPH-LPD suspension and ADM-LPD emulsion.

Analysis of the results in our study revealed that the objective response rate in the DDPH group was significantly higher than that in the ADM group. Moreover, the OS of the patients in the DDPH group was significantly longer than that of the patients in the ADM group. This could be explained as being due to the fact that TACE with ADM cannot be repeated as required because of the high frequency of adverse effects of ADM such as leucopenia, severe vascular changes and occlusion of the hepatic artery[18,31,32]. In fact, the incidences of leucopenia and HAD in the ADM group in our study were significantly higher than those in the DDPH group. Considering the fact that TACE is often repeated in most patients, longer patency of the hepatic artery is preferable for properly deploying the lipiodol mixture and embolic agents into the tumor. In addition, we conclude that anthracyclines such as ADM may be relatively less effective against HCC; this is because of the high expression level of P-glycoprotein, which transports antitumor agents such as anthracyclines or vinca alkaloids from cells with a high active efflux mechanism, in HCC tumors[33].

On the other hand, Pelletier *et al*[34] reported that TACE with CDDP sometimes caused severe complications, such as acute hepatic failure. The treatment also did not produce any significant improvement of the survival rate in this study. Severe complications could be expected with the high doses (2 mg/kg) of CDDP used in their study. Therefore, we performed TACE using DDPH-LPD suspension in our study with half of the dose (50 mg = 1 mg/kg) that they had used. Modification of the CDDP dose used for the treatment to DDPH 50 mg in our study resulted in a lower severity of complications.

Takayasu *et al*[35] reported a nationwide prospective cohort study which was performed in 8510 patients with unresectable HCC who underwent TACE using an emulsion of lipiodol and anticancer agents followed by gelatin sponge particles as an initial treatment. In their report, multivariate analysis for the factors affecting survival showed significant differences in degree of liver damage, AFP, maximum tumor size, number of lesions, and PVTT. In contrast to their report, we could not observe AFP value as a prognostic factor in our multivariate analysis. This may be due to fewer in the study population and a shorter observation period in our study compared with their study. In addition, a cut-off value for AFP of 1000 ng/mL in our study was much higher than that (400 ng/mL) in their study because we aimed to analyze the difference in the effect of TACE with the extent that HCC had progressed. Therefore, we could not observe AFP value as a prognostic factor in our multivariate analysis.

This study was not a well-controlled prospective study. Nevertheless, the patients in the two groups had fairly similar characteristics with regard to age, etiology, Child-Pugh classification, tumor size, number of tumors, PVTT, total bilirubin, albumin, AFP, and DCP. In relation to the differences in the characteristics of the patients, the DDPH group had a significantly higher proportion of males and a more advanced stage in TNM classification than the ADM group. Several investigators[36,37] have shown that TNM classification and tumor stage are independent prognostic factors for survival of patients who are treated by TACE. Therefore, we forecast that the prognosis of the CDDP group was worse than that of the ADM group, because the DDPH group had more advanced stage in TNM classification than the ADM group. However, the OS in the DDPH group was significantly longer than that in the ADM group. Moreover, to avoid the confounding effects of any deviations in the patient characteristics causing an impact on the results, we used the multivariate analysis for comparison of the efficacy between the regimens. The analysis identified the treatment regimen employed for the TACE as one of the most important prognostic factors. Compared to a previous report[18] describing TACE using a suspension of CDDP powder in LPD, the objective response rate and OS in the DDPH group in our study were significantly higher.

Considering these facts, we conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients. To confirm these results, randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD suspension for patients with HCC are mandatory.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Kouko Motodate for preparing serum samples.

COMMENTS

Background

In recent years, transcatheter arterial chemoembolization (TACE) using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been employed commonly for hepatocellular carcinoma (HCC) treatment. However, the tumors have been demonstrated to show a high frequency of recurrence after TACE.

Research frontiers

Cisplatin, a platinum compound, is an effective anticancer agent used in the treatment of various malignancies. Since 2004, a fine-powder formulation of cisplatin (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has also been available as a therapeutic agent for intra-arterial infusion in Japan. Researchers have recently reported that TACE using a suspension of cisplatin powder in LPD may be more effective against unresectable HCC as compared with TACE using ADM-LPD emulsion. Therefore, TACE using DDPH has become widespread in Japanese institutions. However, the efficacy of TACE using DDPH-LPD suspension has not yet been reported.

Innovations and breakthroughs

In this article, the authors reported the effectiveness of TACE using DDPH-LPD suspension compared with that using ADM-LPD emulsion.

Applications

Although randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD suspension for patients with HCC are needed, this study shows that TACE using DDPH-LPD suspension can be a useful treatment strategy for HCC patients.

Terminology

TACE: Transarterial chemoembolization, a procedure in which the blood supply to a tumor is blocked (embolized) and chemotherapy is administered directly into the tumor.

Peer review

Kasai *et al* evaluated the efficacy of TACE using a suspension of DDPH for HCC. The authors indicated that early response rate, progression free survival and overall survival in the DDPH group was significantly higher than that in the ADM group.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3445

World J Gastroenterol 2010 July 21; 16(27): 3445-3449
ISSN 1007-9327 (print)

BRIEF ARTICLE

Association of genetic polymorphisms of aldehyde dehydrogenase-2 with esophageal squamous cell dysplasia

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Supported by The Project of National Natural Science Foundation of China, No. 30571601; the 2004 Key Special Project of Scientific and Technological Development in Shandong Province, China, No. 2004GG1108039; and the 2007 Special Foundation for Postdoctoral Innovation Subject in Shandong Province of China, No. 200702034
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Received: October 18, 2009 Revised: December 30, 2009
Accepted: January 6, 2010
Published online: July 21, 2010

Abstract

AIM: To demonstrate the possible associations between genetic polymorphisms of aldehyde dehydrogenase-2 (*ALDH2*) and esophageal squamous cell dysplasia (ESCD).

METHODS: All participants came from an area of high incidence of esophageal cancer and underwent an endoscopic staining examination; biopsies were taken from a non-staining area of the mucosa and diagnosed by histopathology. Based on the examinations, the subjects were divided into the control group with normal esophageal squamous epithelial cells and the ESCD group. *ALDH2* genotypes of 396 cases were determined including 184 ESCD cases and 212 controls. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated by binary logistic regression models.

RESULTS: The distribution of *ALDH2* genotypes showed significant differences between the two groups. The adjustment factors were gender and age in the logistic regression models. Compared with *2*2/2*2* genotype, *2*1/2*1* genotype was found to be a risk factor for ESCD, and the OR (95% CI) was 4.50 (2.21-9.19). There were significant correlations between *ALDH2* genotypes and alcohol drinking/smoking/history of esophageal cancer.

CONCLUSION: The *ALDH2* polymorphism is significantly associated with ESCD.

Key words: Aldehyde dehydrogenase 2; Polymorphism; Alcohol; Smoking; Esophageal squamous cell dysplasia; History of esophageal cancer

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Zhou YZ, Diao YT, Li H, Li HQ, Ma Q, Cui J. Association of genetic polymorphisms of aldehyde dehydrogenase-2 with esophageal squamous cell dysplasia. *World J Gastroenterol* 2010; 16(27): 3445-3449 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3445.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3445

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of

the most common malignant tumors, and alcohol consumption is a major risk factor for esophageal cancer[1-3]. Alcohol is first oxidized by alcohol dehydrogenase (ADH) to acetaldehyde[4,5], which is then oxidized to acetate by acetaldehyde dehydrogenase (ALDH). The *ALDH2* gene encoding ALDH2 is composed of 13 exons residing on chromosome 12. Deficiency at ALDH2 is a dominant trait that is caused by a single Glu to Lys amino acid substitution at residue 487, a change that can be attributed to a G-to-A transition (dbSNP: rs671) in exon 12 of the gene. This deficient allele (*ALDH2*487Lys*) is common in many East Asian populations[6,7], and approximately 45% of Chinese and Japanese individuals have inactive ALDH2 phenotype[8].

The *ALDH2* alleles encoding the active and inactive subunits are termed "*ALDH2*1*" and "*ALDH2*2*" respectively, and *ALDH2*2* exhibits functional polymorphism that is associated with a lower rate of alcohol dependence[6]. In persons with *ALDH2*2*, the body fails to metabolize acetaldehyde rapidly, leading to excessive accumulation of acetaldehyde, so *ALDH2*2* enhances the risk of esophageal squamous cell carcinoma in East Asian drinkers[9-11].

The stages of the carcinogenic process of esophageal cancer develop from normal esophageal mucosa to esophagitis, esophageal hyperplasia, dysplasia, and then to cancer in situ and early cancer[12]. It is also noteworthy that Japanese and Chinese pathologists prefer to classify "atypical" squamous epithelium dysplasia as a precancerous lesion. Historically, atypical squamous dysplasia has been classified as mild, moderate, or severe[13,14].

Most reports of case-control studies have only consisted of one case-group of esophageal cancer and one normal control group[15,16], and few reports based on population screening data found an association of *ALDH2* with esophageal squamous cell dysplasia (ESCD). Recently, we carried out such a screening survey for esophageal lesions in a high incidence area of esophageal cancer and performed sampling to determine the association of genetic polymorphism of *ALDH2* with the ESCD in this area.

MATERIALS AND METHODS

Study population

The subjects in this study consisted of 184 patients with ESCD and 212 controls with normal esophageal mucosa. All subjects in the present study were selected from the screened participants of Feicheng County between January 2004 and December 2006.

The screening included a cardiograph, ventral ultrasound, and endoscopic examination, which used mucosal stain with 1.2% iodine solution. The biopsies were taken from a non-stained area of mucosa, which then underwent pathologic evaluation carried out by two pathologists. Participating subjects who suffered from cardiovascular, liver and kidney diseases, cancers, and psychiatric disorders were excluded. A uniform questionnaire was used to interview all the subjects to obtain information such as socio-demographic characteristics, alcohol intake, tobacco use, and family history of esophageal carcinoma. The local ethics committee approved the study protocol, and all participants gave their written informed consent.

DNA extraction

A 3-5 mL elbow venous blood sample was collected at 9-10 am after a 12-h fast from each participant. The heparinized sample was centrifuged for 10 min at 3000 r/min to separate plasma and obtain blood cells. Genomic DNA was extracted using phenol-chloroform method and frozen at -20℃.

PCR amplification

The following two pairs of primers were produced by Takara Biotechnology (Dalian Co., Ltd.): F1, 5'-TCATGCCATGGCAACTCCAGC-3'; R1, 5'-CCCACACTCACAGTTTTCTCTTC-3'; F2, 5'-TACGGGCTGCAGGCATACACTA-3'; R2, 5'-TGATCCCCAGCAGGTCCTGAA-3'. F1 and R1 were used to amplify the *ALDH2*1* allele (296 bp), and F2 and R2 to amplify the *ALDH2*2* allele (203 bp). Two 25 microliters reaction tubes were needed for each specimen to amplify *ALDH2*1* (G) and *ALDH2*2* (A) respectively, each containing 30-100 ng DNA, 0.12 mmol/L dNTPs, 12.5 pmol F1 (or R1) primer, 12.5 pmol F2 (or R2) primer, 0.5 U *Taq* polymerase, and 2.5 μL 10 × PCR buffer (containing 15 mmol/L $MgCl_2$). The reaction tubes were heated to 95℃ for 5 min followed by 30 cycles of 95℃ for 60 s, 60℃ for 60 s, 72℃ for 60 s, and 72℃ for 45 s, and then followed by a final extension of 5 min at 72℃. Ten microliters PCR product was used in agarose gel electrophoresis and the electrophoresis result was photographed.

Electrophoresis results

Two lanes were used for each specimen. If one showed 296 bp band and the other showed no band, the corresponding genotype was *ALDH2*1/2*1* (G/G); if one showed 296 bp band and the other showed 203 bp band, the corresponding genotype was *ALDH2*1/2*2* (G/A); and if one showed 203 bp band and the other showed no band, the corresponding genotype was *ALDH2*2/2*2* (A/A).

Statistical analysis

Results for the enumeration of data (e.g. the number of individuals with various genotypes) and comparison of percentages between groups were evaluated with a χ^2 test. Allele frequencies were calculated using allele counting tests for the Hardy-Weinberg equilibrium by the chi-square, while the odd ratio (OR) and 95% confidence intervals (95% CI) were calculated by multinomial logistic regression model. The statistical analysis were made using SPSS program (version 11.5), and $P < 0.05$ (two-sided) was taken as statistically significant.

Table 1 Characteristics of cases and controls *n* (%)

Variables	Controls	Cases	t/χ^2	P^3
Age (yr)	51.01 ± 8.427	54.89 ± 8.443	-4.567	0.000
Income (yuan/yr per person)	1825 ± 1605	1768 ± 1599	0.350	0.726
Height (cm)	163.71 ± 7.374	164.40 ± 7.225	-0.944	0.346
Weight (kg)	67.08 ± 52.848	61.36 ± 8.027	0.671	0.502
Body mass index (kg/m^2)	23.08 ± 2.957	22.68 ± 2.462	1.470	0.142
SBP (mmHg)	132.45 ± 19.852	135.38 ± 20.411	-1.445	0.149
DBP (mmHg)	84.74 ± 13.083	85.27 ± 13.132	-0.406	0.685
Gender				
Male	123 (58.0)	124 (67.4)	3.687	0.055
Female	89 (42.0)	60 (184)		
Age (yr)				
40-49	94 (44.3)	41 (22.3)	22.407	0.000
50-59	81 (38.2)	91 (49.5)		
60-69	37 (17.5)	52 (28.3)		
Education				
Illiteracy	33 (15.6)	34 (18.5)	6.838	0.077
Primary school	54 (25.5)	54 (29.3)		
High school	96 (45.3)	85 (46.2)		
College and above	29 (13.7)	11 (6.0)		
Smoking index[1]				
0	117 (55.2)	81 (44.0)	6.107	0.047
< 500	47 (22.2)	43 (23.4)		
⩾ 500	48 (22.6)	60 (32.6)		
Alcohol drinking status[2]				
0	106 (50.0)	81 (44.0)	2.057	0.358
< 65	48 (22.6)	41 (22.3)		
⩾ 65	58 (27.4)	62 (33.7)		
History of esophageal cancer				
No	166 (84.7)	152 (82.6)	0.302	0.582
Yes	30 (15.3)	32 (17.4)		
ALDH2				
G/G	65 (30.7)	98 (53.3)	25.431	0.000
G/A	106 (50.0)	73 (39.7)		
A/A	41 (19.3)	13 (7.1)		

[1]Smoking index = cigarettes/d × number of smoking years; [2]Alcohol ⩾ 65 g/d = heavy drinker; [3]*t* test and χ^2 test were used for quantitative data variables and categorical data variables respectively. SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

RESULTS

Basic data

All analysis variables are shown in Table 1. Gender, age, smoking, and *ALDH2* genotypes were significantly different between the two groups. The Hardy-Weinberg test for the control group showed the genotype distribution was in equilibrium. Gender and age as potential confounders were adjusted in the logistic models. Other variables including income (yuan/year per person), height, weight, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), alcohol drinking, and family history of esophageal cancer were not significantly different between the two groups.

Association of ALDH2 genotypes with ESCD

As shown in Table 2, after the potential confounders were adjusted, compared with *ALDH 2*2/2*2* genotype, *2*1/2*1* and *2*1/2*2* genotypes were related to having ESCD, and the ORs (95% CI) were 4.50 (2.21-9.19) and 2.19 (1.08-4.43), respectively. Meanwhile, after adjusting for gender and age, no significant association of smoking, alcohol drinking or family history of esophageal cancer with ESCD were observed in the two groups.

Table 2 Association of factors with squamous cell dysplasia of esophagus

Factors	OR (95% CI)[1]	OR (95% CI)[2]
Smoking index		
0	1.00	1.00
< 500	1.32 (0.80-2.18)	1.19 (0.64-2.24)
⩾ 500	1.81 (1.12-2.90)	1.56 (0.83-2.91)
Drinking index		
0	1.00	1.00
< 65	1.12 (0.67-1.86)	0.92 (0.49-1.76)
⩾ 65	1.40 (0.88-2.22)	1.03 (0.55-1.96)
History of esophageal cancer		
No	1.00	1.00
Yes	1.10 (0.66-1.85)	1.08 (0.64-1.83)
ALDH2		
A/A	1.00	1.00
A/G	2.17 (1.09-4.34)	2.19 (1.08-4.43)
G/G	4.76 (2.37-9.56)	4.50 (2.21-9.19)

[1]Crude OR; [2]Adjusted ORs were adjusted for age and gender. OR: Odds ratio; CI: Confidence intervals.

Interaction analysis of the ALDH2 genotypes and environmental factors

The frequency distribution of *ALDH2* genotypes combined with smoking index/alcohol drinking status and history of esophageal cancer are listed in Table 3.

As shown in Table 3, in the no smoking stratum only *ALDH 2*1/2*1* genotype increased the relative risk (OR = 6.97, 95% CI: 1.88-25.87); in the light smoking stratum *2*1/2*2* and *2*1/2*1* increased the relative risk and there was an interaction effect between the genotypes and smoking; and in the heavy smoking stratum either smoking or any genotypes of *ALDH2* were associated with the dysplasia, when compared with *ALDH2*2/2*2* genotype combined with no smoking as baseline.

In the no drinking stratum only *ALDH 2*1/2*1* genotype increased the relative risk (OR = 3.16, 95% CI: 1.19-8.41); and in the light and heavy drinking strata both *2*1/2*2* and *2*1/2*1* increased the relative risk with an interaction effect between the genotypes and drinking, when compared with *ALDH2*2/2*2* genotype combined with no drinking as baseline.

There was a significant interaction between *ALDH2*1/2*1* genotype and family history of esophageal cancer, when compared with *ALDH2*2/2*2* genotype and no family history of esophageal cancer as baseline.

DISCUSSION

Feicheng, a County in Shandong Province of China, was found to be a high incidence area of esophageal cancer. Its mortality rates from esophageal cancer were 63.19, 71.68, 66.87 and 82.33/100 000 in the years 1970-1974,

Table 3 Interaction between *ALDH2* genotypes and environmental factors for esophageal dysplasia *n* (%)

Factors	Genotype	Controls	Cases	OR (95% CI)[1]	OR (95% CI)[2]
Smoking index	*ALDH2*				
0	A/A	18 (8.5)	3 (1.6)	1.00	1.00
0	G/A	62 (29.2)	33 (17.9)	3.19 (0.88-11.64)	3.10 (0.84-11.45)
0	G/G	37 (17.5)	45 (24.5)	7.30 (1.99-26.71)	6.97 (1.88-25.87)
< 500	A/A	17 (8.0)	3 (1.6)	1.06 (0.19-5.99)	0.98 (0.17-5.77)
< 500	G/A	22 (10.4)	19 (10.3)	5.18 (1.32-20.35)	4.70 (1.16-19.07)
< 500	G/G	8 (3.8)	21 (11.4)	15.75 (3.63-68.41)	13.10 (2.85-62.14)
⩾ 500	A/A	6 (2.8)	7 (3.8)	7.00 (1.36-36.01)	6.84 (1.25-37.36)
⩾ 500	G/A	22 (10.4)	21 (11.4)	5.73 (1.47-22.33)	5.03 (1.21-20.82)
⩾ 500	G/G	20 (9.4)	32 (17.4)	9.60 (2.50-36.81)	7.78 (1.92-31.53)
Drinking index	*ALDH2*				
0	A/A	20 (9.4)	8 (4.3)	1.00	1.00
0	G/A	50 (23.6)	34 (18.5)	1.70 (0.67-4.30)	1.98 (0.75-5.25)
0	G/G	36 (17.0)	39 (21.2)	2.71 (1.06-6.91)	3.16 (1.19-8.41)
< 65	A/A	12 (5.7)	3 (1.6)	0.63 (0.14-2.82)	0.65 (0.19-3.09)
< 65	G/A	27 (12.7)	15 (8.2)	1.39 (0.48-3.91)	1.19 (0.40-3.50)
< 65	G/G	9 (4.2)	23 (12.5)	6.39 (2.07-19.69)	5.05 (1.57-16.15)
⩾ 65	A/A	9 (4.2)	2 (1.1)	0.56 (0.10-3.16)	0.43 (0.07-2.52)
⩾ 65	G/A	29 (13.7)	24 (13.0)	2.07 (0.78-5.23)	1.70 (0.60-4.80)
⩾ 65	G/G	20 (9.4)	36 (19.6)	4.50 (1.68-12.06)	3.19 (1.12-9.28)
History of esophageal cancer	*ALDH2*				
No	A/A	31 (15.8)	10 (5.4)	1.00	1.00
No	G/A	82 (41.8)	61 (33.2)	2.31 (1.05-5.06)	2.23 (1.01-4.93)
No	G/G	53 (27.0)	81 (44.0)	47.74 (2.15-10.47)	4.30 (1.93-9.62)
Yes	A/A	9 (4.6)	3 (1.6)	1.03 (0.23-4.58)	0.88 (0.20-4.01)
Yes	G/A	14 (7.1)	12 (6.5)	2.66 (0.93-7.59)	2.37 (0.82-6.89)
Yes	G/G	7 (3.6)	17 (9.2)	7.53 (2.42-23.37)	7.11 (2.25-22.45)

[1]Crude OR; [2]Adjusted ORs were adjusted for age and gender. OR: Odds ratio; CI: Confidence intervals.

1985-1989, 1990-1992, and 1997-1999, respectively[17]. In the present study, the cases and controls came from the same communities of Feicheng County, and they possessed similar environment and customs, so their data are comparable. All diseases were determined by endoscopic and pathological examinations, and the possibility of misclassification was small. Considering the information with little recall bias, we believe the results of this study provide more convincing evidence to elucidate the relationship between *ALDH2* polymorphism and ESCD.

The *ALDH2*2* allele produces an inactive protein subunit, which is unable to metabolize acetaldehyde. Genetic epidemiologic studies have suggested that the *ALDH2*2* allele inhibits the development of alcoholism. The inheritance of alcohol-induced flushing in families also suggested that the trait is dominant, that is, both *ALDH2*1/2*2* and *ALDH2*2/2*2* genotype encode inactive ALDH2[18,19].

The *ALDH2*1/2*1* allele has a dual effect on esophageal cancer. On one hand, it can convert acetaldehyde to acetate and get rid of the carcinogenic role of acetaldehyde. On the other, it decreases the blood level of acetaldehyde and alleviates adverse response to alcohol consumption[20], so individuals who have an *ALDH2*1/2*1* genotype are prone to heavy drinking and an increased risk of esophageal cancer. We found in this study that *ALDH2*1/2*1* genotype was a risk factor for ESCD compared with *ALDH2*2/2*2* genotype. Although individuals with *ALDH2*1/2*1* have a strong alcohol metabolism ability, if the alcohol consumption is beyond the metabolism ability the alcohol becomes a dangerous factor for ESCD.

Most reports have indicated that alcohol increases the risk of ESSC in drinkers with *ALDH2*2/2*2* genotype. This leads us to speculate that alcohol will also increase the risk of ESCD in drinkers with *ALDH2*2/2*2* genotype. However, our result did not confirm this association. The reason may be related to the very low frequency of residents who drink alcohol, in particular who indulge in heavy drinking, in Feicheng County, where the people's living standard is relatively low and the majority of farmers cannot afford to drink wine[21,22].

The main finding in the study was an interaction between the *ALDH2* genotype and smoking/family history of esophageal cancer in cases of ESCD, indicating that a polymorphism of *ALDH2* is involved in the process of some carcinogen metabolism, and that it is helpful to control alcohol and tobacco consumption in high incidence areas of esophageal cancer to reduce ESCD and other esophageal diseases.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors, and alcohol consumption is a major risk factor for esophageal cancer. Acetaldehyde dehydrogenase (ALDH) is related to the risk of ESCC, and esophageal squamous cell dysplasia(ESCD) is one of precancerous lesions, so it is necessary to study the relationship of ALDH and ESCD.

Research frontiers

Most previous reports have indicated that alcohol increases the risk of ESSC in drinkers with *ALDH2*2/2*2* genotype. This leads to speculation that alcohol will also increase the risk of ESCD in drinkers with *ALDH2*2/2*2* genotype. However, this association was not found in this study, but interactions between the *ALDH2* genotype and smoking/family history of esophageal cancer were found in cases of ESCD.

Innovations and breakthroughs

Most reports of case-control studies have only consisted of one case-group of esophageal cancer and one normal control group, and few reports based on population screening data found an association of *ALDH2* with ESCD. In this study, the data of a screening survey for esophageal lesions in a high incidence area of esophageal cancer were used and the association of genetic polymorphisms of *ALDH2* with ESCD in this area was determined.

Applications

It was found that *ALDH2* genotype has interactions with smoking/family history of esophageal cancer for ESCD cases, indicating that a polymorphism of *ALDH2* is involved in the process of some carcinogen metabolism; so it is necessary to control alcohol and tobacco consumption in high incidence areas of esophageal cancer to reduce ESCD and other esophageal diseases.

Terminology

Alcohol is first oxidized by alcohol dehydrogenase to acetaldehyde which is then oxidized to ALDH. The *ALDH2* gene is composed of 13 exons residing on chromosome 12. Deficiency at ALDH2 is a dominant trait that is caused by a single Glu to Lys amino acid substitution at residue 487, a change that can be attributed to a G-to-A transition (dbSNP: rs671) in exon 12 of the gene. The *ALDH2* alleles encoding the active and inactive subunits are termed "*ALDH2*1*" and "*ALDH2*2*" respectively, and *ALDH2*2* exhibits functional polymorphism that is associated with a lower rate of alcohol dependence.

Peer review

This is a well-designed and well-organized study of acetaldehyde dehydrogenase 2 polymorphisms and the risk of esophageal squamous cell dysplasia.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3450

World J Gastroenterol 2010 July 21; 16(27): 3450-3456
ISSN 1007-9327 (print)

BRIEF ARTICLE

Radiofrequency ablation in the treatment of small hepatocellular carcinoma: A meta analysis

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Received: March 31, 2010 Revised: April 28, 2010
Accepted: May 5, 2010
Published online: July 21, 2010

Abstract

AIM: To evaluate survival and recurrence after radiofrequency ablation (RFA) for the treatment of small hepatocellular carcinoma (HCC) using a meta-analysis.

METHODS: Literature on RFA *vs* surgical resection for the treatment of small HCC published between January 1990 and December 2008 was retrieved. A meta-analysis was conducted to estimate pooled survival and recurrence ratios. A fixed or random effect model was established to collect the data.

RESULTS: The differences in overall survival at 1-year, 3-years and at end of follow-up were not statistically significant between the RFA and surgery groups ($P > 0.05$). There were no differences in 1-year and 3-year recurrences between the RFA and surgery groups ($P > 0.05$). However, recurrence in the RFA group was lower than that in the surgery group up to the end of follow-up ($P = 0.03$). Survival was not significantly different. There was a significant difference in recurrences at the end of follow-up after RFA compared with surgical resection.

CONCLUSION: RFA did not decrease the number of overall recurrences, and had no effect on survival when compared with surgical resection in a selected group of patients.

Key words: Hepatectomy; Hepatocellular carcinoma; Meta-analysis; Radiofrequency ablation; Recurrence; Survival

Peer reviewer: Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Liu JG, Wang YJ, Du Z. Radiofrequency ablation in the treatment of small hepatocellular carcinoma: A meta analysis. *World J Gastroenterol* 2010; 16(27): 3450-3456 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3450.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3450

INTRODUCTION

The preferred treatment for hepatocellular carcinoma (HCC) is surgical resection which has a good long-term effect. In recent years, radiofrequency ablation (RFA) has emerged as the latest oriented treatment, especially for HCC, and has become an important treatment following surgical resection and has established its place in the treatment algorithm of liver tumors. The treatment of HCC in patients with chronic liver disease is a major challenge. With the intention of avoiding hepatic failure which can appear after hepatic resection, percutaneous ablative treatments have been proposed. RFA ablation has progressively reached consensus due to its efficacy, tolerability and low-risk[1]. RFA is much less invasive, involves a short hospital stay and has an extremely low associated mortality; however, long-term results are difficult to ascertain, because the majority of reports concern evaluation of the percentage of success in terms of tumor necrosis and few data are available on the overall

and disease-free survival of patients[2-6]. Clear evidence is still needed for RFA to be accepted as an alternative to surgery for resectable HCC on cirrhosis. Few studies have focused on a comparison between the results of surgery and RFA. In order to reduce research bias and differences, we used a meta-analysis to compare survival and recurrences following RFA compared with surgical resection for the treatment of small HCC. This article may provide a reference for clinical practice.

MATERIALS AND METHODS

Data accrual

We carried out an exhaustive Medline, PubMed, CBM and CNKI search of the world literature comparing survival and recurrences following RFA compared with surgical resection for the treatment of small HCC, between the period January 1990 to December 2008 using the key words (radiofrequency, radio-frequency or radio frequency), (surgical resection or hepatectomy) and (liver or hepatic or hepatocellular) in English, French, German, Italian, Spanish, Danish, Dutch, Korean and Chinese. All abstract supplements from published literature were searched manually. Relevant papers were also identified from the reference lists of previous papers which were obtained through the search, and from abstracts from recent international meetings.

In the case of overlap between 2 reports, only the most detailed report was included. Only series with a minimum follow-up of 12 mo were included. Reports about treatments obtained with noncommercial electrodes and treatments with palliative intent (intentional partial debulking) were excluded. When appropriate, authors were contacted to obtain more details about the cases they reported.

In addition, we chose some Chinese articles, as there are many patients with small HCC in China. A good meta-analysis requires these data.

Data extraction and quality assessment

Data were extracted by two or three independent observers using standardized forms. The recorded data included the number of patients, overall survival and recurrence. The quality of all selected articles was ranked in accordance with the score of the non-randomized controlled clinical trial quality evaluation standard (Table 1).

Study selection criteria

Inclusion criteria for this study were as follows: (1) A solitary HCC smaller than 5 cm in diameter or multiple (no more than three) HCC smaller than 5 cm in total diameter; (2) No extrahepatic metastasis; (3) No radiologic evidence of invasion into the major portal/hepatic vein branches; (4) Good liver function with Child-Pugh Class A or B, with no history of encephalopathy, ascites refractory to diuretics or variceal bleeding; (5) No previous treatment of HCC; (6) Patient should be suitable for treatment with either surgical resection or RFA; and (7) No recurrences where no tumor was found by spiral computed tomography and serum α-fetoprotein level when assessed every 3 mo after treatment during the follow-up period.

Statistical analysis

Meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Statistical heterogeneity between trials was evaluated by the Cochran χ^2 test and was considered significant when $P < 0.10$. In the absence of statistically significant heterogeneity, the Mantel-Haenszel method in the fixed-effect model was used for the meta analysis. Otherwise, the DerSimonian and Laird method in the random-effect model was selected.

The odds ratio (OR) with 95% confidence interval (CI) was used to assess treatment efficacy. The combined result was an average OR and 95% CI weighted according to the standard error of the OR of the trial. $P < 0.05$ was considered statistically significant. We used funnel plots to assess the publication bias, and tested for funnel plot asymmetry using Egger's test and Begg's test. All analyses were performed with STATA version 9.0 (Stata Co., College Station, TX, USA) and Review Manager version 4.2.2 (RevMan, Cochrane Collaboration, Oxford, England).

RESULTS

Description of included trials in the meta-analysis

According to exclusion and selected criteria of historical data, 10 studies were selected for the meta analysis, including 787 cases of RFA and 735 cases of surgical resection. However, one publication[7] was removed, because the number of cases continued to expand in another publication[8]. Among the 10 articles selected, 4 (40%) were from China, and corresponded to the high incidence of Hepatitis B virus-associated HCC in China. The characteristics of the 10 clinical trials included are shown in Table 1.

Meta-analysis

The comparison of survival and recurrence following RFA *vs* surgical resection for the treatment of small HCC using the meta-analysis is shown in Figures 1-3 [8-17].

Survival during follow-up 1 year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.95$). Accordingly, a fixed-effect model was used. There was no difference in the 1-year overall survival rate between the RFA group (87.9%) and the surgical resection group (88.6%) with a combined OR of 0.94 (95% CI: 0.65 to 1.36, $P = 0.75$, Figure 1).

Survival during follow-up 3-year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.0002$). Accordingly, a random-effect model was used. There was no difference in the 3-year overall survival rate between the RFA group (62.5%) and the surgical resection group (63.6%) with a combined OR of 0.92 (95% CI: 0.56 to 1.51, $P = 0.73$, Figure 2A).

Table 1 Outcome data and methodological quality of studies included in the meta-analysis

Author	Yr	Study design	RFA (cases)	Hepatectomy (cases)	Journal	Quality evaluation score[1]
Peng *et al*[8]	2008	Retrospective study	251	183	*Zhongguo Shiyong Waike Zazhi*	7
Vivarelli *et al*[9]	2004	Cohort study	58	40	*Ann Surg*	7
Zhang *et al*[10]	2007	Retrospective study	15	29	*Disan Junyi Daxue Xuebao*	7
Zhou *et al*[11]	2007	Retrospective study	47	40	*Gandan Waike Zazhi*	7
Guglielmi *et al*[12]	2008	Retrospective study	109	91	*J Gastrointest Surg*	7
Montorsi *et al*[13]	2005	Cohort study	79	79	*J Gastrointest Surg*	7
Hong *et al*[14]	2005	Cohort study	55	93	*J Clin Gastroenterol*	9
Wakai *et al*[15]	2006	Retrospective study	21	85	*World J Gastroenterol*	7
Cho *et al*[16]	2005	Retrospective study	99	61	*Korean J Hepatol*	9
Gao *et al*[17]	2007	Retrospective study	53	34	*Zhongguo Yixue Yingxiang Jishu Zazhi*	9

[1]The score from the non-randomized controlled clinical trial quality evaluation standard. RFA: Radiofrequency ablation.

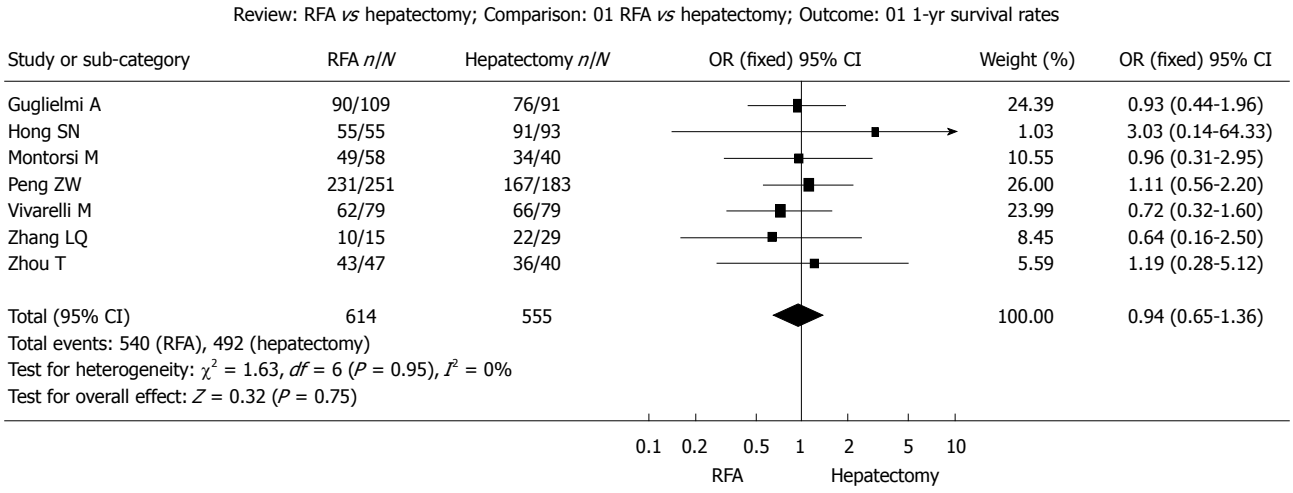

Figure 1 Fixed effect model of odds ratio for survival during follow-up 1-year after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

Survival up to the end of the follow-up period: The χ^2 test of heterogeneity was highly significant ($P < 0.0001$). Accordingly, a random-effect model was used. There was no difference in overall survival rate at the end of follow-up after treatment with RFA (57.4%) compared with surgical resection (60.9%) with a combined OR of 0.82 (95% CI: 0.48 to 1.39, $P = 0.46$, Figure 2B).

Recurrence during follow-up 1-year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.07$). Accordingly, a fixed-effect model was used. There was no difference in recurrence rate during follow-up 1-year after treatment between the RFA group (20.6%) and the surgical resection group (20.9%) with a combined OR of 0.96 (95% CI: 0.69 to 1.33, $P = 0.80$, Figure 3A).

Recurrence during follow-up 3-year after treatment: The χ^2 test of heterogeneity was highly significant ($P < 0.0001$). Accordingly, a random-effect model was used. There was no difference in recurrence rate during follow-up 3-years after treatment between the RFA group (59.4%) and the surgical resection group (60.4%) with a combined OR of 1.19 (95% CI: 0.63 to 2.27, $P = 0.59$, Figure 3B).

Recurrence up to the end of the follow-up period: The χ^2 test of heterogeneity was highly significant ($P = 0.0005$). Accordingly, a random-effect model was used. The recurrence rate up to the end of the follow-up period was significantly higher in the RFA group (66.7%) than in the surgical resection group (52.9%) with a combined OR of 1.73 (95% CI: 1.04 to 2.87, $P = 0.03$, Figure 3C).

Sensitivity analysis and publication bias

Publication bias may exist when no significant findings remain unpublished, thus artificially inflating the apparent magnitude of an effect.

Survival and recurrences following RFA or surgical resection for the treatment of small HCC were calculated by the fixed-effect model and random-effect model, respectively. The results were similar and the combined results were highly reliable.

Funnel plots of the study results are shown in Figure 4A-F. The funnel plots on survival and recurrence following RFA or surgical resection for the treatment of small HCC showed basic symmetry, which suggested no publication bias.

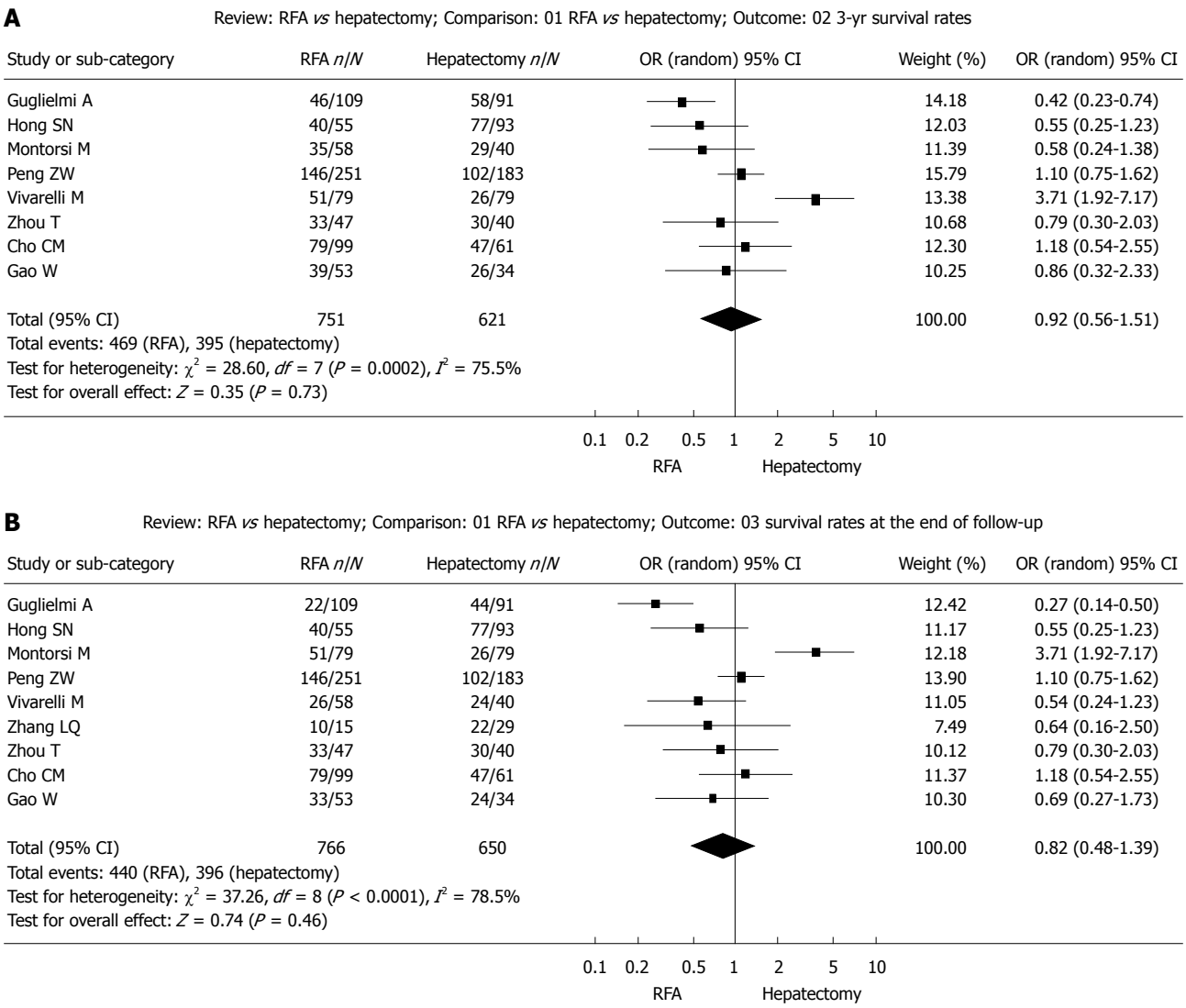

Figure 2 Random effect model of odds ratio for survival of follow-up 3-year (A) and at the end of follow-up (B) after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

DISCUSSION

Hepatocellular carcinoma (HCC) is one of most common malignant tumors of the liver. According to the general condition of patients, tumor location and size and liver function status, surgery can include radical tumor resection, or liver surgery such as local excision. However, there are factors that limit the use of surgical resection. RFA is a relatively new treatment and is now performed more widely, because it results in large coagulated necrosis of the tumor, requires fewer treatment sessions, and achieves higher survival rates[18,19].

RFA has the potential to enhance the long-term survival rate of liver cancer patients worldwide and is of significant importance[20]. Research has indicated that more than 90% of the tumor can be completely destroyed and tumor recurrence *in situ* is effectively inhibited following RFA, which also achieved satisfactory short-term efficacy[21]. Long-term survival following RFA treatment was satisfactory in liver cancer patients as was liver function in those with A-class[22]. The efficacy of RFA was also shown to be related to Child-Pugh grading[23]. Compared with surgery, RFA did not cause significant liver function damage, had a lower rate of complications and was more affordable in terms of treatment costs. The results of this study showed that RFA did not decrease overall recurrences, but had no effect on survival in comparison with surgical resection (i.e. compared with surgical resection, RFA showed no significant difference in the short-term survival rate).

This review has some limitations. Funnel plots can be suggestive of publication bias with lack of negative small RCTs. However, a firm conclusion about bias is difficult to reach as the asymmetry of the funnel plot is minimal. In addition, funnel plots can show asymmetry for reasons other than publication bias. Therefore, our pooled OR might be an overestimate of the true effect. Due to data constraints, this meta-analysis could not analyze the quality of life score and was unable to carry out stratified analyses of other possible confounding factors. If the method is to be more effective, then larger samples and randomized controlled studies with longer

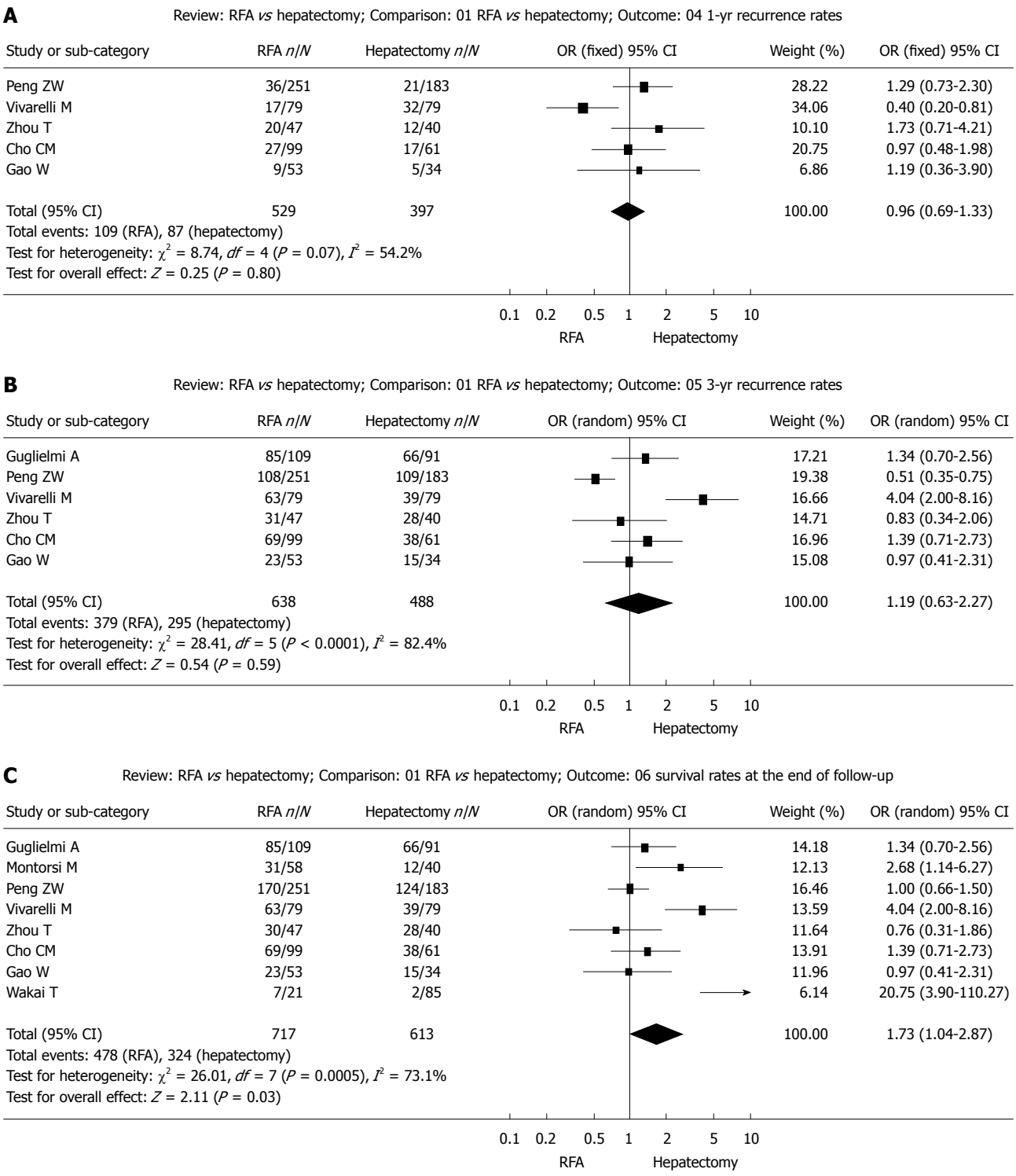

Figure 3 Random effect model of odds ratio for Recurrence of follow-up 1-year (A) and 3-year (B) and the end of follow-up (C) after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

follow-up are required[24]. Chinese article should also be chosen, because there are many patients with small HCC in China. A good meta-analysis requires these data. However, the conclusions of this study also need more detailed data to confirm the results. The search language was limited. The integrity of the data was affected to a certain extent.

In conclusion, with the development of RFA, when conditions permit and under technically assured circumstances, RFA can be performed percutaneously, laparoscopically or during laparotomy, and can partially replace surgical resection. For patients who do not have the opportunity or are unwilling to accept surgical treatment, RFA is an acceptable means of palliative care.

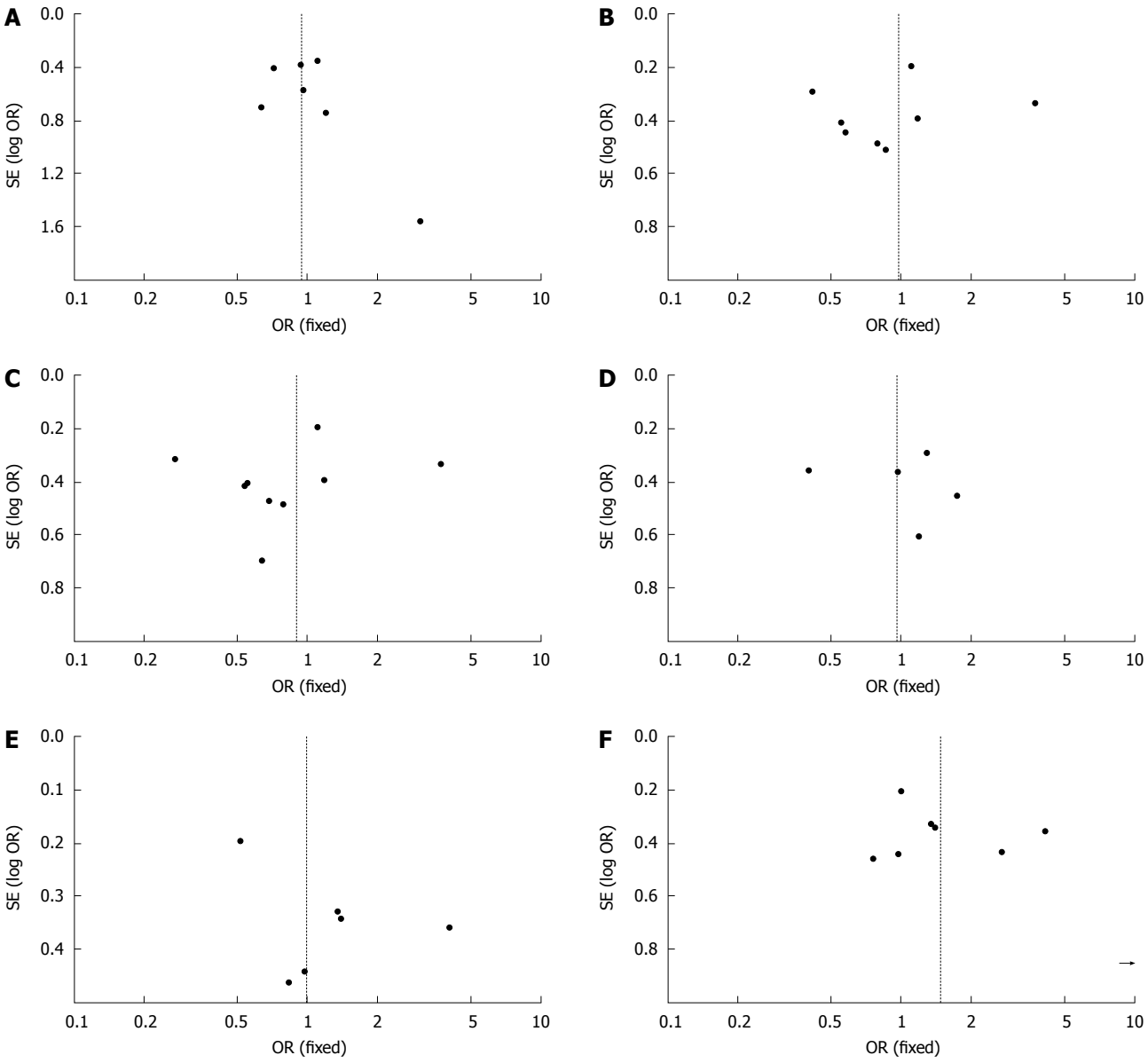

Figure 4 Funnel plots. A: 7 articles in the meta-analysis of survival during follow-up 1-year after treatment; B: 8 articles in the meta-analysis of survival during follow-up 3-years after treatment; C: 9 articles in the meta-analysis of survival up to the end of follow-up; D: 5 articles in the meta-analysis of recurrence during follow-up 1-year after treatment; E: 6 articles in the meta-analysis of recurrence during follow-up 3-years after treatment; F: 8 articles in the meta-analysis of recurrence up to the end of follow-up.

COMMENTS

Background

Over the last decade, radiofrequency thermal ablation (RFA) has established its place in the treatment algorithm of liver tumors. This meta-analysis was designed to evaluate survival and recurrence following RFA for the treatment of small hepatocellular carcinoma (HCC).

Research frontiers

The study evaluated survival and recurrence following RFA for the treatment of HCC using a meta analysis of all relevant controlled studies.

Innovations and breakthroughs

The authors made a comprehensive search of studies dealing with small HCC treated with RFA. The studies were analyzed to determine survival and recurrence after RFA in these patients.

Applications

RFA is an effective technique for the treatment of small HCC and offers an alternative treatment method. This meta-analysis shows that RFA did not decrease overall recurrences, but had no effect on survival in comparison with surgical resection in a selected group of patients. Larger samples and randomized controlled studies with longer follow-up are required.

Peer review

This is an interesting report of RFA *vs* surgical resection for HCC.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3457

World J Gastroenterol 2010 July 21; 16(27): 3457-3464
ISSN 1007-9327 (print)

BRIEF ARTICLE

Impact of human leukocyte antigen mismatching on outcomes of liver transplantation: A meta-analysis

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Received: January 4, 2010 Revised: February 20, 2010

Accepted: February 27, 2010

Published online: July 21, 2010

Abstract

AIM: To assess the effect of human leukocyte antigen (HLA) mismatching on liver graft outcome and acute rejection from a meta-analysis of available cohort studies.

METHODS: Articles in PubMed/MEDLINE, EMBASE and the Cochrane database from January 1970 to June 2009, including non-English literature identified in these databases, were searched. Only studies comparing HLA or sub-phenotype matching with mismatching were extracted. The percentage of graft survival was extracted by "Engauge Digitizer" from survival curves if the raw data were not displayed. A meta-analysis was performed when at least 3 studies provided data.

RESULTS: Sixteen studies met the inclusion criteria. A lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection (relative risk: 0.77, $P = 0.03$). The degree of HLA mismatching (0-2 *vs* 3-6) had no significant effect on 1-year [hazard ratio (HR): 1.04, $P = 0.68$] and 5-year (HR: 1.09, $P = 0.38$) graft survival. In sub-phenotype analysis, the degree of HLA-A, B and DR mismatching (0 *vs* 1-2) had no significant effect on 1-year and 5-year graft survival, either. The HRs and *P*-values were 0.95, 0.71 (HLA-A, 1-year); 1.06, 0.60 (HLA-A, 5-year); 0.77, 0.16 (HLA-B, 1-year); 1.07, 0.56 (HLA-DR, 1-year); 1.18, 0.23 (HLA-DR, 5-year), respectively.

CONCLUSION: The results of this systematic review imply that good HLA compatibility can reduce the incidence of acute rejection in spite of having no influence on graft outcomes. To obtain a short recovery time and minimize rejection post transplantation, HLA matching studies should be considered before the operation.

Key words: Human leukocyte antigen; Mismatching; Liver transplantation; Meta-analysis; Graft rejection

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Lan X, Zhang MM, Pu CL, Guo CB, Kang Q, Li YC, Dai XK, Deng YH, Xiong Q, Ren ZM. Impact of human leukocyte antigen mismatching on outcomes of liver transplantation: A meta-analysis. *World J Gastroenterol* 2010; 16(27): 3457-3464 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3457.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3457

INTRODUCTION

In the past 2 decades, deaths and other complications of organ transplantation have decreased significantly as a re-

sult of improvements in anesthesiology and surgical techniques. In addition, development of immunosuppressive agents and new organ preservation solutions have been shown to play a role in the improved survival rate. However, acute or chronic rejection remains the most important reason of graft failure, especially for patients who suffer from mismatching of human leukocyte antigen (HLA).

The role of HLA matching between donor and recipient in organ transplant rejection and survival has been widely studied and proven to increase graft survival after kidney, heart, and other organ transplantation and to reduce the incidence of acute or chronic rejection[1-6]. In contrast, major histocompatibility complex analysis is not routinely performed in liver transplantation because its importance remains controversial, with different groups reporting disparate results. It was reported that some populations of patients gained benefit from high degrees of HLA matching[7-14]. Concern has been voiced about possible increased likelihood of recurrence of primary disease with good HLA compatibility[15-25].

We therefore performed a systematic review and meta-analysis on the efficacy of HLA mismatching in all published controlled clinical trials on the outcomes of liver transplantation.

MATERIALS AND METHODS

Search strategy

Relevant articles that were published between January 1970 and June 2009 in PubMed/MEDLINE, EMBASE and the Cochrane database, including the non-English literature were identified. The search strategy used the following single text words and combinations: living donor liver transplantation (LDLT), liver transplantation (LT), orthotopic liver transplantation (OLT), human leukocyte antigen (HLA), major histocompatibility complex (MHC), histocompatibility, matching and mismatching. Reference lists of relevant articles were cross checked for other potentially relevant articles.

Selection of trials and quality of the studies

Three separate authors (Lan X, Pu CL and Guo CB) independently reviewed and evaluated all articles for inclusion, which were classified as randomized control trial (RCT), controlled trial (CT), or descriptive study. After the initial article selection, the article dataset was reviewed and updated to capture any articles published between the final consensus review and the final data analysis (Zhang MM). Only cohort studies were indentified because of a lack of RCT.

The scoring system was adapted from Stahl, the Cochrane Collaboration and others[26-29]. This system suits not only RCT but CT or other studies well: (1) Was the trial design clearly stated? (2) Selection bias questions: Was the Patient selection process clearly stated? If the trial was an RCT, were patients randomly allocated to the therapeutic intervention? Were patients and clinicians blinded to the intervention? If the trial was not an RCT, were confounders controlled for? If the trial design was case control were matching procedures clearly described and implemented? Were patient recruitment procedures clearly described? Were the intervention and control groups selected similarly? (3) Performance bias questions: Was the intervention clearly described? Was intervention clearly measured? (4) Attrition bias questions: Were patients followed up? Were they followed up for 2 or more explicitly defined intervals? If patients were lost/dropped out other than because of death, were they accounted for? Were all outcome measures captured at the declared follow-up intervals? (5) Detection bias questions: Were the outcome measures clearly described? Was measurement of the outcome measures blinded? (6) Were appropriate statistical methods used? Were *P*-values clearly stated? Was life table analysis provided, *etc*.; and (7) Was the presentation of data adequate, for example, in the article were endpoints clearly defined i.e. graft survival, patient survival, duration of follow-up, re-transplantation rate, *etc*.? Were survival curves provided or were sufficient data to construct survival curves provided, were donor and recipient variables clearly defined and presented?

These questions were placed on a 3 point scale: unclear/inadequate (0), adequate (1), good (2). Articles were considered for inclusion if their summary score exceeded 30.

Data extraction

Graft loss was measured by hazard ratio (HR) and rejection was measured by relative risk (RR) at 1-year and 5-year in every study by 2 independent reviewers, reconciling any differences by consensus or when in doubt referring it to a third reviewer (Zhang MM) for arbitration. Graft survival rate was extracted for calculating corresponding HR using the formula recommended by Parmar *et al*[30]. Data was extracted by the software "Engauge 4.0" from survival curves if it was not shown in articles directly. Donor/recipient HLA compatibility for HLA class Ⅰ (A and B), and HLA class Ⅱ (DR) was measured as the number of mismatches, locus-specific (0 to 2 mismatches) and overall for the A, B, and DR loci (0 to 6 mismatches).

Meta-analysis

Both HR and RR were compared between 0 with 1-2 mismatches for each locus (mismatches of the HLA-A, B and C loci respectively) and 0-2 with 3-6 mismatches for overall HLA-A, B and DR loci. Comparability of the studies included in each pooled analysis was confirmed by examination of the $\chi^2 Q$ (expressed as a *P*-value) and I^2 statistics of heterogeneity. Statistical heterogeneity was defined as $P < 0.10$ or $I^2 > 50\%$. Lack of over-influence of one individual study to pooled estimates was confirmed by serial omission of each study and examination of the resulting estimate. To account for potential differences that were evident clinically but not identified by statistical tests, random effects models were used for each outcome measure. All statistical analyses were performed using Review Manager 5.0 which was a new program for determining HR.

Table 1 Contents of included studies

Author	Location	Immunosuppression	Number of patients	Contents
Meyer *et al*[9]	France	Cyclosporine, methylprednisolone and azathioprine	162	HLA-A, B and DR (5-yr graft survival); HLA-DR (1- and 5-yr graft survival)
Jakab *et al*[10]	American	NS	631	HLA-A, B and DR (1- and 5-yr graft survival); HLA-A and HLA-B (5-yr graft survival)
Neumanna *et al*[8]	Germany	Cyclosporine, azathioprine and prednisolone	836	HLA-A, B and DR (1- and 5-yr graft survival and rejection); HLA-A and HLA-DR (1- and 5-yr graft survival); HLA-B (1-yr graft survival)
Hashimoto *et al*[11]	Japan	Cyclosporine, methylprednisolone and azathioprine	50	HLA-A, B and DR (1- and 5-yr graft survival)
Langrehr *et al*[12]	Germany	Cyclosporine, azathioprine and prednisolone	165	HLA-A, B and DR (1- and 5-yr graft survival and rejection)
Suehiro *et al*[13]	Japan	Tacrolimus and Steroids	104	HLA-A, B and DR (1- and 5-yr graft survival and rejection)
Harihara *et al*[14]	Japan	Tacrolimus and Steroids	85	HLA-A, B and DR (rejection)
Balan *et al*[7]	American	Cyclosporine, prednisone, and azathioprine or tacrolimus	799	HLA-A, B and DR (1- and 5-yr graft survival); HLA-A (5-yr graft survival)
Sugawara *et al*[16]	Japan	Tacrolimus and methylprednisolone	113	HLA-DR (1-yr graft survival)
Doran *et al*[22]	Germany	NS	446	HLA-A, B and DR (1- yr graft survival); HLA-A and HLA-B (1-yr graft survival)
Poli *et al*[15]	Italy	Cyclosporine, azathioprine and tacrolimus	814	HLA-DR (5-yr graft survival)
Yagihashi *et al*[18]	American	Cyclosporine, azathioprine and tacrolimus	347	HLA-A, HLA-B and HLA-DR (1-yr graft survival)
Nikaein *et al*[21]	American	Cyclosporine and prednisone	701	HLA-A, B and DR (1-yr graft survival); HLA-A (1- and 5-yr graft survival); HLA-B and HLA-DR (1-yr graft survival)
Markus *et al*[20]	American	NS	527	HLA-A (5-yr graft survival); HLA-DR (1-yr graft survival)
Donaldson *et al*[19]	Britain	Cyclosporine, azathioprine	466	HLA-A, B and DR (1-yr graft survival and rejection); HLA-A and HLA-B (1-yr graft survival)
Knechtle *et al*[17]	American	NS	324	HLA-A, B and DR (1-yr graft survival); HLA-A, HLA-B and HLA-DR (1-yr graft survival)

NS: Not specified; HLA: Human leukocyte antigen.

RESULTS

Results of the article selection are described in Figure 1. 1568 potentially relevant articles were identified in the search. The abstracts of these studies were reviewed by 2 independent investigators. One thousand four hundred and forty-two did not meet inclusion criteria as their summary score was less than 30. Publications eligible for analysis included 16 articles: 2 prospective studies[7,8] and 14 retrospective cohort studies[9-22]. Non RCTs were included in our studies. In 4 studies acute rejection rates were compared clearly between 0-2 mismatches and 3-6 mismatches of HLA[8,12-14]. That is too say, specific data could only be extracted in these 4 articles. In 10 and 8 studies 1-year and 5-year survival rates, respectively, were compared between 0-2 mismatches and 3-6 mismatches of HLA[7-10,12,13,15,17,19,21,22]. In 6 and 5 studies 1-year and 5-year survival rates, respectively, were compared or could be extracted from survival curves between 0 mismatches and 1-2 mismatches of the HLA-A epitope[7,8,10,17-22]. In 9 and 5 studies 1-year and 5-year survival rates, respectively, were compared or could be extracted from survival curves between 0 mismatches and 1-2 mismatches of the HLA-DR epitope[8,9,16-22]. In 6 studies 1-year survival rates were compared between 0 mismatches and 1-2 mismatches of the HLA-B epitope[8,17-19,21,22]. Although 0 mismatches of the HLA-B epitope were compared with 1-2 mismatches in 5-year survival rates in 3 articles, the statistical heterogeneity was $P = 0.004$ and $I^2 = 82\%$ in the meta-analysis. Hence, the HR of the HLA-B epitope in 5-year survival rates was not included in our discussion. Details of these studies are described in Table 1.

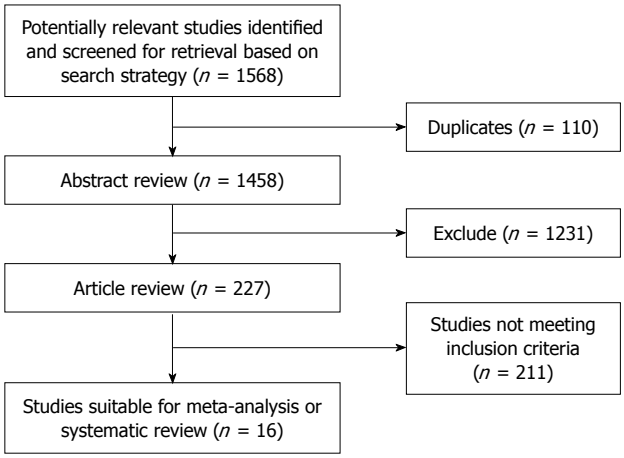


Figure 1 Selection of articles.

Table 2 Methodological quality of the controlled trials

Study	Selection criteria specified	Study design	Score	Other causing of death report	Dropouts explained	Funding
Meyer C	Yes	RCS	30	No	No	NS
Jakab SS	Yes	RCS	32	Yes	Yes	NS
Neumanna UP	Yes	PCS	31	No	No	NS
Morioka D	Yes	RCS	30	No	No	NS
Langrehr JM	Yes	RCS	33	Yes	Yes	NS
Suehiro T	Yes	RCS	30	Yes	No	NS
Harihara Y	Yes	RCS	30	No	No	NS
Vijayan B	Yes	PCS	35	Yes	Yes	NS
Sugawara Y	Yes	RCS	30	Yes	No	NS
Doran	Yes	RCS	30	No	No	NS
Poli F	Yes	RCS	31	No	No	NS
Yagihashi A	Yes	RCS	30	No	No	NS
Afzal N	Yes	RCS	33	No	No	NS
Markus BH	Yes	RCS	32	No	Yes	NS
Donaldson P	Yes	RCS	32	No	Yes	NS
Knechtle SJ	Yes	RCS	31	No	Yes	NS

RCS: Retrospective cohort studies; NS: Not specified.

The methodological quality of the studies was assessed using a validated tool as described above (Table 2).

Meta-analysis of HLA epitope

HLA-A, B and DR (0-2 mismatches *vs* 3-6 mismatches): In the studies included in the meta-analysis, a total of 4260 patients were included in 10 articles (1-year graft survival) and 3180 patients were included in 8 articles (5-year graft survival). No differences between 0-2 mismatches and 3-6 mismatches of HLA-A, B, and DR epitopes were seen in terms of 1-year graft survival [HR: 1.04, 95% confidence interval (CI): 0.86-1.25, $P = 0.68$] and 5-year graft survival (HR: 1.09, 95% CI: 0.90-1.32, $P = 0.38$, Figure 2).

HLA-A epitopes (0 mismatch *vs* 1-2 mismatches): Of the studies included in the meta-analysis, there were a total of 2049 patients in 6 articles (1-year graft survival) and 2138 patients in 5 articles (5-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-A epitopes were seen in terms of 1-year graft survival (HR: 0.95, 95% CI: 0.72-1.25, $P = 0.71$) and 5-year graft survival (HR: 1.06, 95% CI: 0.85-1.34, $P = 0.60$, Figure 2).

HLA-B epitopes (0 mismatch *vs* 1-2 mismatches): A total of 1969 patients were included in 6 articles (1-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-B epitopes were seen in terms of 1-year graft survival (HR: 0.77, 95% CI: 0.53-1.11, $P = 0.16$, Figure 2).

HLA-DR epitopes (0 mismatch *vs* 1-2 mismatches): A total of 2688 patients were included in 9 articles (1-year graft survival) and 2175 patients were included in 5 articles (5-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-DR epitopes were seen in terms of 1-year graft survival (HR: 1.07, 95% CI: 0.84-1.37, $P = 0.56$) and 5-year graft survival (HR: 1.18, 95% CI: 0.90-1.54, $P = 0.23$, Figure 2).

HLA epitopes and acute rejection

A total of 1268 patients were included in 4 articles (acute rejection within 3 mo after transplantation). Significant differences between 0-2 mismatches and 3-6 mismatches of HLA-A, B and DR epitopes were seen in terms of acute rejection (RR: 0.77, 95% CI: 0.61-0.97, $P = 0.03$, Figure 2).

DISCUSSION

This is the first systematic review and meta-analysis on the effect of HLA mismatching in short and long term liver graft outcome and acute rejection. We identified and analyzed 16 unique cohort studies and all HLA locus-specific analyses were performed by standard lymphocytotoxicity tests with confirmation by polymerase chain reaction, with HLA-A, B and DR locus mismatches being compared. The results clearly showed that a lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection. The degree of HLA mismatching (0-2 *vs* 3-6) had no significant effect on 1-year and 5-year graft survival. Furthermore, we found no difference between 0 mismatches and 1-2 mismatches in 1-year and 5-year graft survival of HLA-A, HLA-B and HLA-DR on subgroup analysis.

The role of HLA matching between donor and recipient in liver transplant rejection and graft survival has been determined in some cohort studies and there still is no consensus view[7-15,31]. This systematic review analyzed the different data of various studies and has given our own results. However, the main objective in performing this analysis was to assess the necessity of donor-recipient HLA matching before liver transplantation.

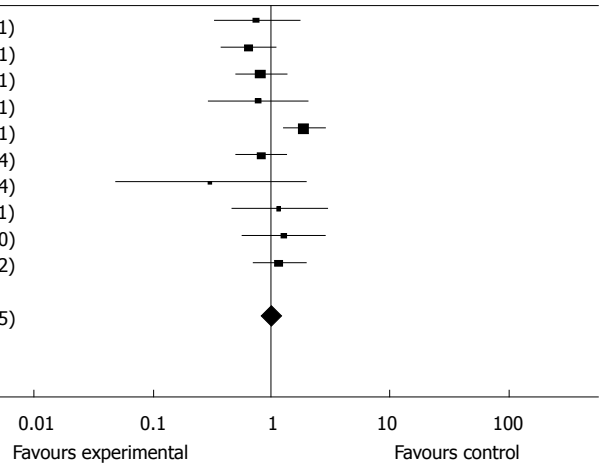
As the role of HLA matching between donor and recipient in organ transplant rejection and survival had been proven to increase graft survival after kidney and heart transplantation, it has been debated whether these matches affected the outcomes of the liver graft similarly. In liver transplantation, organ allocation relies mostly on ABO blood group, recipients' body weight, and clinical urgency, and the outcome of liver grafts relies mostly on complications after transplantation; HLA matching is usually not taken into account and the literature is inconsistent on the role of this parameter. In fact, any complications after transplantation are associated with graft outcome and rejection. Liver artery thrombosis, venous thromboembolic complications, seventh-day syndrome, primary graft nonfunction, and serious infection can decrease survival[32-36]. Compared to HLA mismatching, these complication are more important for long term graft survival.

Although the liver graft was considered to be a kind of immune-free organ, in our meta-analysis a lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection. It has become clear in recent years that mismatching of HLA in liver grafts led to endothelialitis induced by the recipient's natural killer cells and so rejection was instigated. The association of acute rejection with

A

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Nikaein A 1994	5.3	0.76 (0.34, 1.71)
Morioka D 2007	12.0	0.65 (0.38, 1.11)
Doran TJ 2000	16.2	0.83 (0.52, 1.31)
Langrehr JM 2006	3.9	0.78 (0.30, 2.01)
Donaldson P 1993	22.5	1.90 (1.28, 2.81)
Jakab SS 2007	15.3	0.83 (0.52, 1.34)
Knechtle SJ 1993	1.0	0.31 (0.05, 1.94)
Suehiro T 2005	4.1	1.17 (0.47, 2.91)
Neumann UP 2002	5.6	1.27 (0.58, 2.80)
Balan V 2008	14.1	1.17 (0.72, 1.92)
Total (95% CI)	100.0	1.04 (0.86, 1.25)

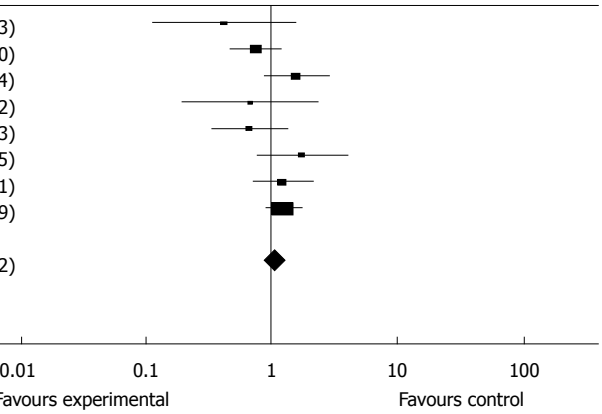
Heterogeneity: χ^2 = 16.95, *df* = 9 (*P* = 0.11); I^2= 47%
Test for overall effect *Z* = 0.42 (*P* = 0.68)



B

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Meyer C 1997	2.2	0.42 (0.12, 1.53)
Morioka D 2007	17.6	0.76 (0.48, 1.20)
Poli F 2001	10.7	1.57 (0.87, 2.84)
Langrehr JM 2006	2.4	0.67 (0.19, 2.32)
Jakab SS 2007	8.0	0.67 (0.34, 1.33)
Suehiro T 2005	5.7	1.76 (0.78, 3.95)
Neumann UP 2002	13.2	1.24 (0.73, 2.11)
Balan V 2008	40.1	1.25 (0.92, 1.69)
Total (95% CI)	100.0	1.09 (0.09, 1.32)

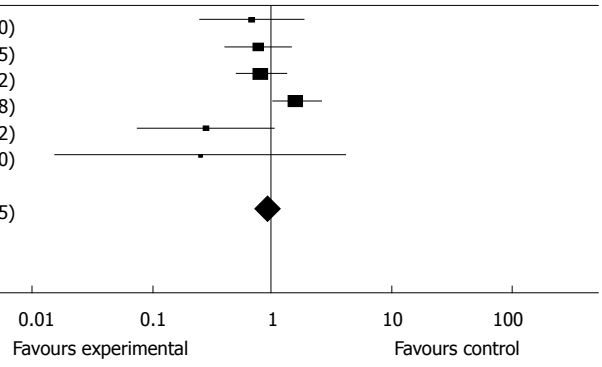
Heterogeneity: χ^2 = 10.77, *df* = 7 (*P* = 0.15); I^2= 35%
Test for overall effect *Z* = 0.88 (*P* = 0.38)



C

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Yagihashi A 1992	7.5	0.70 (0.26, 1.90)
Nlkaeln A 1994	19.6	0.78 (0.42, 1.45)
Jakab SS2007	33.3	0.82 (0.51, 1.32)
Donaldson P 1993	33.9	1.61 (1.00, 2.58)
Knechtle SJ 1993	4.6	0.29 (0.08, 1.02)
Neumann UP 2002	1.0	0.26 (0.02, 4.10)
Total (95% CI)	100.0	0.95 (0.72, 1.25)

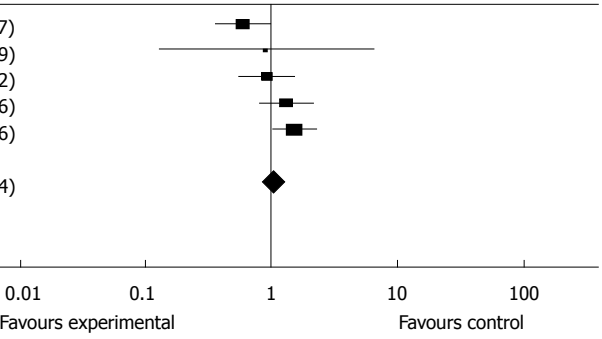
Heterogeneity: χ^2 = 10.18, *df* = 5 (*P* = 0.10); I^2= 49%
Test for overall effect *Z* = 0.37 (*P* = 0.71)



D

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Balan V 2008	22.9	0.60 (0.37, 0.97)
Neumann UP 2002	1.4	0.91 (0.13, 6.49)
Jakab SS 2007	21.1	0.93 (0.56, 1.52)
Markus BH 1988	21.8	1.32 (0.81, 2.16)
Nikaein A 1994	32.9	1.52 (1.02, 2.26)
Total (95% CI)	100.0	1.06 (0.85, 1.34)

Heterogeneity: χ^2 = 5.13, *df* = 3 (*P* = 0.16); I^2= 42%
Test for overall effect *Z* = 0.53 (*P* = 0.60)



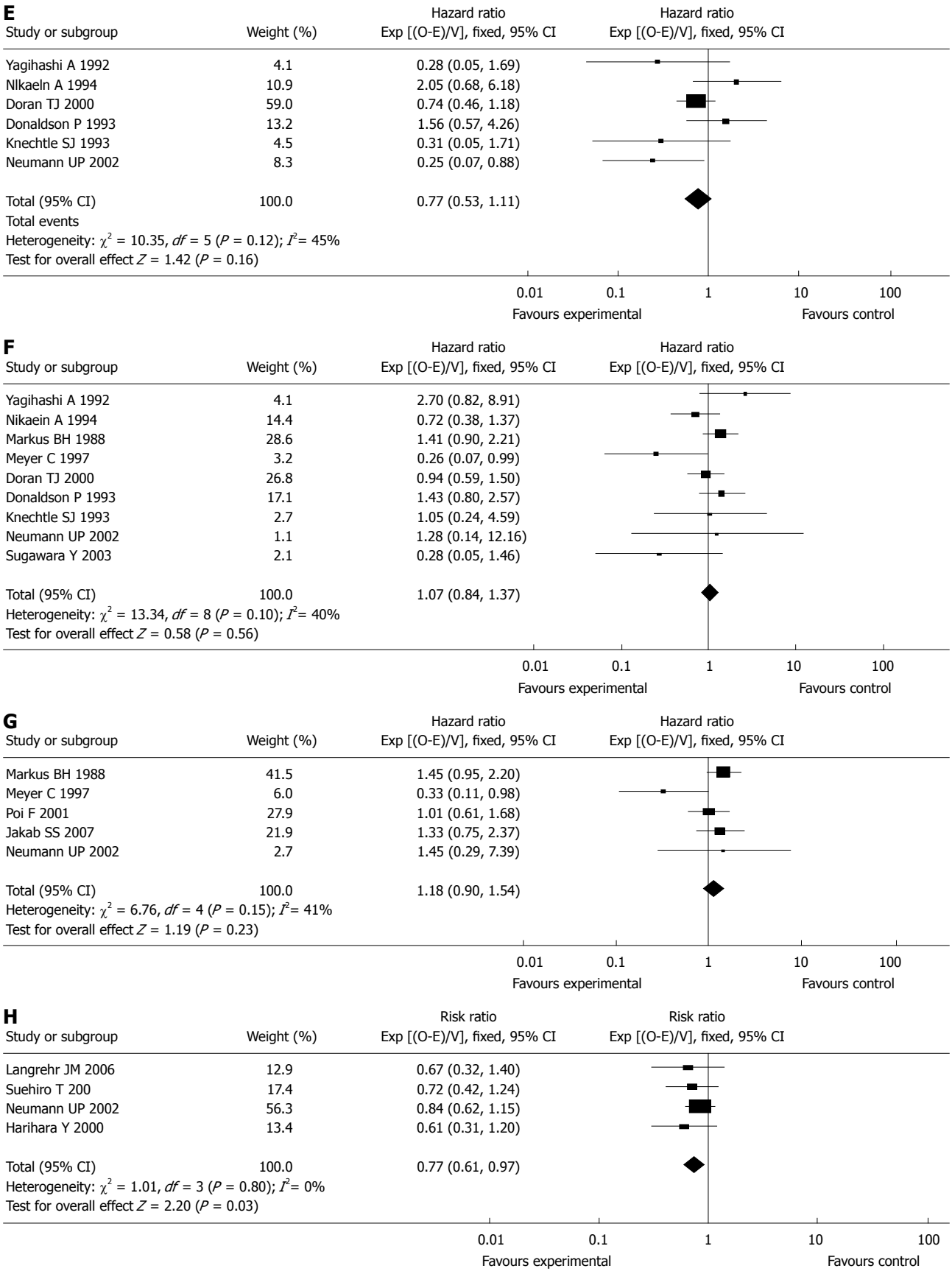


Figure 2 Meta-analysis of cohort trials comparing the effect of different mismatches of human leukocyte antigen epitopes on graft survival and acute rejection. A: 0-2 *vs* 3-6 mismatches of human leukocyte antigen (HLA)-A, B, DR epitopes on 1-year graft survival; B: 0-2 *vs* 3-6 mismatches of HLA-A, B, DR epitopes on 5-year graft survival; C: 0 *vs* 1-2 mismatches of HLA-A epitopes on 1-year graft survival; D: 0 *vs* 1-2 mismatches of HLA-A epitopes on 5-year graft survival; E: 0 *vs* 1-2 mismatches of HLA-B epitopes on 1-year graft survival; F: 0 *vs* 1-2 mismatches of HLA-DR epitopes on 1-year graft survival; G: 0 *vs* 1-2 mismatches of HLA-DR epitopes on 5-year graft survival; H: 0-2 *vs* 3-6 mismatches of HLA-A, B and DR epitopes on acute rejection.

3 other risk factors (cold ischemia time greater than 15 h, pretransplantation elevation of aspartate transaminase, and older donor age) are less readily explained, but suggest that nonallogeneic and allogeneic immunological injury may be related. Both a long cold ischemia time and older donor age predispose an allograft liver to injury shortly after transplantation, which evokes immunological reactions that are not necessarily triggered by allogeneic differences.

Although a meta-analysis may provide a high level of scientific evidence, it is important to realize the limitations of interpreting results of meta-analyses. One major limitation to the meta-analysis is that inferences are based on aggregate analysis of relatively heterogeneous studies. We acknowledge the potential heterogeneity of combining studies from different centers in different geographic locations with different treatment protocols. In our systematic review, results obtained from each study were considered to be homogeneous (heterogeneity test was $P > 0.10$ and $I^2 < 50\%$ in all available studies) in spite of there being no RCTs in this meta-analysis. Although we did not investigate through meta-regression any differences in the use of immunosuppressants or differences in study centers, the treatment protocols were nearly the same: cyclosporine or tacrolimus, azathioprine and prednisolone and no mycophenolate mofetil were used (Table 1).

Additionally, some studies did not report results with the measures that we chose for data extraction. It is the second limitation we must deal with. Survival rates under 1-year or 5-year were extracted by special software from survival curves if they was not shown in articles directly. We did not even obtain any data from some cohort studies, but including or excluding these articles also did not affect our conclusions.

The length of post transplantation follow-up was another limitation of many of the trials that we analyzed. Although most trials reported follow-up of some patients up to 5 years or even longer, some reported follow-up only to 1 year or 6 mo. Long-term graft survival, including HBV, HCV and hepatocellular carcinoma recurrence, may only become apparent or more pronounced after many years of post liver transplantation follow-up, and hence we may have underestimated the mortality in our study. In other words, we may have overestimated the role of HLA mismatching in liver graft loss.

Despite these limitations, our meta-analysis suggests that a lower number of HLA mismatching did reduce the incidence of acute rejection. The degree of HLA mismatching had no significant effect on 1-year and 5-year graft survival. Performing good donor-recipient HLA matching appears to be associated with a reduction in the incidence of acute rejection. Thus to obtain a shorter recovery time and avoid more rejection post transplantation, HLA matching examinations should be considered before surgery.

COMMENTS

Background

The role of human leukocyte antigen (HLA) matching between donor and recipient in organ transplant rejection and survival has been widely studied and proven to increase graft survival and to reduce the incidence of acute or chronic rejection. In contrast, major histocompatibility complex analysis is not routinely performed in liver transplantation because its importance remains controversial.

Research frontiers

Different groups have reported disparate results on the effect of HLA matching: some patients acquired benefit from high degrees of HLA matching but concern has been voiced about a greater likelihood of recurrence of primary disease with good HLA compatibility.

Innovations and breakthroughs

This is the first systematic review and meta-analysis on the effect of HLA mismatching in short and long term liver graft outcome and acute rejection. Importantly, the authors have some different conclusions compared to traditional views. Good donor-recipient HLA matching appears to be associated with a reduction in the incidence of acute rejection although there is no effect on 1-year and 5-year survival rates.

Applications

The percentage of graft survival was extracted by "Engauge Digitizer" from survival curves if the raw data was not presented. All statistical analyses were performed using Review Manager 5.0 which was a new program for determining HR.

Peer review

The authors aimed to assess the effect of HLA mismatching in liver graft outcome and acute rejection from available cohort studies by a systematic review and meta-analysis. The design of the study is rational and reliable, and the statistical methods used are appropriate. The article is also well organized. The conclusion may provide reliable and valuable information for clinical practice.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3465

World J Gastroenterol 2010 July 21; 16(27): 3465-3471
ISSN 1007-9327 (print)

BRIEF ARTICLE

Precise prediction model and simplified scoring system for sustained combined response to interferon-α

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Received: January 20, 2010 Revised: February 22, 2010
Accepted: March 1, 2010
Published online: July 21, 2010

Abstract

AIM: To establish a predictive algorithm which may serve for selecting optimal candidates for interferon-α (IFN-α) treatment.

METHODS: A total of 474 IFN-α treated hepatitis B virus e antigen (HBeAg)-positive patients were enrolled in the present study. The patients' baseline characteristics, such as age, gender, blood tests, activity grading (G) of intrahepatic inflammation, score (S) of liver fibrosis, hepatitis B virus (HBV) DNA and genotype were evaluated; therapy duration and response of each patient at the 24th wk after cessation of IFN-α treatment were also recorded. A predictive algorithm and scoring system for a sustained combined response (CR) to IFN-α therapy were established. About 10% of the patients were randomly drawn as the test set. Responses to IFN-α therapy were divided into CR, partial response (PR) and non-response (NR). The mixed set of PR and NR was recorded as PR+NR.

RESULTS: Stratified by therapy duration, the most significant baseline predictive factors were alanine aminotransferase (ALT), HBV DNA level, aspartate aminotransferase (AST), HBV genotype, S, G, age and gender. According to the established model, the accuracies for sustained CR and PR+NR, respectively, were 86.4% and 93.0% for the training set, 81.5% and 91.0% for the test set. For the scoring system, the sensitivity and specificity were 78.8% and 80.6%, respectively. There were positive correlations between ALT and AST, and G and S, respectively.

CONCLUSION: With these models, practitioners may be able to propose individualized decisions that have an integrated foundation on both evidence-based medicine and personal characteristics.

Key words: Chronic hepatitis B; Interferon-α; Patient selection; Predictive model; Scoring system; Treatment outcome

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Mao QG, Pan JS, Fang KN, Zhang RM, Hong QY, Song MN, Zhu JP, Huang WQ, Chen LM, Hong MZ. Precise prediction model and simplified scoring system for sustained combined response to interferon-α. *World J Gastroenterol* 2010; 16(27): 3465-3471 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3465.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3465

INTRODUCTION

Chronic hepatitis B (CHB) is one of the most refractory diseases that mankind faces and is a serious global public health problem. Hepatitis B virus (HBV) infection often leads to acute or chronic hepatitis B, hepatocellular carcinoma (HCC) and other complications. Of approximately 350 million carriers of HBV worldwide, about 1 million die from chronic complications, such as cirrhosis, HCC or both every year[1]. According to Liu *et al*[2], there is a 9% rate of HBV surface antigen (HBsAg) in the general population in China. In the United States, there are an estimated 1.25 million HBV carriers[3]. Currently, available antiviral options can be divided into 2 types, interferon-α (IFN-α) and nucleoside/nucleotide analogues, including conventional IFN-α, pegylated IFN-α (PEG-IFN), lamivudine, adefovir dipivoxil, entecavir, telbivudine, *etc*. IFN-α is one of the major choices; however, some factors greatly hinder its wide range of applications. First of all, the expense of antiviral therapy is considerable in either underdeveloped or developing countries; in addition, there are several side effects, such as fatigue, flu-like syndrome and others; ultimately, the most important aspect is that only a proportion of patients may achieve a response after therapy[4,5]. Thus, antiviral therapy is not generally accepted by patients.

Predicting the efficacy of IFN-α is crucial before attempting treatment for CHB patients. Some factors, such as HBV genotype A or B, lower viral load, higher serum alanine aminotransferase (ALT) levels, higher grading (G) of intrahepatic inflammation, and lower staging (S) of liver fibrosis, have been identified to be the predictors of the outcome of IFN-α therapy in HBV e antigen (HBeAg)-positive patients[3,6,7]. Female gender, short course of disease, having mild liver fibrosis, having good compliance with therapy, absence of co-infection and an early virological response at the 12th wk also indicate a good therapeutic outcome[8]. Sometimes, however, patients do not have all the "positive" predictors. They may also have one or more "negative" predictors. For example, a patient infected by HBV of genotype C, has a high viral load, accompanied by high ALT and high G. Is he suitable for IFN-α treatment? As pointed out by the European Association for the Study of the Liver, the HBV genotype has a poor individual predictive value, and currently, genotype alone should not define the choice of treatment[7]. These types of issues may be challenging for both practitioners and patients. Owing to the trials that had rigorous designs, many patients have benefited from evidence-based medicine developed in the last several decades. However, evidence-based medicine aims at the resolution of issues which came from individuals with a common background whereas individual information is not always taken into account. The current research aims at making a sensible decision that has an integrated foundation in both evidence-based medicine and personal characteristics.

We therefore conducted the present study to determine (1) baseline predictive factors for the response to IFN-α; (2) what was the relationship between these predictive factors; (3) whether a predictive algorithm for IFN-α treatment of CHB can be derived from these factors; and (4) what was the efficacy of the model.

MATERIALS AND METHODS

Patients

During the period between July 2005 and November 2008, all HBeAg-positive CHB patients were followed up for their response to IFN-α if they initially started IFN-α treatment in our Liver Division, the 174th Hospital of the PLA, the Traditional Chinese Medicine Hospital of Xiamen, Zhongshan Hospital Xiamen University, Xiamen, or Macheng Hospital, Hubei. Patients were recruited according to the guidelines in China[8], and were administrated with 5 MU of conventional IFN-α every other day for 24 wk or longer. The patients' baseline information was collected, including age, gender, blood tests, G, S, HBV DNA, genotype, *etc*. Several studies have indicated the potential benefits of extended duration of IFN-α or PEG-IFN therapy regarding a sustained response[9,10] or suppression of chronic complications[11]. Thus, duration was also recorded for balancing the effect of therapy span. Patients were excluded if they had HCC on presentation or other concomitant diseases including hepatitis A, C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis and alcoholic liver disease. Patients with the following conditions were also excluded: pregnancy, mental disorders (such as severe depression), uncontrolled epilepsy, alcohol abuse, narcotic abuse, uncontrolled autoimmune disorders, decompensated liver cirrhosis, symptomatic heart disease, neutrophil count below 1.0×10^9/L and/or platelet count below 50×10^9/L before treatment, had received or were receiving any other form of established treatment for CHB. Finally, 474 patients were included in the current study. For the treatment of HBeAg-positive CHB, a combined response (CR) was defined as ALT levels returning to normal, undetectable HBV DNA, and HBeAg seroconversion; partial response (PR) was defined as ALT levels returning to normal, HBV DNA $< 10^5$ copies/mL, but no seroconversion; whereas non-response (NR) refers to no CR or PR observations[8]. The mixed set of PR and NR was recorded as PR+NR. A sustained response was defined as the response at the 24th wk after cessation of IFN-α treatment.

Monitoring of patients

Patients were followed up every 1-2 mo by monitoring HBsAg status, HBeAg/anti-HBe status, HBV DNA level,

ALT, aspartate aminotransferase (AST), α-fetoprotein (AFP), complete blood count and mental status. Complete blood counts were taken once every 1-2 wk for the first month, then once per month until cessation of treatment. Other tests, such as thyroid function, blood glucose, routine urinalysis, were taken once every 3 mo. For patients who had abnormal thyroid function at baseline, appropriate therapy was initiated, and thyroid function was closely monitored during antiviral therapy. If there was evidence of a depressive disorder or suicidal tendency, treatment was stopped and patients were closely monitored. Ultrasound of the liver was scheduled for patients with AFP levels greater than 20 ng/mL. Patients were suggested to stop IFN-α administration if CR or NR occurred after therapy for 24 wk, or if severe side effects developed during the course of treatment. Patients' choices were also taken into account.

Determination of HBV genotypes and HBV DNA levels

Sera from patients on presentation were taken for the following tests: (1) HBV genotyping performed by the polymerase chain reaction (PCR)-fluorescence detection kit for HBV genotype B, C according to the manufacturer's instructions (Bioselex, Hangzhou, China); and (2) HBV DNA levels were determined by quantitative fluorescence PCR on the ABI 7000 (Applied Biosystems), with a lower limit of detection of 1000 copies/mL. HBV DNA levels below the lower detection limit were regarded as negative for statistical calculations.

Statistical analysis

Statistical analyses were performed using version R 2.8.1 (a language and environment for statistical computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org). The inter-variable correlation was determined by the Spearman rank correlation coefficient. The Gini index based on random forest methodology was used to determine whether the identified variables were associated with therapy outcomes. In the present study, the response to IFN-α treatment (dependent variable) was ordinal data. If the independent variable was ordinal data (such as ALT, AST, G, S, *etc.*), Kendall's *tau-b* test was adopted to test the statistical significance between independent variable and dependent variable. For the nominal independent variable (gender, genotype, *etc.*), the Pearson χ^2 test was used.

About 10% of patients were randomly selected as the test set, and the remaining patients were employed as the training set. The predictive model was constructed with a support vector machine (SVM) package for the R platform. Accuracies for CR and PR+NR in the training set and test set were calculated. The above process was repeated 300 times and mean accuracy was calculated. Performance of the constructed predictive algorithm was evaluated by the mean accuracies for CR and PR+NR for the training set and test set. The scoring system for sustained CR (SCR) was derived from our observations (Table 1) with computer-aided minor adjustment according to other data[3,7,12,13]. The weight for every level in each factor was rounded to the nearest integer.

Table 1 Baseline demographic and virological data of the study population

Factor	*n* (range)
Sex (M:F)	345:129
Age (yr)	29.8 (10-58)
ALT (U/L)	250 (16-1908)
AST (U/L)	146 (24-1304)
Genotype (A:B:C)[1]	51:212:211
HBV DNA (log copies/mL)	7.35 (5.00-9.83)
Fibrosis staging, S (0:1:2:3:4)	10:154:157:114:39
Histology activity index, G (1:2: 3:4)	39:215:169:51

Continuous variables are expressed as median (range). [1]B, C refers to genotype B, C, respectively; genotype B and C co-infection and other genotypes were named as A. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

The area under the curve (AUC) was then calculated for measuring the overall prediction accuracy. A 95% confidence interval (CI) for an AUC was obtained by sampling the 474 patients for 1000 bootstrap samples with the confidence limits calculated as the 2.5th and 97.5th percentiles. The scoring system was assessed by the leave-one-out cross-validation in order to assess the performance of new data[14]. To ease clinical employment of the SCR score, cut-off values were determined by maximizing the Youden index, i.e. sensitivity + specificity - 1, calculated from the receiver operating characteristic curves analysis. Accuracy of using the optimal cut-off values was assessed by the sensitivity, specificity, predictive values and likelihood ratios. Their 95% CIs were obtained from 1000 bootstrap samples. The cut-off values were also cross-validated by the leave-one-out method.

RESULTS

Demographics

A total of 474 CHB patients were enrolled. The baseline demographics, liver function tests, liver biochemistry, histology data and virological data are listed in Table 1. As shown in Table 2, the ratios of CR, PR and NR at the 24th wk after cessation of IFN-α therapy were 34.4%, 45.1% and 20.5%, respectively. It should be pointed out that genotype A in the current research refers to co-infection of genotype B and C, and other genotypes beside B and C.

Patients' factors and treatment factor for the response to IFN-α therapy

As shown in Table 2, female patients had a higher chance of a CR compared to male patients (41.1% *vs* 31.9%, $P < 0.001$; Kendall's *tau-b* test). Genotype B had a preferential effect on CR (45.3% and 25.1% for genotype B and genotype C, respectively, $P < 0.001$, Pearson χ^2 test). ALT and AST had a positive reciprocal relationship with treatment response ($P < 0.001$; Kendall's *tau-b* test) (Table 2).

Table 2 Individual factors of patients with diverse responses at the 24th week after cease of interferon-α therapy

	CR	PR	NR
Sex (M:F)	110:53	163:51	72:25
Age [0-14):(15-24):(25-44):(≥ 45), yr]	3:64:123:5	5:47:148:14	2:21:71:3
ALT [(1-2):(2-3):(3-5):(5-10):(≥ 10), ULN]	7:5:27:75:49	27:41:71:44:31	19:31:31:13:3
AST [(0-1):(1-2):(2-3):(3-5):(5-10):(≥ 10), ULN]	1:22:30:49:43:18	12:76:54:38:24:10	9:50:18:14:6:0
Genotype (A:B:C)	14:96:53	30:101:83	7:15:75
HBV DNA [(5-5.99):(6-6.99):(7-7.99):(8-8.99):(≥ 9), log copies/mL]	21:55:56:26:5	19:65:79:49:2	5:31:37:21:3
Fibrosis staging, S (0:1:2:3:4)	2:54:51:48:8	7:68:71:50:18	1:32:35:16:13
Histology activity index, G (1:2:3:4)	11:71:60:21	22:95:77:20	6:49:32:10
Responses (CR:PR:NR)	163	214	97

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ULN: Upper limit of normal; HBV: Hepatitis B virus; CR: Combined response; PR: Partial response; NR: No response.

Table 3 Inter-variable correlations determined by the Spearman rank correlation coefficient

	Gender	Age	Grading	Staging	ALT	AST	DNA[1]	Genotype	Duration	Y F6 m[2]
Gender	1.00									
Age	0.06	1.00								
Grading	-0.11[a]	0.05	1.00							
Staging	-0.06	0.08	0.74[b]	1.00						
ALT	0.05	-0.01	0.13[b]	0.02	1.00					
AST	-0.13[b]	-0.04	0.25[b]	0.17[b]	0.73[b]	1.00				
DNA[1]	-0.09	0.04	-0.04	-0.10[a]	0.07	0.09[a]	1.00			
Genotype	0.09[a]	0.00	-0.01	0.01	0.09	0.03	-0.12[b]	1.00		
Duration	0.05	-0.03	-0.02	0.03	0.00	-0.04	0.03	0.02	1.00	
Y F6 m	0.05	-0.01	-0.03	0.00	-0.39[b]	-0.35[b]	0.06	0.25[b]	-0.07	1.00

[a]$P < 0.05$, [b]$P < 0.01$; [1]log copies/mL; [2]Response after 6 mo of follow-up. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Inter-variable correlations

The correlations between variables were determined by the Spearman rank correlation coefficient. As shown in Table 3, there were positive reciprocal relationships between G and S (0.74, $P < 0.01$), ALT and AST (0.73, $P < 0.01$). Baseline ALT (0.39, $P < 0.01$), AST (0.35, $P < 0.01$) and genotype (0.25, $P < 0.01$) had a substantial predictive effect on the sustained response. The correlations between G and S, ALT and AST are illustrated in Figure 1A and B.

Multivariate analysis for factors associated with the response to IFN-α therapy

Stratified by duration of IFN-α therapy, Gini index analysis showed that baseline predictors, from highly significant to least significant, were ALT, HBV DNA in log copies/mL, AST, genotype, S, G, age and gender (Table 4).

Predictive algorithm for the SCR to IFN-α therapy

Based on SVM, a predictive model was developed for the SCR to IFN-α therapy. According to the established model, the accuracies for SCR and PR+NR respectively were 86.4% and 93.0% for the training set, 81.5% and 91.0% for the test set.

Predictive scoring system for the SCR to IFN-α therapy

Based on our data provided in Table 2 with computer-aided minor adjustment according to other data[3,7,12,13], a predictive scoring system was developed for the SCR to IFN-α therapy. The odds ratio of SCR score was 15.25 (95% CI: 9.65-24.68, $P < 0.001$) indicating that the scoring system had an excellent prediction performance. By optimizing with the Youden's index, the optimal cut-off for the prediction of SCR was 169. This cut-off had good sensitivity and specificity and had been accurately validated by the leave-one-out validation (Table 5). The AUCs were as high as 0.797 (95% CI: 0.773-0.812) for SCR prediction (Figure 2A). The odds ratio of SCR according to the scoring system is depicted in Figure 2B.

Table 4 Significance of baseline factors for sustained combined response to interferon-α therapy

Variable	CR	PR	NR	Mean decrease accuracy	Mean decrease Gini
ALT	2.242	0.860	2.611	1.363	42.806
Duration	0.536	0.599	0.251	0.507	36.340
HBV DNA	0.603	0.742	0.015	0.553	35.713
AST	1.045	-0.243	0.955	0.502	35.488
Genotype	1.101	1.083	3.095	1.269	30.462
Staging	0.737	-0.058	-0.147	0.205	29.400
Grading	-0.033	0.330	-0.035	-0.182	23.283
Age	-0.069	0.094	0.160	0.050	20.944
Gender	-0.125	0.478	-0.084	0.186	14.396

CR: Combined response; PR: Partial response; NR: No response; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

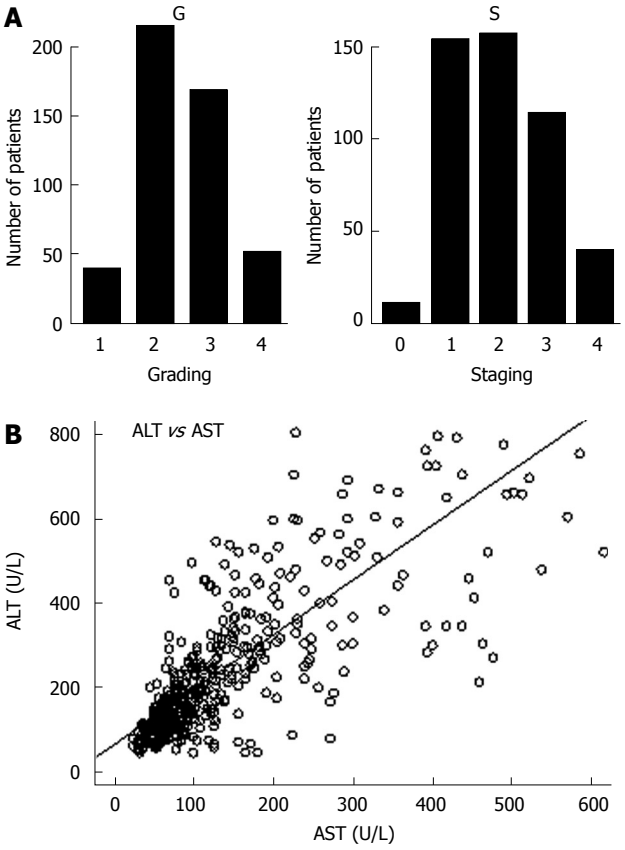

Figure 1 Inter-variable correlations between grading and staging, alanine aminotransferase and aspartate aminotransferase. A: Positive correlation between grading (G) and staging (S); B: Positive correlation between alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Table 5 Optimal cut-off values by maximizing the Youden index and their accuracies for the sustained combined response score derived from whole study population and validated with leave-one-out cross-validation

	Value	95% CI
Total study population		
Optimal cut-off	169	
Sensitivity (%)	78.79	71.93-84.33
Specificity (%)	80.58	75.81-84.61
Positive predictive value (%)	68.42	61.50-74.61
Negative predictive value (%)	87.68	83.34-91.00
Positive likelihood ratio	4.06	3.19-5.16
Negative likelihood ratio	0.26	0.20-0.36
Odds ratio	15.25	9.65-24.68
Accuracy (%)	79.96	76.12-83.31
Youden index	0.594	0.5913-0.5961
AUC	0.797	0.773-0.812
Leave-one-out cross-validation		
Optimal cut-off	169	
Sensitivity (%)	78.18	71.28-83.80
Specificity (%)	79.94	75.11-84.02
Positive predictive value (%)	67.54	60.61-73.78
Negative predictive value (%)	87.28	82.89-90.67
Positive likelihood ratio	3.90	3.08-4.94
Negative likelihood ratio	0.27	0.20-0.37
Odds ratio	14.13	8.98-22.73
Accuracy (%)	79.32	75.45-82.73
Youden index	0.581	0.5787-0.5836
AUC	0.79	0.779-0.807

AUC: Area under the curve.

DISCUSSION

In summary, the main findings and results of the current research include: (1) development of an accurate predictive model for the SCR to IFN-α therapy; (2) deduction of a scoring system for SCR as the data mining method may be unavailable to most the clinicians; (3) identification of positive reciprocal relationships between G and S, ALT and AST; (4) a predictive role of baseline ALT, AST and genotype for SCR; and (5) baseline predictive factors, listed from the greatest to the least significant, are ALT, HBV DNA, AST, genotype, S, G, age and gender.

The predictive factors for the response to IFN-α therapy have been extensively investigated; however, response prediction for individual patients remains uncertain. First, evidence-based medicine aims at a therapeutic strategy for patients with a similar background rather than a given patient. Additionally, a given individual may have "positive" predictive factors and "negative" predictive factors at the same time. Thus, patients or even clinicians may be perplexed by the probability estimation of therapy outcome. This leads to significant profligacy of health resources and a delay in treating patients who need an appropriate antiviral intervention. Fortunately, the present study may facilitate the management of these difficulties. One of the limitations of the current study is that most of the genotypes are B and C, or co-infection of B and C, which is in accordance with the report by Zeng *et al*[15]. There are not enough patients infected by other genotypes of HBV for statistical analysis. Another limitation is that treatment with conventional IFN-α rather than PEG-IFN was evaluated in the present research. Although PEG-IFN was extensively prescribed in developed countries, its application was greatly hindered by its high cost in developing countries. In China, there is great disparity in the prescription costs of IFN-α and PEG-IFN. We cannot recruit enough patients administrated with PEG-IFN for statistical analysis. However, IFN-α and PEG-IFN share the same bioactive molecule *in vivo* and similar baseline predictors[16]. Therefore, using our scoring system, which was easily employed in clinical practice, the response to PEG-IFN therapy may be predicted with reasonable accuracy. If statistical packages were available, higher predictive accuracy could be achieved.

In line with several studies[12,17], genotypes B or C have dramatically different effects on treatment response. Apart from genotypes, the present study also found that increasing HBV DNA levels were associated with a stepwise decrease in the response, which was similar to that of S; in contrast, increasing ALT, AST and G played an opposite role. Patients with higher ALT, AST and G tended to have better outcomes. According to the inter-variable correlation analysis, there were significantly positive reciprocal relationships between ALT and AST, G and S, respectively. The correlation between ALT and AST sounds reasonable, which may be a result of parallel release of intracellular contents after immune injury.

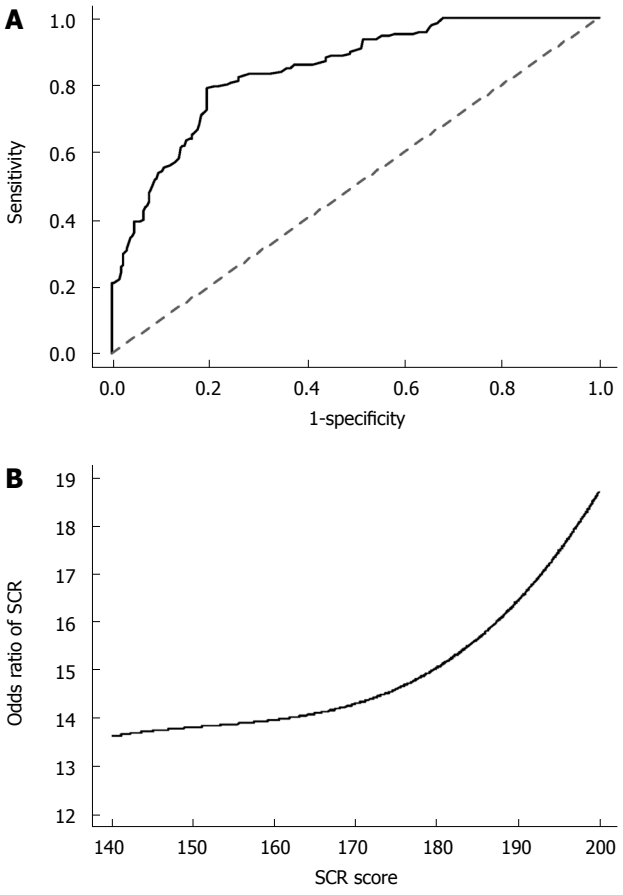

Figure 2 Predictive performance of the scoring system for sustained combined response. A: Receiver-operator characteristics curve of the scoring system for sustained combined response (SCR); B: Odds ratio of the scoring system for SCR.

Repeated intrahepatic immunologic inflammation triggers fibrinogen secretion from hepatic stellate cells and eventually leads to cirrhosis, which may partly explain the correlation between G and S. The performed study indicated the most important predictive factor was baseline ALT, followed by HBV DNA level, AST, genotype, S, G, age and gender. Interestingly, the Gini index of S was slightly higher than that of G, which may be due to the higher variation of G than that of S though they have a high correlation as mentioned before. Baseline AST also had a considerable predictive role for SCR. Although ALT is regarded as a specific index for HBV-induced inflammation, AST rather than ALT manifests a correlation with fibrosis stage[18]. Data by Brook *et al*[19] also indicated AST > 85 U/L is a predictive factor for the response to IFN-α therapy.

We developed a predictive model that was shown to have parallel accuracies for both a training set and test set by adjusting kernel parameters, which ensured that satisfactory sensitivity may be achieved for samples out of the observation pool. In other words, the established model would have a reasonable predictive accuracy for patients who were not enrolled in the current research. In addition, a scoring system for SCR was developed to identify patients who may have SCR if the score was greater or equal to the optimal cut-off value of 169. This score was validated by the stringent leave-one-out statistical analysis with high sensitivity and specificity of 78.2% and 79.9%, respectively, for the prediction of SCR. Using these SCR scores, the practitioner can calculate the prognosis of a patient on presentation, which is important for devising individual management of the patient. The practitioner can also identify very high-risk patients who should be recommended for treatment by nucleoside/nucleotide analogues to obtain good results.

In clinical practice, we are not aware of any predictive score for the SCR to IFN-α therapy in HBeAg-positive CHB patients with the integration of potential predictive factors. Our novel predictive algorithm and SCR score may serve as an excellent reference for clinicians to decide who should undergo IFN-α therapy. With these models, practitioners would be able to propose individualized treatment paradigms that have an integrated foundation in both evidence-based medicine and personal characteristics. It has extensive potential clinical use to identify CHB patients who have a high potential of a SCR to IFN-α therapy. These patients should be suggested to be treated by IFN-α to delay or prevent lethal complications of CHB such as liver cirrhosis and HCC.

ACKNOWLEDGMENTS

We are much indebted to Professor Bayasi Guleng and Miss Qi Li for discussion and comments on the manuscript.

COMMENTS

Background

Predicting the efficacy of interferon-α (IFN-α) is crucial before attempting treatment of chronic hepatitis B (CHB) patients. Though predictors of the responses to IFN-α have been well identified, patients frequently have "positive" and "negative" predictors at the same time. Therefore, it is very difficult to predict the treatment response before IFN-α therapy for a specific patient.

Research frontiers

Evidence-based medicine aims at the resolution of issues which came from individuals with a common background whereas individual information can not always be taken into account. The current research aims at sensible decision-making that has an integrated foundation on both evidence-based medicine and personal characteristics.

Innovations and breakthroughs

The current research successfully integrated the patients' personal characteristics into the foundations of evidence-based medicine. A precise prediction model and a simplified scoring system for a sustained combined response (SCR) to IFN-α were generated.

Applications

It has extensive clinical use to identify CHB patients who have a high potential of a SCR to IFN-α therapy. With these predictive models, practitioners would be able to propose individualized treatments that have an integrated foundation in both evidence-based medicine and personal characteristics.

Peer review

This is an interesting report of a prediction model and simplified scoring system for SCR to IFN.

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S- Editor Wang JL **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i27.3472

World J Gastroenterol 2010 July 21; 16(27): 3472-3474
ISSN 1007-9327 (print)

CASE REPORT

Anterograde jejunojejunal intussusception resulted in acute efferent loop syndrome after subtotal gastrectomy

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Received: February 12, 2010 Revised: April 7, 2010
Accepted: April 14, 2010
Published online: July 21, 2010

Abstract

Postoperative intussusception is an unusual clinical entity in adults, and is rarely encountered as a complication following gastric surgery. The most common type after gastric surgery is retrograde jejunogastric intussusception, and jejunojejunal intussusception has been rarely reported. We report a case of anterograde jejunojejunal intussusception after radical subtotal gastrectomy with Billroth Ⅱ anastomosis in a 38-year-old Korean woman with early gastric cancer, and include a review of the literature on this unusual complication.

Key words: Intussusception; Postoperative complications; Gastrectomy

Peer reviewer: Eren Ersoy, MD, Associate Professor, Department of General Surgery, Ankara Ataturk Research and Educational Hospital, Bilkent, Ankara 06810, Turkey

Kwak JM, Kim J, Suh SO. Anterograde jejunojejunal intussusception resulted in acute efferent loop syndrome after subtotal gastrectomy. *World J Gastroenterol* 2010; 16(27): 3472-3474 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3472.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3472

INTRODUCTION

Postoperative intussusception is an unusual clinical entity in adults, and is rarely encountered as a complication following gastric surgery. The reported incidence is under 0.1% in patients who undergo gastric surgery[1]. The most common type after gastric surgery is jejunogastric intussusception[2]. Jejunojejunal intussusception has been rarely reported, and all previously reported cases have been observed after Roux-en-Y gastro- or esophagojejunostomy. Here, we report a case of anterograde jejunojejunal intussusception that developed at the efferent limb after subtotal gastrectomy with Billroth Ⅱ reconstruction in a woman with early gastric cancer. We also include a review of the literature on this unusual complication. To the best of our knowledge, this is the first case report of jejunojejunal intussusception after partial gastrectomy with Billroth Ⅱ type gastrojejunostomy.

CASE REPORT

A 38-year-old woman was referred to our outpatient clinic for biopsy-proven adenocarcinoma of the stomach. The patient had no significant medical history. On gastroduodenoscopic examination, type Ⅱc early gastric cancer was found at the lesser curvature of the body. Moderately differentiated adenocarcinoma was confirmed by performing a biopsy of the lesion. Staging work-up including abdominal computed tomography (CT) revealed no metastasis.

Radical subtotal gastrectomy with Billroth Ⅱ reconstruction, including complete dissection of the perigastric nodes plus the lymph node along the left gastric and hepatic arteries, was performed. The lesser curvature of the stomach and the duodenal stump were closed with staples. Antecolic, isoperistatic gastrojejunostomy was performed using Albert-Lembert sutures. A nasogastric tube was placed in the remnant stomach.

On the first postoperative day (POD), the patient did not complain of abnormal symptoms. However, on POD 2, she started to complain of recurrent episodes of abdominal colicky pain, which did not subside for over 5 d,

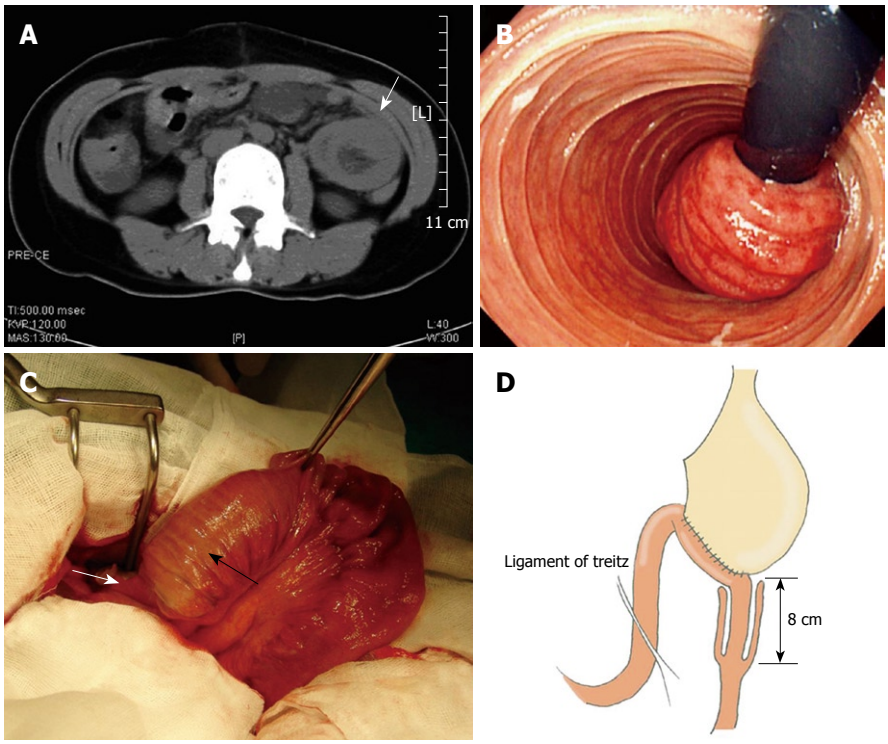

Figure 1 Radiologic, endoscopic and intraoperative findings of anterograde jejunojejunal intussusception. A: Abdominal computed tomography showed a non-homogeneous mass (arrow) in the left upper quadrant of the abdomen; B: A J-turn view of gastrofibroscopic examination demonstrated a congested jejunal mass (intussusceptum); C: Intraoperative findings revealed that the intussusception started just below the gastrojejunostomy anastomosis. The black arrow indicates the intussuscipiens, and the white arrow indicates the intussusceptum; D: Schematic diagram of the intussusception at the efferent-loop.

without a definite cause. During that time, 500-900 mL bile-containing gastric juice was drained *via* the nasogastric tube daily. Serial plain abdominal radiography and laboratory tests showed no remarkable findings. The blood tests were unremarkable except for elevated white blood cell count up to 16000/μL. On POD 6, the patient complained of intermittent abdominal cramping, and abdominal tenderness in the left upper quadrant of the abdomen on physical examination. Non-enhanced CT was carried out and revealed a non-homogeneous jejunal mass below the anastomosis site (Figure 1A). Gastrofibroscopic examination was performed subsequently. The entrance of the efferent loop was narrow and edematous, but the gastrofibroscope was able to pass through the efferent loop. A congested jejunal mass was observed on the J-turn image (Figure 1B). Emergency laparotomy was performed, on suspicion of a jejunojejunal intussusception.

On exploration, an anterograde jejunojejunal intussusception was found at about 5 cm distal to the gastrojejunostomy anastomosis line (Figure 1C and D). Manual reduction was performed carefully and the intussusception came loose subsequently. The proximal jejununal segment (intussusceptum), about 8 cm in length, did not recover from ischemia. The primary gastrojejunostomy anastomosis, which was non-viable, was removed, and a Roux-en-Y gastrojejunostomy was formed. On pathological examination, no identifiable leading point for the intussusception was present. She was discharged without further complications or abnormal symptoms on POD 11 after the second operation.

DISCUSSION

Intussusception is primarily a disease of infancy and childhood, and only about 5% of cases occur in adults[3]. Although childhood intussusception is idiopathic in 90% of cases, adult intussusception has an organic lesion as a leading cause in 70%-90% of cases, and > 50% of the lesions have been reported to be malignant[4,5].

Although postoperative intussusception is a rare clinical entity in both age groups, it is also more common in the pediatric population than in adults. It accounts for 5%-10% of cases of postoperative ileus in infancy and childhood[6], and only 1% of cases in adults[7].

Intussusception is an extremely rare complication after gastric surgery; the incidence is reported to be < 0.1%[1]. Since the first case of jejunogastric intussusception after gastrojejunostomy was reported by Bozzi[8] in 1914, a large number of isolated cases have been reported, and fewer than 200 cases of postoperative intussusception after gastric surgery have been reported in the English-language literature[2]. Retrograde jejunogastric intussusception is the most common type after gastric surgery[9]. Rarer cases of jejunojejunal[10-14], jejunoduodenal[15] or duodenogastric intussusception[16], or intussusception through a Braun anastomosis[17] also have been reported after gastric surgery.

All cases of jejunojejunal intussusception have been observed after Roux-en-Y gastro- or esophagojejunostomy. To the best of our knowledge, this is the first case report of jejunojejunal intussusception after partial gastrectomy with Billroth II type gastrojejunostomy. However, it should be pointed out that jejunojejunal intussusception might not be such a rare problem after Roux-en-Y gastric bypass for morbidly obese patients[18]. Simper *et al*[18] recently have reported 22 cases (0.15%) of postoperative jejunojejunal intussusception after more than 15000 Roux-en-Y gastric bypasses.

In the present case, neither functional nor mechanical causes were identified as leading causes of intussusception. Although most adult intussusceptions are caused

by a definable structural lesion[5], definite anatomical or pathological causes are rarely found in cases following gastric surgery[2]. A variety of postoperative conditions, such as adhesions around the suture lines[3,19], a long intestinal tube[20], increased intra-abdominal pressure[1], shortening of the jejunal mesentery[21], and reverse peristalsis[19,22], have been proposed as possible mechanisms of intussusception after gastric surgery, but none has been confirmed. Functional causes include reverse peristalsis, which is triggered by an anastomosed jejunal loop being irritated by hydrochloric acid, and atonic stomach, which is caused by vagotomy[2].

Diagnosis of postoperative intussusception is difficult in adults. The classic symptom triad of intussusception (pain, palpable mass, and currant-jelly stool) rarely occurs in adults[3]. Furthermore, usual symptoms encountered in acute postoperative intussusception are easily confused with postoperative ileus or adhesion[23,24]. For these reasons, diagnosis was delayed in our case, which rapidly progressed to incarceration and strangulation of the involved efferent limb. Thus, a high index of clinical suspicion is necessary for early diagnosis of this potentially lethal complication. Upper gastrointestinal endoscopy and abdominal CT are highly diagnostic in the setting of urgent, high-level intestinal obstruction[25].

Endoscopic reduction of jejunogastric intussusception has been suggested in a few selected cases[26]; however, this is associated with a significant risk of recurrence[27]. Surgery is the mainstay of treatment in jejunojejunal intussusception, and should be individualized for each patient. Surgical procedures include reduction, resection, and revision of the intussusception and takedown of the previous anastomosis with construction of a new anastomosis, depending on the operative findings. If the affected segment loses viability, resection of the non-viable segment is inevitable. Ozdogan *et al*[13] have suggested that the operation should be conservative, provided that the bowel is viable; manual reduction is the only required treatment, and other preventive measures are not necessary.

We report a case of anterograde jejunojejunal intussusception that caused acute efferent loop syndrome after partial gastrectomy. Acute jejunojejunal intussusception after gastric surgery is an extremely rare clinical entity that requires a high index of clinical suspicion for early diagnosis and prompt surgical management. Thus, intussusception should be considered as one of the possible causes of high-level intestinal obstruction in the immediate postoperative period after partial gastrectomy.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2010 July 21; 16(27): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 July 21; 16(27): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

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January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

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Prague, Czech Republic
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World J Gastroenterol 2010 July 21; 16(27): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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

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ISSN 1007-9327
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Volume 16 Number 28
July 28, 2010
World J Gastroenterol
2010 July 28; 16(28): 3475-3602

Online Submissions
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 28 July 28, 2010

EDITORIAL

3475 Colorectal cancer and pollution
El-Tawil AM

3478 Radiofrequency ablation of locally advanced pancreatic adenocarcinoma: An overview
D'Onofrio M, Barbi E, Girelli R, Martone E, Gallotti A, Salvia R, Martini PT, Bassi C, Pederzoli P, Pozzi Mucelli R

REVIEW

3484 Role of surgery in colorectal liver metastases: Too early or too late?
Dimitroulis D, Nikiteas N, Troupis T, Patsouras D, Skandalakis P, Kouraklis G

3491 Protein-protein interaction map is a key gateway into liver regeneration
Xie C, Gao J, Zhu RZ, Yuan YS, He HL, Huang QS, Han W, Yu Y

ORIGINAL ARTICLE

3499 Hypoxia inducible factor-1α accumulation in steatotic liver preservation: Role of nitric oxide
Zaouali MA, Ben Mosbah I, Boncompagni E, Ben Abdennebi H, Mitjavila MT, Bartrons R, Freitas I, Rimola A, Roselló-Catafau J

3510 Accuracy of ultrasound to identify chronic liver disease
Allan R, Thoirs K, Phillips M

3521 Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma
Akutsu N, Yamamoto H, Sasaki S, Taniguchi H, Arimura Y, Imai K, Shinomura Y

3529 Probiotic Bio-Three induces Th1 and anti-inflammatory effects in PBMC and dendritic cells
Hua MC, Lin TY, Lai MW, Kong MS, Chang HJ, Chen CC

BRIEF ARTICLE

3541 Hepcidin levels in hereditary hyperferritinemia: Insights into the iron-sensing mechanism in hepatocytes
Arnold J, Sangwaiya A, Manglam V, Thursz M, Beaumont C, Kannengiesser C, Busbridge M

3546 Bacteriolytic therapy of experimental pancreatic carcinoma
Maletzki C, Gock M, Klier U, Klar E, Linnebacher M

3553 Promoter methylation status of *hMLH1*, *MGMT*, and *CDKN2A/p16* in colorectal adenomas
Psofaki V, Kalogera C, Tzambouras N, Stephanou D, Tsianos E, Seferiadis K, Kolios G

3561 Primary site resection is superior for incurable metastatic colorectal cancer
Tanoue Y, Tanaka N, Nomura Y

3567 Rational therapeutic strategy for T2 gallbladder carcinoma based on tumor spread
Kohya N, Kitahara K, Miyazaki K

3573 Proton pump inhibitors as a risk factor for recurrence of *Clostridium-difficile*-associated diarrhea
Kim JW, Lee KL, Jeong JB, Kim BG, Shin S, Kim JS, Jung HC, Song IS

3578 Correlation between *pre-miR-146a* C/G polymorphism and gastric cancer risk in Chinese population
Zeng Y, Sun QM, Liu NN, Dong GH, Chen J, Yang L, Wang B

3584 Enhancement of antitumor vaccine in ablated hepatocellular carcinoma by high-intensity focused ultrasound
Zhang Y, Deng J, Feng J, Wu F

CASE REPORT

3592 Sustained treatment response of metastatic hepatocellular carcinoma with bevacizumab and sorafenib
Wich C, Agaimy A, Strobel D, Wissniowski TT, Hartmann A, Ocker M

3597 Placement of removable metal biliary stent in post-orthotopic liver transplantation anastomotic stricture
Tee HP, James MW, Kaffes AJ

LETTERS TO THE EDITOR

3601 Role of endoscopic ultrasound/SpyScope in diagnosis and treatment of choledocholithiasis in pregnancy
Girotra M, Jani N

World Journal of Gastroenterology
Volume 16 Number 28 July 28, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
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SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.,
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
July 28, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm. If you do not have web access please contact the editorial office.

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3475

World J Gastroenterol 2010 July 28; 16(28): 3475-3477
ISSN 1007-9327 (print)

EDITORIAL

Colorectal cancer and pollution

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Received: March 26, 2010 Revised: April 21, 2010
Accepted: April 28, 2010
Published online: July 28, 2010

Abstract

The incidence of colorectal carcinoma is increasing in young patients, in contrast to the well established wisdom that it is exclusively diagnosed in patients older than 40 years. In this survey, we examined all possible risk factors, and we recommend a number of measures for early detection in young patients who are at risk of developing this malignant tumor.

Key words: Colorectal adenocarcinoma; Food contamination; Pesticides; Young patients; Free radicals

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El-Tawil AM. Colorectal cancer and pollution. *World J Gastroenterol* 2010; 16(28): 3475-3477 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3475.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3475

INTRODUCTION

Colorectal cancer continues to be one of the most common human malignancies, afflicting nearly one million individuals worldwide every year. The disease can be considered endemic in all western and industrialized countries, but there are indications that, in the near future, colorectal neoplasms will become frequent also in populations that at present show a low incidence of the disease[1]. Reports from Asian and African countries have demonstrated that annual diagnosis of colorectal cancer is increasing[2-5] and cases have been identified in those aged < 40 years old[6]. However, hereditary factors could not be recognized as risk factors in these cases[6] and high dietary intake of meats and fats could not be blamed as risk factors in these developing countries because of a lack of supply of either to the majority of the population[6-8]. Therefore, this phenomenon requires further investigation. It is likely to be due to chemical contamination of food and drink.

CHLORINATION OF DRINKING WATER AND DEVELOPMENT OF BOWEL CANCER

It is likely that chlorination of drinking water plays a role in the above phenomenon. During the chlorination process, chlorine reacts with organic materials in the water to produce a complex mixture of halogenated and non-halogenated by-products, the concentration and distribution of which vary with characteristics of the raw water and the treatment process[9]. A large number of halogenated chemical species have been identified, including trihalomethanes, halogenated acetonitriles, halogenated acids, haloketones, and haloaldehydes[10]. Trihalomethanes are the most frequently occurring by-products[11,12] and are routinely measured in public water supplies, thus making them a useful marker for the level of chlorination by-products in treated water. Chronic exposure to disinfected surface waters is considered one of the contributing factors to the development of urinary bladder and colon cancer[13-15]. Mutagenic activity of chlorinated water may be due to the presence of chemicals produced from reactions of chlorine with natural substances released by the breakdown of vegetation in the source water[16]. The chlorinated hydroxyfuranone [e.g. 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-

furanone], for example, has been shown to be responsible for a majority of this mutagenic activity[5,12]. 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone has been reported to occur at much lower concentrations in drinking water than other disinfection by-products, yet it might be a significant public health concern because it is the most potent mutagen currently identified in drinking water[17]. Two years of treatment with this compound in drinking water resulted in a multi-organ carcinogenic response in male and female Wistar rats[18]. Moreover, reliable evidence from epidemiological studies has suggested an increase in the risk of urinary bladder, colon, and rectal cancer among people using disinfected drinking water that contains high levels of trihalomethanes[15,19]. King *et al*[20] have reported that increasing exposure to different drinking water constituents is associated with increasing risk of colon cancer in men. In this population, several halogenated chemical species have shown a tendency for increasing colon cancer risk across different exposure categories due to increasing exposure, which reached at least a 53% elevation in risk for the highest exposure categories. Long-term (approximate 35 years) exposure to trihalomethanes at a level of approximate 75 mg/L was associated with a doubled colon cancer risk among men (OR: 2.10, 95% CI: 1.21-3.66). The highest quartile of cumulative trihalomethanes-years exposure was associated with an OR of 1.74 (95% CI: 1.25-2.43)[20]. The continuous representation of cumulative trihalomethanes exposure was associated with a 17% increase in risk for each 1000 mg/L-years (95% CI: 6-29)[20].

FOOD CONTAMINATION AND DEVELOPMENT OF BOWEL TUMORS

Polychlorinated biphenyls are possible sources of food contamination. They are synthetic organochlorine compounds that are used in industrial and commercial processes[19,21]. However, dioxins, commonly referred to as dibenzodioxins and dibenzofurans, are organochlorine by-products of waste burning, paper lightening, pesticide production, and production of polyvinyl chloride plastics[21,22]. In the United States, major dietary sources of polychlorinated biphenyls and dioxins are beef, chicken, and pork, dairy products, vegetables, fish and shellfish, and eggs[23]. Animal experiments and some evidence in humans have indicated that polychlorinated biphenyls and dioxins are carcinogenic. This is likely to be related to effects on the aryl hydrocarbon receptor, a transcription factor that affects gene expression[24,25].

Food contamination with pesticides is another example. High levels of different types of pesticides in the collected serum samples from patients with colorectal carcinoma and their relatives have been reported[26-29]. However, no significant correlation between these chemicals and development of this neoplasm has been recognized. Also, higher concentrations of these chemicals in the samples from the healthy relatives[29] might have precluded any possibility of any significant link. Nevertheless, there is increasing evidence that systemic oxidative stress plays an important role in the development and progression of cardiovascular disease and cancer[30].

Oxidative stress is defined as a state in which the level of toxic reactive oxygen intermediates (free radicals) overcomes the endogenous antioxidant defenses of the host, such as the lipid-soluble antioxidants. Oxidative stress can result, therefore, from either an excess of free radical production or depletion of antioxidant defenses. For example, in the absence of adequate levels of lipid-soluble antioxidants, increased free radical production may cause functional and structural damage by reacting with lipoproteins, thus resulting in lipid peroxidation with the formation of degradation products, such as malondialdehyde, which are themselves carcinogenic. The mechanism by which overproduction of free radicals can lead to development of a chronic non-infectious disease has been fully described previously[31].

Recent studies have reported that various pesticides can induce oxidative stress in different tissues[32-35]. The significantly reported positive correlation between different tumor stages, concentrations of lipid peroxidation products, and lack of antioxidants in the serum of patients with colorectal cancer[36-38] gives hope for screening those patients who are at risk for development of these tumors, and for detecting these malignancies at an early stage.

The failure to link measurements of pesticides in the serum of patients with diagnosed colorectal adenocarcinoma to other measurements of lipid peroxidation products, (e.g. malondialdehyde) antioxidants, (e.g. vitamin C, zinc) and copper/zinc ratio in the blood likely explains the confusing outcome of the published reports.

IMPLICATIONS IN CLINICAL PRACTICE AND IN RESEARCH

Well-structured studies need to be designed to assess a possible link between levels of pesticides in those at risk of developing bowel tumors with the levels of lipid peroxidation products for early tumor detection.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3478

World J Gastroenterol 2010 July 28; 16(28): 3478-3483
ISSN 1007-9327 (print)

EDITORIAL

Radiofrequency ablation of locally advanced pancreatic adenocarcinoma: An overview

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Received: March 10, 2010 Revised: April 2, 2010
Accepted: April 9, 2010
Published online: July 28, 2010

Abstract

Radiofrequency ablation (RFA) of pancreatic neoplasms is restricted to locally advanced, non-resectable but non-metastatic tumors. RFA of pancreatic tumors is nowadays an ultrasound-guided procedure performed during laparotomy in open surgery. Intraoperative ultrasound covers the mandatory role of staging, evaluation of feasibility, guidance and monitoring of the procedure. Different types of needle can be used. The first aim in the evaluation of RFA as a treatment for locally advanced pancreatic ductal adenocarcinoma, in order of evaluation but not of importance, is to determine the feasibility of the procedure. The second aim is to establish the effect of RFA on tumoral mass in terms of necrosis and cytoreduction. The most important aim, third in order of evaluation, is the potential improvement of quality of life and survival rate. Nowadays, only a few studies assess the feasibility of the procedure. The present paper is an overview of RFA for pancreatic adenocarcinoma.

Key words: Radiofrequency ablation; Pancreatic adenocarcinoma; Intraoperative ultrasound; Contrast-enhanced ultrasound; Perfusion computed tomography

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D'Onofrio M, Barbi E, Girelli R, Martone E, Gallotti A, Salvia R, Martini PT, Bassi C, Pederzoli P, Pozzi Mucelli R. Radiofrequency ablation of locally advanced pancreatic adenocarcinoma: An overview. *World J Gastroenterol* 2010; 16(28): 3478-3483 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3478.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3478

INTRODUCTION

Ductal adenocarcinoma is the most common primary malignancy of the pancreas, and represents 80% of malignant pancreatic tumors[1-3]. Pain, anorexia and weight loss are common symptoms, even in early stages. Tumors of the pancreatic head frequently cause obstruction of the common bile duct and present with jaundice. Tumors of the body/tail of the pancreas may grow to a large size before obvious symptoms appear[4]. Macroscopically, pancreatic ductal adenocarcinoma is a white/yellow and firm mass with infiltrating epithelium that recapitulates ductal struc-

tures. Microscopically, it is composed of infiltrating glands surrounded by dense and reactive fibrous tissue[4]. Highly aggressive adenocarcinomas are characterized by the presence of intratumoral fibrosis and necrosis, typically with a reduction of the microvascular density and perfusion[5].

Diagnostic imaging aims to correctly detect and characterize the tumor, thus playing an important role in the management of pancreatic ductal adenocarcinoma[6]. Conventional ultrasonography (US) is often the initial noninvasive imaging modality chosen for the first evaluation of the pancreas, as it is inexpensive, easy to perform and widely available[7]. The detection of a solid hypoechoic mass should be considered to be a ductal adenocarcinoma until proven otherwise. Contrast-enhanced US can better characterize and stage pancreatic tumors already detected by US[8], thus differentiating between solid and cystic lesions and influencing the choice of further examinations. A solid hypovascular pancreatic mass has to be considered a ductal adenocarcinoma until proven otherwise and requires computed tomography (CT) confirmation and staging. more than 95% of cases, pancreatic ductal adenocarcinoma is diagnosed at an advanced stage[5], with locally advanced (presence of perineural and vascular invasion) or metastatic disease (commonly in the liver, lungs, peritoneum and adrenal glands)[2,3,6,7]. Prognosis and treatment approach depend on the resectability or non-resectablility of the lesion at presentation[6]. Thus, only 10%-20% of patients are candidates for surgery[4], whereas in most cases, there is worse survival[8,9] and only palliative therapies are feasible.

Palliative therapies for advanced pancreatic adenocarcinoma consist of chemotherapy to give both local and systemic effect and radiotherapy for local effect. Endo-prosthesis positioning, biliary and pancreatic anastomosis, or ethanol ablation of the celiac plexus for pain relief represent further palliative procedures.

Radiofrequency ablation (RFA) of pancreatic ductal adenocarcinoma builds on its positive experiences in the liver. In fact, ablation therapy performed on the liver, usually minimally invasive through a percutaneous approach, provides extremely favorable results[10,11]. Thus, the first aim in the evaluation of RFA as a treatment for locally advanced pancreatic ductal adenocarcinoma, in order of evaluation but not of importance, is to determine the feasibility of the procedure. Nowadays, only few studies that are mainly concerned with the feasibility and complications of the procedure have been reported[12,13]. The second aim is to establish the effect of RFA on tumoral mass in terms of necrosis and cytoreduction. This is the same endpoint of radiotherapy, but with a possible better effect in terms of extent and type of tumor necrosis for RFA, even if performed during open surgery. The most important aim, third in order of evaluation, is the improvement of quality of life and survival rate.

INDICATIONS

RFA of pancreatic neoplasms is restricted to locally advanced, non-resectable but non-metastatic tumors. The resectability of a lesion represents an absolute exclusion criterion, because surgical resection is the treatment of choice. The presence of metastatic spread again represents an exclusion criterion. At preoperative imaging, the eligibility is related to the presence of a locally advanced, non-resectable pancreatic solid mass in the absence of any sign of metastatic spread, including ascites.

The anatomical complexity of the pancreatic and peripancreatic regions in which the pancreatic ductal adenocarcinoma grows makes the procedure of RFA different from that in other regions. In fact, independent from tumor size, the necrotic area must not overcome the lesion owing to the required safety margins in respect to the contiguous main vascular and digestive structures. Therefore, RFA has become a palliative treatment and could be included in a combined therapeutic plan.

The indications depend on the different clinical presentations related to the site of the obstructive or non-obstructive tumors. Non-obstructive tumors are usually located in the body/tail of the pancreas. These neoplasms do not need immediate surgical treatment and neoadjuvant chemotherapy represents the first choice. The absence of distant metastases and a good local response to therapy are expected at the post-treatment restaging evaluation. Resectable masses are treated surgically, whereas RFA can be performed in cases of non-resectable tumor. Obstructive tumors are usually located in the pancreatic head and frequently cause obstructive jaundice that promptly requires endoprosthesis positioning or derivative treatment and subsequent neoadjuvant chemotherapy. The presence of a resectable mass leads to surgical treatment, whereas RFA can be performed in cases of inveterate non-resectable tumor.

RFA TECHNIQUE

RFA of pancreatic tumors is nowadays an ultrasound-guided procedure that is performed during laparotomy in open surgery (Figure 1). Intraoperative ultrasound (IOUS) covers the mandatory role of staging, evaluation of feasibility, guidance and monitoring of the procedure (Figure 2).

The malignant nature of the lesion has to be confirmed pathologically before the procedure. Then, presurgical imaging staging needs confirmation. In particular, metastatic spread in the peritoneum (under inspection and extemporary histology of suspicious lesions, eventually present) and liver (under IOUS with extemporary cytology of doubtful lesions, eventually present) must be excluded. RFA will not be performed if the lesion is found resectable at IOUS, or if metastatic liver lesions are detected. The occurrence of very small lesions or those that envelop the main vessels without a true mass is a contraindication for the procedure. However, the possibility of RFA must be at first evaluated at preoperative imaging.

IOUS has to confirm the safety and feasibility of the RFA procedure without any risk of damage to the contiguous vascular and digestive structures, especially the duodenum. Tumor shape and diameters technically influence the procedure (approach, choice of needle and opening of the electrodes).

Two main types of needle are now available. In a needle with expandable electrodes, the electrodes can be opened from the top (Figure 3A) or the back (Figure 3B)

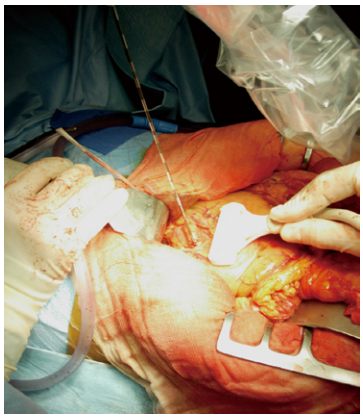

Figure 1 Radiofrequency ablation procedure. Ultrasound-guided radiofrequency ablation performed during laparotomy in open surgery.

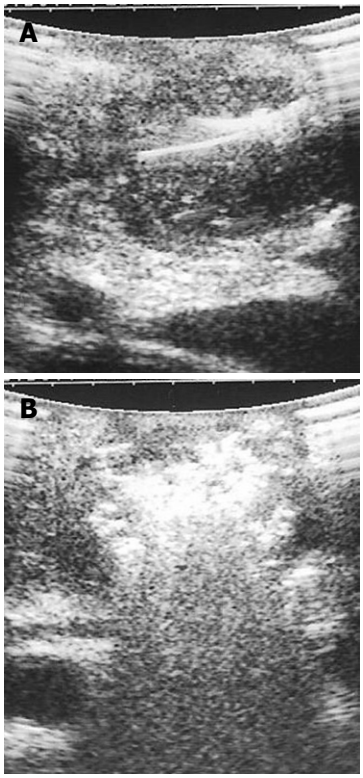

Figure 2 Intraoperative ultrasound monitoring. A: Needle with expandable electrodes is opened in the pancreatic hypoechoic mass under intraoperative ultrasound guidance; B: During radiofrequency ablation, the ablation zone becomes hyperechoic.

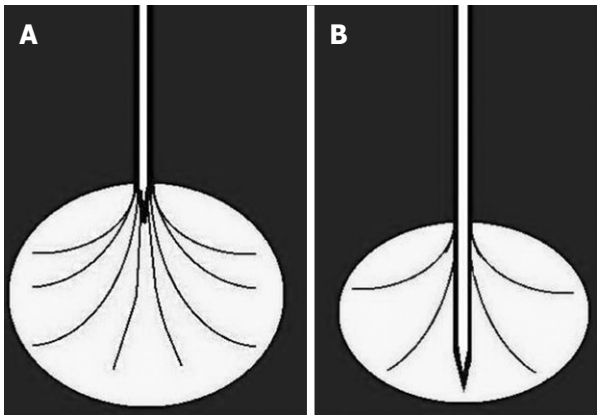

Figure 3 Needle with expandable electrodes. The electrodes can be opened into the lesion from the top (A) or from the back (B) of the needle.

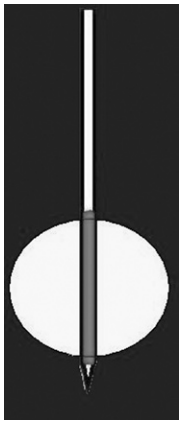

Figure 4 Needle with single electrode. Single electrode of the needle in the lesion.

of the needle. In the first case, the tip of the needle has to stop immediately prior to the lesion and electrodes (up to nine) open up and widen into the lesion. It is also possible to treat small tumors because the device can be opened at different degrees in relation to the size of the requested ablation zone. However, the flexibility of the electrode is not a negligible disadvantage in the treatment of very hard tumors. In the second case, by using a needle with electrodes coming from the back during the opening procedure, the electrodes (usually four) open up at about 2 cm behind the tip and move towards the outside. After complete opening, the electrodes arrive at the same level as the central needle tip. As a consequence, for correct positioning, the needle has to pass completely through the lesion, and the presence of the central needle for at least 2 cm within the mass assures the electrodes enter the neoplastic tissue. The central needle crossing throughout the mass and the stiffer electrodes guarantee excellent stability, even in the presence of hard tumor. On the other hand, the required introduction of the needle throughout the lesion for at least 2 cm can represent a disadvantage with respect to the dimensions and location of the tumor.

In a needle with a single electrode (Figure 4), the length of the area that has to be treated depends on the uncovered portion of the electrode. The width of the treated area depends on both the length of the uncovered portion and the time of ablation. The first type of needle produces a spherical/ovoid necrotic area, with a diameter ranging from 2 to 5-6 cm, depending on the needle and the electrode opening. The second type produces a cylindrical necrotic area ranging from 1 to 3 cm, depending on the extension of the uncovered metallic portion of the needle.

As reported above, the shape and dimension of the tumor influence the choice of the needle. For example, in the case of rounded lesions, even if located in the head of the pancreas, the first type have to be chosen, whereas, to treat ovoid lesions, the second type of needle is preferred. The latter is also preferable for very small lesions with particularly difficult access and/or location; choosing the needle with a lower caliper (17 rather than 14 gauge) and

taking advantage of the cool tip for safer placement.

After laparotomy, the gastrocolic ligament is divided to access the pancreas. For most tumors localized in the pancreatic head, after Kocher maneuver a cold wet gauze is placed over the inferior vena cava to protect it from heat, to reduce maximally the risk of complications. The duodenum is continuously perfused with cold saline solution through a nasogastric tube. Simultaneously, instillation of cool water to the areas around the tumor has to be performed during ablation.

IOUS guides the ablation procedure, mainly during the needle positioning and opening of the electrodes into the lesion (Figure 2A). During treatment, the tip of the needle and the electrodes must be kept at almost 5 mm from the sensitive structures such as the duodenum and peri-pancreatic vessels, as previously reported[13-15]. The correct needle positioning and electrode opening are followed by setting of the parameters for the procedure. These parameters differ depending on the system used. The time setting usually ranges from 5 to 10 min. The power supply affects the temperature directly, and the treated volume indirectly. Some systems also allow evaluation of impedance, which increases with the development of necrosis during the procedure. The temperature at the tip of each electrode can be monitored, thus assuring a more uniform distribution of the temperature in the mass. The temperature setting depends on the treatment aims. Since protein denaturation begins at 50-60℃, the higher temperatures used during the procedure achieve homogeneous necrosis. On the other hand, the use of too high temperatures (105℃) increases the risk of complications, without a favorable effect. Hence, during the ablation procedure of a pancreatic mass, middle-range temperatures are usually applied (90℃). During the procedure, monitored with ultrasound, the tumor gradually becomes hyperechoic owing to the gas produced inside the treated lesion (Figure 2B). This sign can be used to confirm the radiofrequency effect that monitors the integrity of the sensitive surrounding structures, as described previously[10]. At the end of the procedure, the electrodes have to be closed and the needle removed.

The post-treatment IOUS evaluation has to assess the volume of the resultant hyperechoic treated area. The presence of incomplete necrosis with a significant hypoechoic neoplastic remnant can be treated again. When technically possible, a biliary and gastric bypass is required for pancreatic head tumors[12]. In particular, Siriwardena *et al*[16] have recommended that no patient should undergo laparotomy simply for ablation, but the procedure should be used only in patients in whom palliative bypass is required or non-resectable disease is found at surgery. As a consequence, intraoperative RFA of tumor of the pancreatic body, which is non-resectable at imaging, seems unacceptable at this time, but it could be justifiable if positive survival results in large populations are found.

Moreover, in the future, endoscopic or percutaneous approaches for ablation of these tumors, under local anesthesia with sedation, are expected, as occurs in other regions.

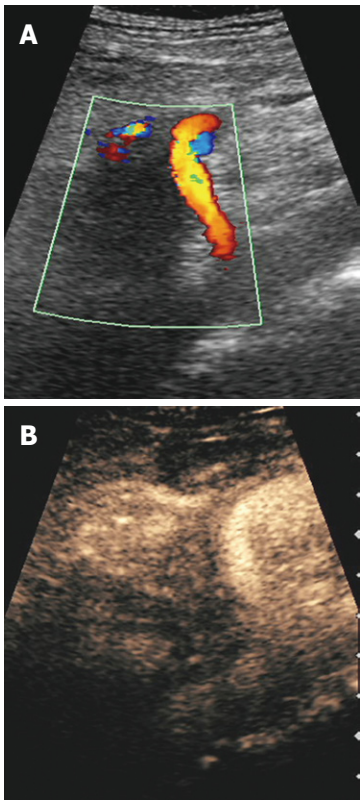

Figure 5 Pancreatic ductal adenocarcinoma after radiotherapy. A: Longitudinal color Doppler scan of the lesion, with hypoechoic infiltration of the superior mesenteric artery; B: Longitudinal contrast-enhanced ultrasonography scan of the hypovascular lesion.

FEASIBILITY AND COMPLICATIONS

Only a few studies have focused on the feasibility and complications of RFA, and none of these have reported major intraoperative complications[12,13,16,17]. Hadjicostas *et al*[17], based on results obtained in four patients, have concluded that RFA seems to be a feasible, potentially safe and promising option in patients with advanced and non-resectable pancreatic cancer. Girelli *et al*[12], based on results obtained in 50 patients, showed that RFA of locally advanced pancreatic cancer is feasible and relatively well tolerated, with a 24% complication rate. In a previously published series of 16 patients, however, a mortality rate of 25% was reported, with all deaths in patients treated for pancreatic head tumor and complicated with massive gastrointestinal hemorrhage[13]. However, the temperature applied exceeded 90℃ and no protective/refrigeration practices were utilized. On the contrary, in the Girelli series in which the procedure was performed with protective/refrigeration practices, the reported mortality rate was 2%. Moreover, in the same series in the second part of the study, by using a temperature of 90℃, the complication rate decreased to 8%[12].

Postoperative observation (clinical surveillance, laboratory tests and imaging studies) is mandatory because of the potential for major or minor, early or later complications. The most frequent complications encountered in the earlier postoperative period (within 1 wk) are fluid

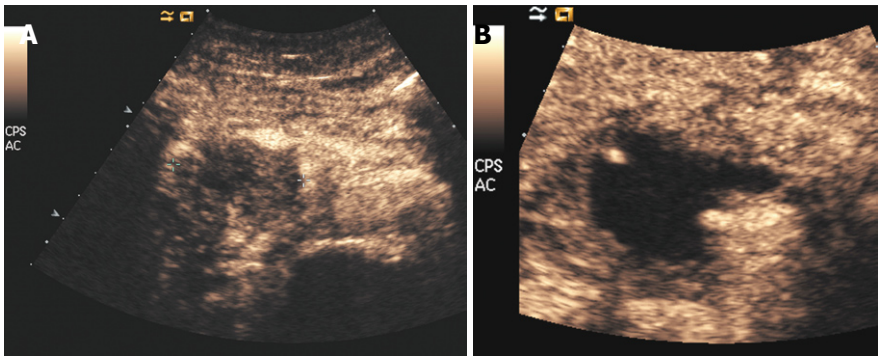

Figure 6 Pancreatic ductal adenocarcinoma before and after radiofrequency ablation. A: Axial contrast-enhanced ultrasonography (US) scan of the hypovascular lesion; B: Axial contrast-enhanced US scan of the avascular lesion after radiofrequency ablation.

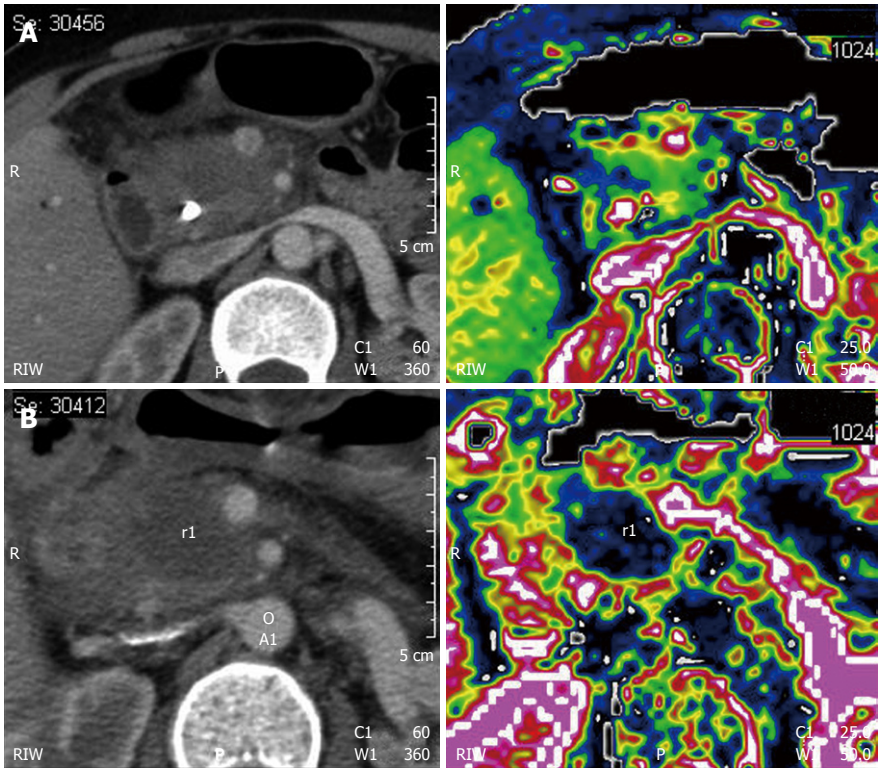

Figure 7 Pancreatic ductal adenocarcinoma before and after radiofrequency ablation. A: Contrast-enhanced computed tomography (CT) of pancreatic head lesion that appears hypodense and vascularized at perfusion CT (right side); B: Contrast-enhanced CT of the lesion after radiofrequency ablation, which appears hypodense and avascular at perfusion CT (right side).

collection, pancreatic fistula, duodenal perforation and vascular damage. At later times, digestive or abdominal bleeding, infections or abscesses are more common. Severe acute pancreatitis is a rare complication[17]: in Girelli's study, there was only one case, and none was reported in Wu's study[12,13]. Major complications frequently are present with RFA of pancreatic head tumors, mainly owing to the closeness of the duodenum. These lesions are more difficult to treat, as reported previously[13].

TECHNICAL RESULTS AND POSTOPERATIVE IMAGING

The necrotic area obtained by RFA is complete. In comparison with radiotherapy (Figure 5), the RFA-treated area (Figure 6) is completely avascular at perfusion imaging. Immediate ultrasound evaluation is useful for identification of possible fluid collections. In the presence of clinical suspicion of major complications, CT is mandatory. Dynamic studies after the administration of contrast agents, usually starting 1 mo after the procedure, are performed to detect the intratumoral necrotic area produced by RFA.

Postoperative imaging of RFA of the pancreas differs from that of the liver. With hepatocellular carcinoma, detection of the ablated area after treatment is immediate because of the hypervascular nature of the tumor. On the contrary, ductal adenocarcinoma is markedly hypovascular, such that after the procedure, the identification of the necrotic area with respect to residual viable tumor tissue can be difficult (Figure 7). It is more important to assess the type and extent of the post-ablation necrotic area, rather than the presence of remnant tumor. In treating pancreatic tumors, the presence of residual viable tumor at the periphery of the treated area is an intrinsic aspect of the procedure. This is the other important difference in comparison with RFA of the liver, in which the lesion must be covered by the necrotic area. On the contrary, in RFA of the pancreas, the necrotic area must be included in the tumor (Figure 8).

All these particular features of pancreatic RFA have to be considered during postoperative imaging. Precise and

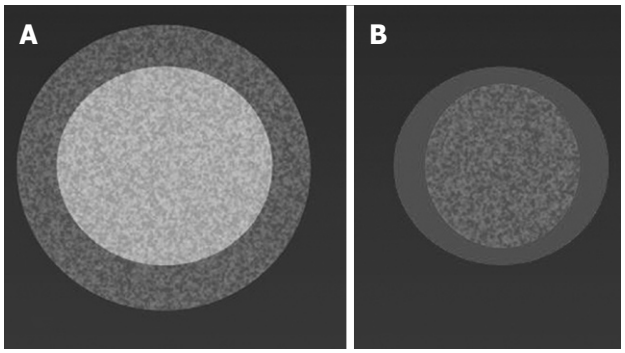

Figure 8 Ablation zone on target lesion. A: Necrotic ablation zone (dotted grey) must covered the hepatocellular carcinoma (white); B: Necrotic ablation zone (dotted grey) must be included in the pancreatic ductal adenocarcinoma (grey).

accurate dynamic imaging evaluation, such as contrast-enhanced US or perfusion CT (Figures 6 and 7), is necessary.

CLINICAL RESULTS AND FUTURE VIEW

RFA of locally advanced pancreatic neoplasms is currently performed under ultrasound guidance in open surgery during laparotomy, with the palliative aim of tumor reduction in a combined therapeutic plan. Minimally invasive laparoscopic or percutaneous approaches, as in other regions, are expected.

The primary endpoint, represented by improvement in quality of life, has been achieved, given that the treatments obtain excellent pain relief[12,13,17]. In particular, in the series of Wu *et al*[13], pain relief was reported in 50% of cases, and in 68% in the series of Girelli[12]. Pain relief seems not to be related to ablation necrotic volume. Girelli *et al*[12] have reported a decreased of CA 19-9 concentration 7 d after surgery.

Regarding the more ambitious clinical endpoint, significant results about improved survival are still missing. At this time, only one study has investigated survival in a group of 25 consecutive patients, which showed a significant difference in survival between RFA and control groups in patients with stage Ⅲ disease[18]. Further studies are needed to validate the reported preliminary results, to evaluate a possible correlation with tumor markers eventually expressed during the preoperative period, and particularly, to validate the technique.

CONCLUSION

RFA of locally advanced, non-resectable but non-metastatic, pancreatic tumors is a feasible palliative treatment that leads to tumor reduction and improved quality of life. Further studies are needed to validate therapeutic strategies and associations for the best possible results on survival.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3484

World J Gastroenterol 2010 July 28; 16(28): 3484-3490
ISSN 1007-9327 (print)

REVIEW

Role of surgery in colorectal liver metastases: Too early or too late?

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Received: February 2, 2010 Revised: April 28, 2010
Accepted: May 7, 2010
Published online: July 28, 2010

Abstract

As colorectal cancer and colorectal liver metastases become a serious public health problem, new treatment modalities are needed in order to achieve better results. In the last decade there has been very important progress in oncology, with new and more effective chemotherapeutic agents administered alone or in combination improving the resectability rate in up to 40% of patients with colorectal liver metastases. Advances in interventional radiology, in particular, with the use of portal vein embolization and radiofrequency thermal ablation are new strategies allowing major liver resections and treatment of small liver metastases or early recurrences. Surgery, however, remains the gold standard strategy with intention to treat. In this review article we will describe the advanced role of surgery in the multidisciplinary approach to colorectal liver metastases, and the clinical problems the liver surgeon has to deal with, such as the resectability of the metastases, the presence of bilobar liver lesions and extrahepatic disease, the impact of chemotherapy in already resectable liver metastases, the problem of vanishing metastases after chemotherapy and the dilemma of staged or combined liver and colon operations and which organ first in the clinical scenario of synchronous colorectal liver metastases.

Key words: Metastases; Liver metastases; Colorectal cancer; Surgery

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Dimitroulis D, Nikiteas N, Troupis T, Patsouras D, Skandalakis P, Kouraklis G. Role of surgery in colorectal liver metastases: Too early or too late? *World J Gastroenterol* 2010; 16(28): 3484-3490 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3484.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3484

INTRODUCTION

Colorectal cancer (CRC) is an important public health problem: there are nearly one million new cases diagnosed worldwide each year and half a million deaths. Recent reports show that CRC is the third most frequent cancer in the Western world while in the USA it is the most frequent form of cancer among persons aged 75 years and older[1]. Approximately 25% of these patients have liver metastases at the time of diagnosis [colorectal liver metastases (CRLM)] and 25% will develop, CRLM during the course of the disease. Eventually two-thirds of patients

with CRLM will die because of liver metastases[2]. Given that the majority of malignancies occur in elderly people and with ageing of the population in mind the above epidemiological observations make the need for prevention and improved treatment strategies very urgent.

In the last decade there has been very important progress in 3 different fields regarding the treatment of CRLM: new and more effective chemotherapeutic agents in oncology administered alone or in combination; an advanced role of interventional radiology with portal vein embolization and radiofrequency ablation; and last, but not least, new strategies and manipulation techniques for safer hepatic resections. The combination of these improvements makes the role of a multidisciplinary approach in the patient with CRLM the only therapeutic modality, which has gradually but effectively improved the resectability rate of metastases to 20%-30% of cases and has resulted in 5-year survival of 35%-50% for selected cases[3,4].

In this review we will discuss the improvement in chemotherapy and interventional radiology as well as the new and advanced role of surgery in this multidisciplinary approach in order to establish treatment strategies and to gain better results in disease-free and overall survival in patients with CRLM.

CHEMOTHERAPY-ONCOLOGY

Chemotherapeutic agents have substantially changed over the last decade. Two new agents, irinotecan and oxaliplatin are used in combination with the traditional regimen of 5-fluorouracil (5-FU) and leucovorin (LV). Irinotecan is an inhibitor of topoisomerase I and its combination with 5-FU and LV is called Folfiri while oxaliplatin is a non-nephrotoxic platinum complex which in combination with 5-FU and LV is called Folfox. While traditional chemotherapy was of limited efficacy, with response rates not exceeding 25%, the new combinations allowed a tumor response in approximately 40%-50% of patients[5,6]. Another very important issue is that the new chemotherapeutic combinations have been reported to facilitate the resection of 9%-40% of initially unresectable metastases, with data emerging from randomized trials suggesting that the addition of targeted agents and a third cytotoxic drug might improve these results even more[7,8]. The use of a triple combination of irinotecan, oxaliplatin and 5-FU or LV seems to further increase the efficacy of systemic chemotherapy, as 3 different studies described a response rate of up to 70% and improvement in the overall survival of up to 26 mo[9-11]. All these studies showed that patients who can undergo surgical resection for their CRLM have better survival rates compared to patients treated only with chemotherapy.

Besides oxaliplatin and irinotecan, the introduction of two new targeted agents opens a new era in the oncological treatment of colorectal liver metastases. Cetuximab is a monoclonal antibody against the epithelial growth factor receptor and bevacizumab is a humanized antibody against the vascular endothelial growth factor (VEGF). The use of the above-mentioned targeted agents in combination with Folfox or Folfiri has increased the resectability rate in patients whose tumors were previously considered unresectable and has also improved the overall survival rate in patients with CRLM as mentioned above. Hurwitz *et al*[12] published a randomized controlled trial where 813 patients with CRLM were allocated to either Folfiri or Folfiri plus bevacizumab. The addition of bevacizumab was associated with a statistically significant increase in median survival, progression-free survival and overall tumor response rate. There were also other studies where the addition of bevacizumab to Folfox was associated with an increased tumor response rate and median overall survival[13]. However, these studies were not randomized.

However, apart from these positive effects of chemotherapy on resectability and survival rates, there were also some severe adverse effects on the liver, as histological lesions are known to occur in the liver parenchyma following chemotherapy, with the type of lesion being specific for the agent used. Sinusoidal obstruction syndrome is characterized by erythrocytic congestion and can be accompanied by perisinusoidal fibrosis and fibrotic venous occlusion. This syndrome has been described in association with oxaliplatin, and the incidence of these histological changes is between 20% and 29%[14,15]. The administration of oxaliplatin-based chemotherapy can also be associated with vascular lesions in the liver such as hemorrhagic centrilobular necrosis, and carry a higher risk of operative bleeding and transfusion requirement, as well as impaired liver regeneration and increased post-hepatectomy mortality[16,17]. The second adverse effect is a form of steatohepatitis that has been related to irinotecan administration. Chemotherapy-associated steatohepatitis is characterized by the simultaneous presence of severe steatosis, lobular inflammation and ballooning of the hepatocytes[18]. Analysis of the impact of steatosis on surgical outcome suggests that morbidity is increased and that there is also an increased rate of infectious complications[19,20]. Steatohepatitis linked to irinotecan treatment is associated with increased 90-d mortality because of liver failure after surgery[18,21].

At this point there is an important question raised whether the above-mentioned chemotherapy-linked liver damage is related to the duration of treatment or not. There are 2 pertinent studies in the literature which clearly showed that the morbidity rate was related to the number of cycles of chemotherapy administered[17,21]. More recent data on postoperative complications in the EORTC 40983/EPOC study show that 3 mo of preoperative chemotherapy with Folfox-4 had a relatively mild impact on surgical outcome[22]. Another issue regarding the safety of preoperative chemotherapy is the potent effect of the monoclonal antibody bevacizumab, as targeting VEGF could augment hepatic damage and diminish regeneration after resection. One study showed that there was no major effect of bevacizumab on the incidence of postoperative complications if stopped at least 5 wk prior to surgery[23]. Data from another study described that the use of this agent may even reduce the

incidence of liver failure after hepatic resection[24]. One can conclude that neoadjuvant chemotherapy can induce liver injury but with little clinical impact if patients are not overtreated and if a proper time interval is maintained between chemotherapy and surgery[25].

INTERVENTIONAL RADIOLOGY

Advances in interventional radiology have contributed to the treatment of patients with CRLM, in particular the use of portal vein embolization (PVE) and radiofrequency thermal ablation (RFA). Since 1982 when Makuuchi *et al*[26] first used PVE in order to provoke compensatory hypertrophy of the future remnant liver in patients planned for major hepatic resections, much experience has been gained and this technique is today available in every specialized hepatobiliary center[27,28]. In patients planned for major hepatectomy and with an otherwise normal liver, preoperative PVE is recommended when the ratio of the remnant liver to total liver volume is estimated to be less than 30%, whereas in patients with neoadjuvant chemotherapy this ratio is considered to be 40%[29,30]. PVE is a safe procedure, but movement of the embolic material to the main portal vein or into branches that supply the future remnant liver remains a risk.

RFA was initially and widely used for local treatment of hepatocellular carcinoma and recently has gained popularity for the management of CRLM, where its indications are still under debate. Critical review of the results of RFA shows that RFA must be restricted in cases with a maximum of 3 lesions with the size of the biggest lesion less than 3 cm[31]. Another limitation for the use of RFA in the management of CRLM is the anatomic location of the lesion. When the metastases are near big vessels the risk of incomplete ablation is increased as the heat effect is minimized[32]. Because of the above-mentioned limitations there is still no place for RFA in patients with resectable CRLM. The use of RFA is actually limited in cases with early recurrence after resection, detected as small lesions, because it is not mandatory to stop chemotherapy[33].

THE ROLE OF SURGERY

The liver surgeon of the multidisciplinary team for the treatment of CRLM will face some clinical problems and scenarios such as the resectability of the metastases, the presence of bilobar liver lesions or extrahepatic disease, the impact of neoadjuvant chemotherapy in the case of resectable metastases, the problem of vanishing metastases after chemotherapy, and the dilemma of staged or combined liver and colon resections in the clinical scenario of synchronous CRLM. For historical reasons we mention that liver metastases were classified as unresectable if they were large in size, bilobar or there were more than 4[34,35]. Today there has been substantial progress in liver surgery owing to improved preoperative diagnosis and intraoperative and postoperative care, and new strategies are being developed in order to achieve larger and

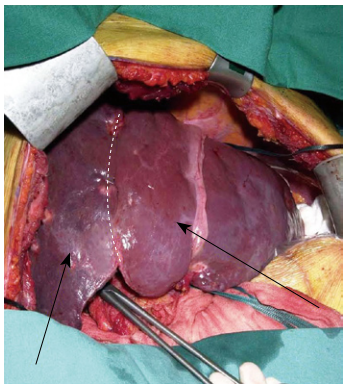

Figure 1 Atrophy of the right hemiliver (right arrow) and hypertrophy of the left hemiliver (left arrow) after right portal vein ligation.

safer resections. Many tumors that were previously considered unresectable are now amenable to complete resection. In 2007, Figueras *et al*[36] published a study where no predefined criteria of resectability were mentioned with regard to number, size, location of the tumor or presence of extrahepatic disease. The only prerequisite is that the resection was possible and that the potential liver remnant was able to sustain metabolic, synthetic and detoxifying functions[37]. It is in general accepted that where possible it is better to remove a metastasis than to leave it and that even R1 resections might become an acceptable clinical strategy provided that they confer meaningful patient benefit[25].

The presence of bilobar CRLM is a challenging issue for the liver surgeon as this problem requires extended resections. As mentioned above, interventional radiology with PVE is an important preoperative tool in order to increase the volume of the remnant liver after an extended resection. However, the legitimate concern that metastases in the non-embolized hemiliver might grow more rapidly after right PVE[38] has led to the proposal of a 2-stage procedure. In the first stage all visible metastases in the left hemiliver are removed in association with right portal vein ligation. In the second stage, which takes place between 2 and 4 wk after the first stage, an extended right hemihepatectomy is performed[37] (Figure 1).

Despite the great effort that has been made both in preoperative liver volume manipulation and in intraoperative and postoperative care there are still many patients who present to the liver surgeon with initially unresectable metastases. Current treatment practice for these patients is a combination of classical chemotherapy with the targeted agents cetuximab and bevacizumab, which facilitates resection in 9%-40% of lesions initially considered unresectable[7]. As a consequence, 5-year survival rates of 50% after combined treatment are becoming increasingly common[36]. The next step is to define the most effective chemotherapeutic regimen for this treatment strategy. Currently, data from randomized trials are beginning to show the added benefit conferred by the targeted agent cetuximab on response and resection rates achieved with standard first-line therapies in patients with advanced CRC[39,40]. The rate of surgery with curative intent was higher in patients who received Folfiri plus cetuximab in comparison with Folfiri alone and the R0 resection was also increased[40]. In this setting the requirement for a

delay between the end of chemotherapy treatment and the planning of surgery, as well as the presence of K-ras mutation in the primary tumor are 2 very important considerations for the management of these complex cases[41-43]. Mutations in this gene have been shown to be predictive of reduced disease-free and overall survival. Furthermore, it has been demonstrated that those with K-ras mutations do not benefit in the same manner from the traditional chemotherapeutic regimens as those with the wild type. Analysis of recent data show that patients with wild type K-ras had a significant advantage in terms of tumor response with the addition of bevacizumab compared to standard Folfiri treatment. Further studies are needed in the era of biomarkers in order to achieve better tumor response rates and increased survival[44,45]. At this point it is important to mention that the presence of extrahepatic disease is no longer considered in the criteria for unresectability, provided that it is also resectable[46].

The conventional way of thinking in patients with resectable synchronous CRLM is to offer an upfront operation, and the reason for this attitude is the fear that the CRLM will not respond to chemotherapy and that during the time of chemotherapy the tumors will grow beyond the possibility of surgical cure. However, chemotherapy before surgery, even in patients with resectable CRLM, can increase the complete resection rate, facilitate limited hepatectomies, improve postoperative recovery, treat micrometastases, provide a test for chemoresponsiveness, identify aggressive disease and spare ineffective chemotherapy. All the above parameters are supported by the results of the EORTC 40983 study where the progression-free survival rate at 3 years was increased by 8.1% in those patients who received perioperative chemotherapy when compared with surgery alone. Furthermore, single-center non-randomized studies support the use of neoadjuvant protocols for resectable CRLM[47]. A specific problem that has emerged with the use of effective neoadjuvant chemotherapy regimens is known as the "missing" or "vanishing" metastases. This terminology reflects lesions that were present on initial radiological examinations and can no longer be identified by imaging performed after chemotherapy. Because these lesions are very difficult to localize and resect during surgery the situation is well characterized by the sentence "when the dream of the oncologist becomes the nightmare of the surgeon". While a publication suggested that missing metastases are cured in 70% of cases[48], another study showed persistent microscopic or macroscopic residual disease or very early recurrence in 83% of cases with a complete radiological response[49]. Thus a complete radiological response does not mean a complete histological response and some authors suggest that when the detailed intraoperative ultrasound examination fails to detect missing metastases, the corresponding parenchymal region should be resected in the basis of vascular landmarks[33].

The final but very important issue that the liver surgeon has to deal with is whether to proceed to combined (liver and colon) or staged surgery and if he chooses a staged procedure which organ first. The ideal solution for this complicated problem would be a simultaneous colon and liver operation. The advantage of the combined procedure is that we have one operation with less psychological considerations for the patient, less financial costs and shorter hospitalization time. On the other hand the advantages of the staged procedure are that there is no accumulation of the risks of liver and bowel resections at the same time, a neoadjuvant chemotherapy may be given before liver resection, and an extended hepatectomy or difficult bowel resection can be performed with the full attention of the surgical team focused on the liver or bowel disease. However, the critical issue for decision-making is the patient's safety. Considering the initial experience with simultaneous *versus* staged resections, a French multicenter study showed an operative mortality of 7% for simultaneous *vs* 2% for staged surgery[50], while in a single center US study the mortality was 12% for simultaneous *vs* 4% for staged resections[51]. It was also shown in several studies that simultaneous operations can be performed without death[52-55]. These studies, however, were retrospective and patients for simultaneous resection were selected by experienced hepatobiliary surgeons. In conclusion, simultaneous liver and bowel operations can be performed on selected patients but should be avoided in cases of major hepatectomies, in elderly patients, and in difficult rectal surgery.

In the case of a staged operative procedure, the standard treatment recommendations in the pertinent literature until now were resection of the primary tumor followed by chemotherapy for 3-6 mo and then liver surgery. Given that liver metastases rather than the primary tumor determine survival the above-mentioned standard approach has some disadvantages. Firstly, chemotherapy which is effective against liver metastases cannot be given during the treatment of the primary tumor, especially if complications of colorectal surgery are encountered as the risk for an anastomotic leak in rectal surgery varies from 6% to 12%[56,57]. Even if the colon surgery runs uneventfully the recommended treatment for locally advanced rectal cancer is a long course of radio-chemotherapy (5 wk). Surgery is usually planned 6-10 wk after finishing neoadjuvant therapy. During these 3 mo no treatment is given to hepatic metastases and these may progress beyond cure[58]. The second disadvantage is that there rapid growth of metastases described after removal of the primary tumor in several mouse models[59,60]. The underlying mechanism for these experimental data seems to be the loss of primary tumor-induced inhibition of angiogenesis in the metastases. It was also demonstrated in human CRLM that vascular density increased after resection of the primary tumor[61]. However, the clinical significance of these experimental demonstrations is still unknown.

These two major disadvantages gave impetus to a new approach for the treatment of CRLM. This new approach is the "liver first" approach, where, after initial treatment with chemotherapy, the liver metastases were operated first followed by removal of the primary tumor. The new reverse approach includes the risk that during the period

between chemotherapy and liver resection the primary tumor might become obstructive. This rare possibility can easily be solved by performing Hartmann's procedure. There have already been pertinent studies published in the literature showing that the "liver first" approach is a safe procedure and brings excellent results[58,62]. The results of these studies suggest that patients with advanced synchronous CRLM and non-obstructive primary tumors can be safely and effectively treated with highly effective neoadjuvant chemotherapy towards the metastases, followed by liver surgery and, finally, colorectal resection.

CONCLUSION

Many changes have occurred in the treatment of CRLM in the last decade. Progress in neoadjuvant chemotherapy, new targeted agents and improvement of interventional radiology with PVE and RFA as well as diagnostic imaging provide useful tools. However, since surgery is still the only treatment modality that has curative potential on its own, this may appear the most attractive approach in some situations, even if resistance to medical treatment generally means an unfavorable prognosis. The 2-stage hepatectomy as well as the "liver first" approach seem to create new treatment strategies and improve the rate of survival of patients in whom an R0 resection can be achieved with curative intention. The multidisciplinary treatment modality in these complex cases of patients with CRLM helps to make the best treatment selection for patients in order to offer each of them the best strategy.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3491

World J Gastroenterol 2010 July 28; 16(28): 3491-3498
ISSN 1007-9327 (print)

REVIEW

Protein-protein interaction map is a key gateway into liver regeneration

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Supported by Chinese Human Liver Proteome Project, No. 2004BA711A19-08 and National 863 Project, No. 2007AA02Z100
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Received: February 2, 2010 Revised: April 6, 2010
Accepted: April 13, 2010
Published online: July 28, 2010

Abstract

Recent studies indicate that the process of liver regeneration involves multiple signaling pathways and a variety of genes, cytokines and growth factors. Protein-protein interactions (PPIs) play a role in nearly all events that take place within the cell and PPI maps should be helpful in further understanding the process of liver regeneration. In this review, we discuss recent progress in understanding the PPIs that occur during liver regeneration especially those in the transforming growth factor β signaling pathways. We believe the use of large-scale PPI maps for integrating the information already known about the liver regeneration is a useful approach in understanding liver regeneration from the standpoint of systems biology.

Key words: Liver regeneration; Protein-protein interaction; Protein-protein interaction maps; Transforming growth factor β

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Xie C, Gao J, Zhu RZ, Yuan YS, He HL, Huang QS, Han W, Yu Y. Protein-protein interaction map is a key gateway into liver regeneration. *World J Gastroenterol* 2010; 16(28): 3491-3498 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3491.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3491

INTRODUCTION

Liver has the capacity to regenerate by a process of compensatory growth following injury and various molecular and cellular pathways are involved in this process[1-4]. It is still difficult to understand precisely how the process of liver regeneration is regulated. Previous studies of liver regeneration have been made at the functional, cellular, molecular or gene level[5]. It is generally believed that most cellular processes are determined by protein-protein interactions (PPIs)[6,7] and, therefore, PPIs maps provide a valuable framework for a better understanding of the functional organization of the proteome during liver regeneration[2,8,9]. Various methods have been used to study the functions of specific proteins during liver regeneration. In this review, we describe the use of high-throughput experimental methods and algorithmic predictions to unravel the complex processes of liver regeneration through the use of PPI maps.

LIVER REGENERATION

Source of liver cells and genes analysis in liver regeneration processes

Liver regeneration can be seen as a timely sequence of

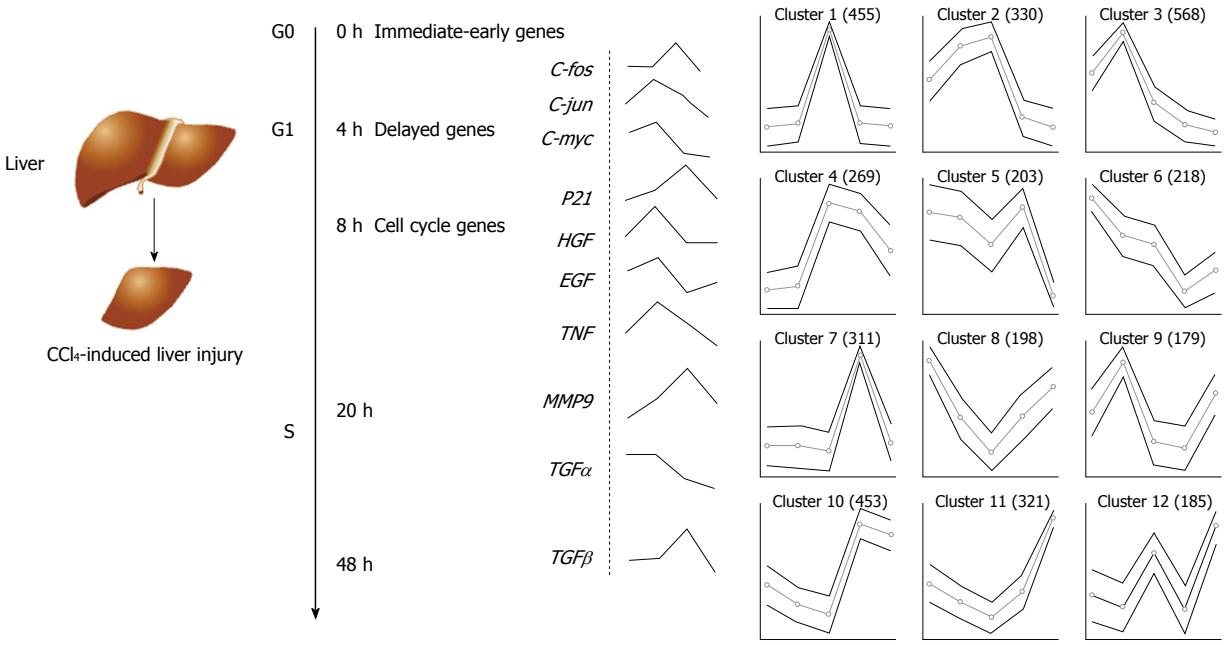

Figure 1 Hepatocyte cell cycle during liver regeneration and clustering algorithm of gene expressions in gene chip during mouse liver regeneration following CCl_4-induced liver injury. Some important genes are activated in the regenerating liver after partial hepatectomy or hepatocellular injury induced by CCl_4. Liver regeneration can be divided into four phases: G0: Corresponds to approximately the first 4 h; G1: Quiescent cells enter the cell cycle during production of hepatocyte growth factor (HGF), epidermal growth factor (EGF), tumor necrosis factor (TNF), *etc.*; S: Chromosomal DNA is replicated and peak DNA production occurs at approximately 24 h; then, after 48 h or more, the process of regeneration is terminated. The curves following the gene products and the dashed line represent the changes in gene expressions measured in our lab using Gene Chip® mouse 430 2.0 during liver regeneration following CCl_4-induced liver injury. Changes on the level of gene expression were log2-transformed using signal value at 0 h time-point as baseline. Genes with their expression levels varying at least 2-fold between any two time-points were subjected to hierarchical clustering analysis. TGF: Transforming growth factor; MMP9: Matrix metalloproteinase 9.

Table 1 Liver cell types and their functions during liver regeneration

Cell types	Functions
Hepatocytes	Organized in single cell plates; perform metabolic and detoxification function; can secrete HGF, IL-6, proteases and protease inhibitors
Sinusoidal endothelial cells	Involved in endocytosis and metabolism of molecules; can produce TGFβ, HGF, IL-6 and nitric oxide
Biliary epithelial cells	Can promote fibrogenesis by attraction of hepatic stellate cells and can secrete cytokines such as MCP-1 and IL-6
Kupffer cells	Major producers of cytokines such as TNF and IL-6
Hepatic stellate cells	Store vitamin A and secrete laminins, collagens and growth factor: HGF, EGF, TGFβ and cytokines IL-6; also produce MMPs
Oval cells	Can differentiate to biliary and hepatocytes lineage

HGF: Hepatocyte growth factor; IL-6: Interleukin-6; EGF: Epidermal growth factor; TGF: Transforming growth factor; MCP: Monocyte chemotactic protein; MMP: Matrix metalloproteinase; TNF: Tumor necrosis factor.

morphological events; resulting in the reconstitution of the lost liver mass following surgical resection or carbon tetrachloride (CCl_4) induced injury. Depending on the nature of the regenerative processes, several sources of liver cells are involved[4,10,11] (Table 1).

After partial hepatectomy (PH) or toxic forms of liver injury, some immediate early genes are expressed simultaneously in the liver. C-fos, c-jun and c-myc are up-regulated immediately and they activate hepatic non-parenchymal cells. Then tumor necrosis factor α, epidermal growth factor, hepatocyte growth factor and transforming growth factor α (TGFα) are released to provide the cooperative signals for the hepatocytes cell cycle to move from G0 through G1 to S phase, leading to DNA synthesis, and hepatocyte proliferation. TGFβ, which controls hepatocyte DNA synthesis, is blocked during the proliferative phase, but is again restored at the end of the process of regeneration. TGFβ is believed to be a key factor in returning hepatocytes to the quiescent state and ending liver regeneration[1,3,4,12].

Our laboratory has used a mouse model of CCl_4-induced liver injury to investigate changes of gene expression during the process of liver regeneration (Figure 1). Changes in the expression of 3642 genes were detected during the process of liver regeneration by microarray analysis[13] (Figure 1). Genes whose expression levels varied at least 2-fold at any time-point were subjected to self-organizing analysis, using Cluster 3.0 (Stanford University). Their specific functions of each cluster were analyzed and will be described elsewhere (manuscript in preparation).

Termination response during liver regeneration

Many research studies have focused on the regulation of the initiation and proliferation phases of liver regeneration. Although the molecular mechanisms for termination of liver regeneration are still not completely understood, TGFβ and activins, which belong to the TGFβ superfam-

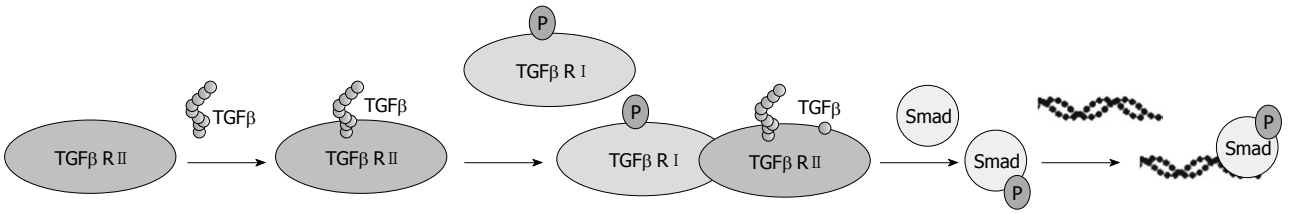

Figure 2 The classical and basic view of transforming growth factor β signaling pathway during liver regeneration. Generally speaking, transforming growth factor β (TGFβ) superfamily ligands bind to a type Ⅱ receptor (RⅡ), which recruits and phosphorylates a RⅠ. The RⅠ then phosphorylates receptor-regulated Smads. Then complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression.

ily, appear to be important[14]. TGFβ and activin A may bind to their high-affinity cell surface type Ⅱ receptor (TGFβRⅡ) and ActRⅡ or ActRⅡb, respectively and TGFβ inhibits G1 to S phase transition in hepatocytes. TGFβ levels rise rapidly after CCl_4-induced injury and, therefore, TGFβ is known to have growth inhibitory effects on liver regeneration. So, at its simplest, the basic TGFβ signals through its Type Ⅰ and Type Ⅱ receptors (TGFβRⅠ and TGFβRⅡ) cause phosphorylation of Smad proteins and activate the Smads complex that controls transcription (Figure 2). Dierssen *et al*[15] demonstrated that gp130-dependent Stat3 activation and concomitant suppressor of cytokine signaling 3 (Socs3) is involved in timing of DNA synthesis during liver regeneration. Also, Riehle *et al*[16] showed that Socs3 modulates several signaling pathways and involved in physiological proliferative processes and protects hepatocyte proliferation in liver regeneration. Therefore, there is more to be understood about the TGFβ signaling pathway and its effects in termination of regeneration, but this still should underscore the complexity of other related pathways and their contribution to the process of termination of liver regeneration[17]. Further studies need to be conducted on understanding the mechanism of liver regeneration.

PPIs AND CORRESPONDING MAPS

PPIs refer to the association of protein molecules and PPI maps/networks summarize large amounts of PPI data, both from experiments and prediction.

There are a multitude of methods to detect PPI including biochemical, physical/biophysical and theoretical methods. The yeast two-hybrid (Y2H) technology is one of the most reliable and feasible experimental biochemical methods to detect PPI since it was established in 1989[18]. The improvement of Y2H techniques can provide high-throughput methods which are widely used in proteome studies of PPIs. Co-immunoprecipitation (CoIP) is also considered to be the gold standard assay for PPIs, especially when it is performed with endogenous proteins. Pull-down assays are a common variation and immunoprecipitation and immunoelectrophoresis are used identically, although this approach is more amenable for an initial screening of interacting proteins[19]. Fluorescence resonance energy transfer is a common biophysical technique used for observing the interactions of two different proteins[20]. There are many ways to detect PPI and each of the approaches has its own strengths and weaknesses, therefore, it is wise to determine the specific method after weighing the advantages and disadvantages.

Visualization of PPI is a popular application and already, the map of PPI among *Saccharomyces cerevisiae*[21], *Drosophila melanogaster*[22], *Caenorhabditis elegans*[6], constructed through a series of experiments and algorithmic predictions, has proved its usefulness for analyzing complex gene regulation and cellular behavior[23]. Similar efforts for PPI maps for human are ongoing and the first human PPI map was constructed from more than 3000 PPIs[24] (Table 2). Recently, the size of PPI maps in different organisms were estimated (with the PPI map for humans being nearly 650 000 PPIs) and the size is strongly believed to have correlation with the organism's apparent biological complexity[25].

Although the PPI maps are still far from complete, whether its deciphered from DNA parts are molecules, cells, or living organisms, a PPI map provides an insight into the systems biology. Our current challenge is to know how large-scale PPI maps possible functions and to understand how cells operate in an integrated manner to carry out phenotypic functions, besides increasing the coverage and accuracy of the existing and novel PPI data sets[39,40].

PEELING THE PPI MAPS DURING LIVER REGENERATION

During liver regeneration, cytokine, growth factors and metabolic pathways were both active after PH or CCl_4-induced liver injury, and the pathways interacted with each other[2]; although, there is indeed a flow of information *via* interactions between DNA, RNA and proteins on which this review will mainly focus. More research about PPI maps is on the level of proteome (Table 2), and these PPI maps will reveal the connectivity of the proteome. If we construct a PPI map during liver regeneration, it will reflect the particular cellular or unique signaling pathway status. As to the PPI map analysis, the so called small-word and scale-free behavior are considered, which indicated that in the maps only few nodes (stand for proteins) are highly connected with others (hub protein) and most of the nodes are connected with only a few nodes (low degree)[41]. In this processes, to capture the changes in protein connectivity and find the key signaling pathways, especially those that interact, is most attractive. For the mechanisms of liver regeneration to be completely understood, a multitude of PPI maps must be coordinated[37].

Table 2 Some protein-protein interaction maps established in recent years

Researcher	Yr	Level	Model organisms	Method	PPI numbers
Bartel *et al*[26]	1996	Proteome	*T7 phage*	Y2H	25
Uetz *et al*[21]	2000	Proteome	*Saccharomyces cerevisiae*	Y2H	1389
Ito *et al*[27]	2000	Proteome	*Saccharomyces cerevisiae*	Y2H	183
Walhout *et al*[28]	2000	Proteome	Caenorhabditis elegans	Y2H	148
Ito *et al*[29]	2001	Proteome	*Saccharomyces cerevisiae*	Y2H	4549
Ho *et al*[30]	2002	Proteome	*Saccharomyces cerevisiae*	MS	367
Giot *et al*[22]	2003	Proteome	Drosophila melanogaster	Y2H	20240
Colland *et al*[31]	2004	Pathway	Caenorhabditis elegans	Y2H	755
Stanyon *et al*[32]	2004	Cell cycle	Drosophila melanogaster	Y2H	20000
Li *et al*[6]	2004	Proteome	Caenorhabditis elegans	Y2H	3955
Lehner *et al*[33]	2004	mRNA	Human	Y2H	247
Formstecher *et al*[34]	2005	Proteome	Drosophila melanogaster	Y2H	2300
Stelzl *et al*[24]	2005	Proteome	Human	Y2H	3083
Rual *et al*[8]	2005	Proteome	Human	Y2H	2529
Ewing *et al*[35]	2007	Proteome	Human	MS	24540
Parrish *et al*[36]	2007	Proteome	*Camp. jejuni.*	Y2H	11687
Gao *et al*[37]	2008	Liver regeneration	Human	Y2H	64
Chen *et al*[38]	2008	Protein degradation	Human	Y2H	114

Y2H: Yeast two-hybrid; MS: Mass spectrometry; PPI: Protein-protein interaction.

PPI maps and TGFβ signaling pathway

Understanding the processes and mechanisms of liver regeneration involves recognizing components in liver regeneration system, the dynamic change of these components and their interactions[42].

In this review, it is pointed out that PPI maps (or PPI data) are closely correlated with TGFβ regulated Smad signaling pathways during liver regeneration. Colland *et al*[31] have used Y2H technique identified 755 interactions, mainly in a focused analysis of TGFβ signaling pathways and have constructed the PPI maps. They used this method to analyse LMO4, HYPA, KIAA1196 and LAPTm5 proteins, which are additional proteins involved in regulation of TGFβ signaling pathways. Also, they present an integrated approach for the identification of new factors implicated in TGFβ signaling pathway involved in several human pathologies and in the termination of liver regeneration. From this point of view, we can apply this strategy to study liver regeneration.

This review focuses on PPI maps and liver regeneration, and pays attention to the TGFβ signaling pathway. The PPI maps were constructed containing proteins related to the TGFβ signaling pathway and some of these proteins may have potential functions on the termination of liver regeneration (Figure 3). It can be easily used to find the key proteins in this process and additional experiments should be done to validate this hypothesis. Regardless, it is confirmed that the PPI map is an effective tool to study liver regeneration.

A PPI maps acting during liver cell proliferation

Gao *et al*[37] constructed a PPI map of transcription factors acting during liver regeneration which contains 32 regulatory proteins. Among them, 27 transcription factor genes that might have roles in the control of liver regeneration and five other genes that encode signal transducers might modulate transcription. After using a matrix mating Y2H technique, a PPI map in which all the components are related with liver cell proliferation was constructed (Figure 4) and some of the interactions were validated by α-glutathione S-transferase pull-down and CoIP assays. From this PPI map, Gao *et al*[37] pointed out that ATF3, a member of the mammalian activation transcription factor/cAMP responsive element-binding protein family of transcription factors, interacts with FHL2 which may be an important interaction during liver regeneration, especially for liver cell proliferation. When it comes to the termination response during liver regeneration, FHL2 and ATF3 may form a complex which abolishes its function on DNA synthesis and might terminate the liver regeneration. Also, it is possible that FHL2 may interact with Stat3 to inhibit its function in activating downstream gene expression that is necessary to terminate the liver regeneration. Nearly all the interactions in this map are growth repressors during liver regeneration and this is one of the ways in which the termination of hepatocyte proliferation and liver regeneration is regulated. Although this is one hypothesis for termination of liver regeneration, there is still growing evidence which shows that it is feasible to understand liver regeneration.

This PPI map is only a small-scale map and already can make sense of termination of liver regeneration. It would be no exaggeration to say that if large-scale, more complicated PPI maps are constructed it will greatly help us to know more about mechanism of liver regeneration.

FUTURE PROSPECTS

During the last decade, several efforts had been made to demonstrate the mechanism of liver regeneration. But unfortunately, an important gap is the lack of understanding of liver regeneration and therefore, some difficulties appear in liver cancer treatments or liver transplantation and drug development, which remain to be

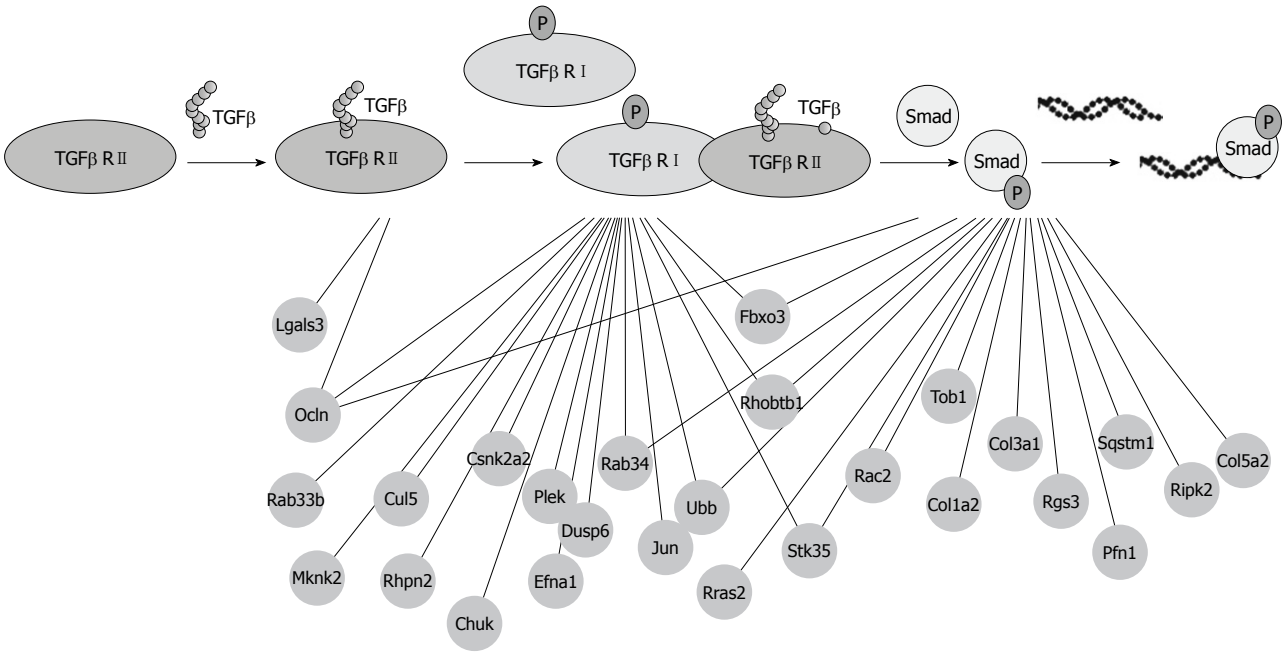

Figure 3 A protein-protein interaction comprising the transforming growth factor β signaling pathway. This figure just lists the protein-protein interactions which correlated with transforming growth factor β type Ⅰ receptor (TGFβ R Ⅰ), TGFβ R Ⅱ and Smads and all the proteins that directly interact with these three proteins which indicate that additional partners are not represented in this figure.

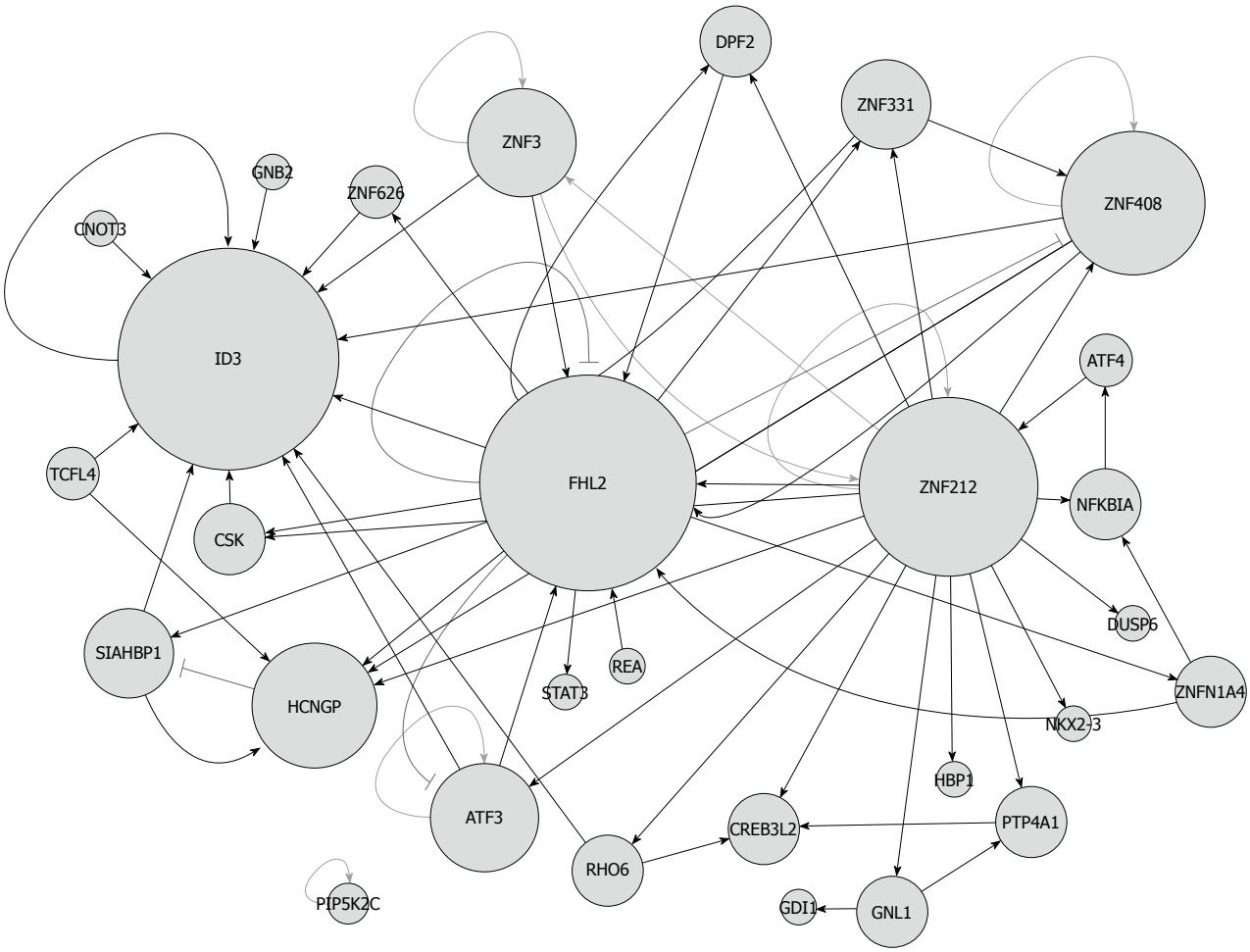

Figure 4 Protein-protein interaction maps comprising the transforming growth factor β signaling pathway of transcription factors associated with liver cell proliferation. The protein-protein interaction maps consist of different stages of liver regeneration (0 d, 0.5 d, 1.5 d, 4.5 d and 7 d after CCl_4-induced liver injury).

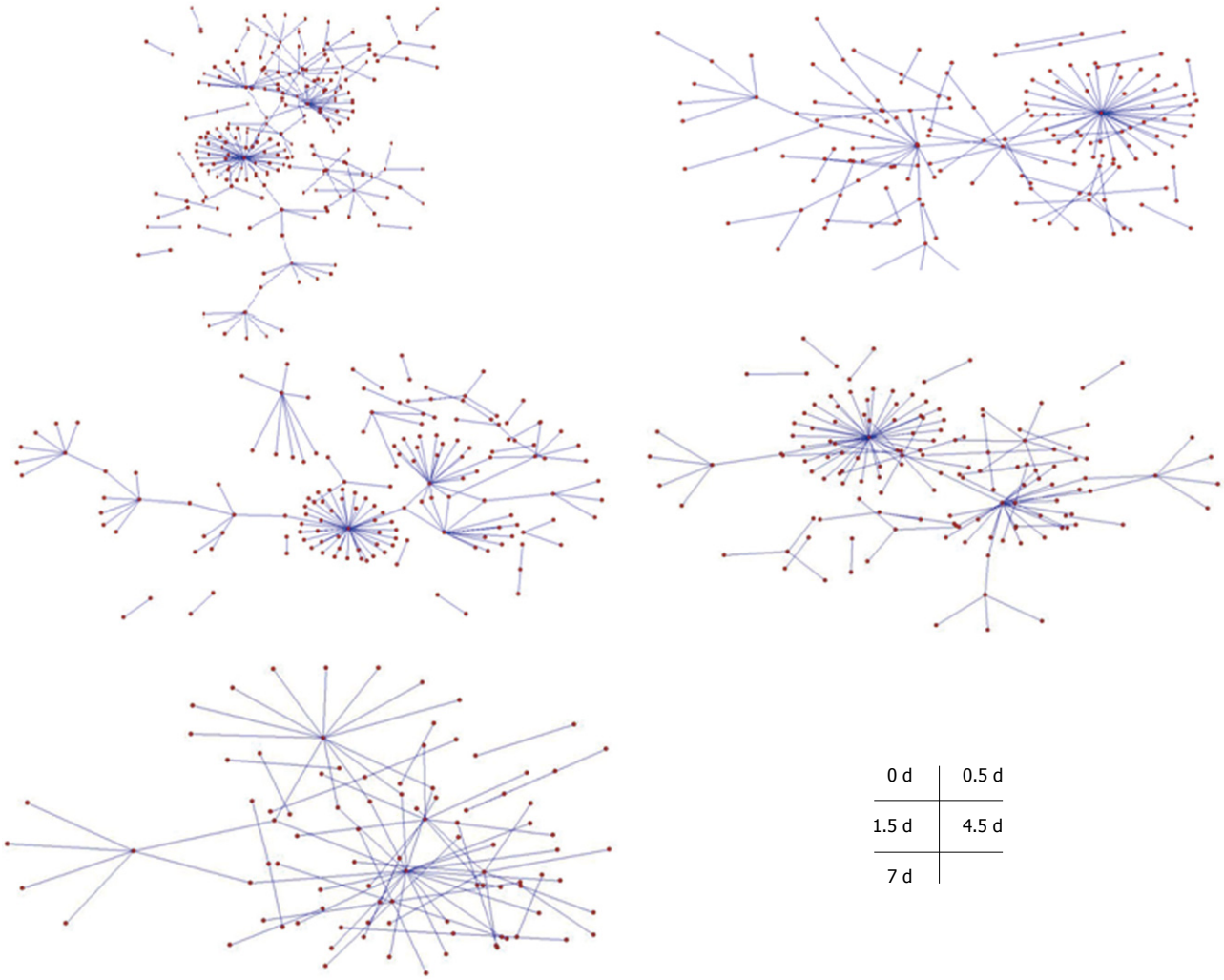

Figure 5 A protein-protein interaction map of transcription factors associated with liver cell proliferation. Node stands for protein and the edge means the two proteins are interactive; the node size symbolizes the degree of this protein (the more highly connected with others, the bigger node); the edge target arrow shape and color: delta and blue, interactions are only validated by yeast two-hybrid (Y2H); diamond and green means validated by Y2H and α-glutathione S-transferase pull-down; T and black means validated by Y2H and co-immunoprecipitation.

solved. Constructing PPI maps is a powerful step toward addressing these challenges. The most important profiles shall be discussed.

Integration of PPI data druing liver regeneration

Different high-throughput PPI data are difficult to cover even if in the same species, also, it has a higher rate of false positives than that of small-scale data[44]. However, it may be useful to increase the capacity of false positive identification in order to find out the real PPI from the noise data. The first step is to collect the correct and reliable PPI data and find several criteria which can be used to evaluate the PPI data sets. By integrating small-scale and large-scale PPI data, PPI databases have emerged and it is generally believed that a PPI database is a symbol of the level of PPIs. Although a series of PPI databases such as BIND (http://bind.ca), MIPS (http://mips.gsf.de) and DIP (http://dip.doe-mbi.ucla.edu) are popular and helpful, there are still no PPI databases for liver cells, liver regeneration or specific liver diseases, such as liver cancer. PPI data should be collected, evaluated, retrieved and systemically stored (including the detailed information for PPIs).

With liver regeneration PPI data which is integrated into the databases and is made readily accessible through the internet, researchers will be able to quickly locate the PPIs for their proteins of interest during liver regeneration, also, they will get the interpretation of PPIs in detail, which means any type of available information on proteins or protein domains can be verified. Meanwhile, we should also explore a tool that allows easy navigation in this complex of PPI databases especially for liver regeneration.

Already, the Y2H system has increasingly been applied in high-throughput applications intended to map genome-scale PPI for liver regeneration and is definitely believed to be an effective way to construct large-scale PPI maps during liver regeneration. Also, the low coverage and experimental bias call for development of computational methods to predict PPIs[45], and mining of existing inter-

action data to infer additional interactions is also a trend to enlarge the PPI database. Nowadays, lots of PPI data are obtained from different organisms and we can get PPI from interacting proteins to exhibit similar phylogenetic trees[46]. As to PPIs during liver regeneration, much more computational methods and algorithms must be fixed in order to predict PPIs during liver regeneration, also it may from signaling pathways level or proteome level.

Static and dynamic architecture of PPI maps during liver regeneration

Static PPI maps during liver regeneration, especially the PPI maps on signaling pathways, will help us to understand the different phases and different gene changes of liver regeneration. In order to understand the mechanism of liver regeneration, the PPI map furthered the understanding of the architecture of cellular machinery and revealed fundamental properties[47]. The large-scale and static PPI maps show us some information and they are quite important to understand the spatiotemporal existence of PPIs. Obviously, PPI maps are dynamic and not static, but unfortunately nearly all the PPI maps are static and do not consider the PPI strength and spatiotemporal existence let alone the types of PPI maps and do not reflect the actual situation in liver cells. Therefore, dynamic PPI maps are of more importance for cell signaling and dictate timing and intensity of map outputs. Our lab has also tried to construct dynamic PPI maps during mouse liver regeneration.

We picked up 5 major time points (0 d, 0.5 d, 1.5 d, 4.5 d and 7 d) during the mouse liver regeneration process after CCl_4-induced liver injury, in which all the proteins are transcription factors associated with TGFβ signaling pathway and constructed 5 PPI maps (Figure 5). It is easy to identify the PPIs change in the whole process of liver regeneration including numbers and the protein category. The numbers of PPIs increased at the beginning and then decreased. A detailed analysis of these PPI maps are in progress, which is a mark of the beginning of dynamic PPI maps construction.

Though no large-scale data sets are yet available on liver PPI map dynamics, the time dimension can be added by projecting time series of liver regeneration mRNA expression data onto transcription factors, allowing one method to interpret dynamic PPI maps. Researchers also need to consider addressing where and when interactions take place in different phases of liver regeneration and how they regulate the process. It will be a great help for us to know about liver regeneration if we know the full range of PPIs, from static to dynamic, and this is a useful method for studying liver regeneration over the next few years which will hopefully improve our ability to understand the PPI maps during liver regeneration.

Liver regeneration remains a fascinating project and the exact cellular and molecular mechanisms are still a mystery to us. In order to understand this phenomenon, many methods must integrate and we are just beginning to appreciate the relationship between PPIs and liver regeneration. We strongly believe that PPI maps from systems biology is a key gateway for deciphering liver regeneration. Certainly, like the sequencing of the human genome, the construction of a PPI map during liver regeneration will represent a major step along the path towards understanding the mechanisms of liver regeneration.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3499

World J Gastroenterol 2010 July 28; 16(28): 3499-3509
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Hypoxia inducible factor-1α accumulation in steatotic liver preservation: Role of nitric oxide

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Supported by The Ministerio de de Sanidad y Consumo (PI 081988), CIBER-EHD, Instituto Carlos III, Madrid and Ministerio de Asuntos Exteriores y de Cooperación Internacionales (A/020255/08 and A/02987/09), Madrid
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Received: January 14, 2010 Revised: February 16, 2010
Accepted: February 23, 2010
Published online: July 28, 2010

Abstract

AIM: To examine the relevance of hypoxia inducible factor (HIF-1) and nitric oxide (NO) on the preservation of fatty liver against cold ischemia-reperfusion injury (IRI).

METHODS: We used an isolated perfused rat liver model and we evaluated HIF-1α in steatotic and non-steatotic livers preserved for 24 h at 4℃ in University of Wisconsin and IGL-1 solutions, and then subjected to 2 h of normothermic reperfusion. After normoxic reperfusion, liver enzymes, bile production, bromosulfophthalein clearance, as well as HIF-1α and NO [endothelial NO synthase (eNOS) activity and nitrites/nitrates] were also measured. Other factors associated with the higher susceptibility of steatotic livers to IRI, such as mitochondrial damage and vascular resistance were evaluated.

RESULTS: A significant increase in HIF-1α was found in steatotic and non-steatotic livers preserved in IGL-1 after cold storage. Livers preserved in IGL-1 showed a significant attenuation of liver injury and improvement in liver function parameters. These benefits were enhanced by the addition of trimetazidine (an anti-ischemic drug), which induces NO and eNOS activation, to IGL-1 solution. In normoxic reperfusion, the presence of NO favors HIF-1α accumulation, promoting also the activation of other cytoprotective genes, such as heme-oxygenase-1.

CONCLUSION: We found evidence for the role of the HIF-1α/NO system in fatty liver preservation, especially when IGL-1 solution is used.

Key words: Fatty liver; Tissue preservation; Hypoxia inducible factor-1α; IGL-1; Nitric oxide; Trimetazidine

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Zaouali MA, Ben Mosbah I, Boncompagni E, Ben Abdennebi H, Mitjavila MT, Bartrons R, Freitas I, Rimola A, Roselló-Catafau J. Hypoxia inducible factor-1α accumulation in steatotic liver preservation: Role of nitric oxide. *World J Gastroenterol* 2010; 16(28): 3499-3509 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3499.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3499

INTRODUCTION

During the last decade, the dramatic shortage of organs has obliged physicians to consider the transplantation of liver grafts with moderate steatosis[1-3]. Liver steatosis results from the abnormal accumulation of fat in the cytoplasm of hepatocytes. This causes alterations in the microcirculation with a higher risk of graft dysfunction or primary nonfunction. Steatotic liver grafts thus show poor tolerance to ischemia-reperfusion injury (IRI) associated with transplantation[3-5]. The composition of preservation solutions is critical for the quality of liver grafts during cold ischemia. Although University Wisconsin solution (UW) is the gold standard for abdominal organs, its use has been questioned because it contains hydroxyl-ethyl starch (HES), and a high concentration of K^+ ions[6-9]. It is has been reported that HES could be responsible for red blood cell aggregation[10-12].

Recently, IGL-1 solution has been proposed as an effective alternative to UW for steatotic liver preservation[12]. It has a lower concentration of K^+ ions and contains polyethylene glycol as osmotic support instead of HES. The benefits of IGL-1 are due in part to its capacity to increase the levels of nitric oxide (NO)[12], which protects the liver against IRI[13], and thus mitigates the alterations to hepatic microcirculation[12].

Cold ischemia graft preservation is characterized by a lack of oxygen supply to the liver. In these conditions, hypoxia inducible factor 1 (HIF-1) regulates the adaptive response of the organ to the changes in oxygenation induced during preservation[14]. HIF-1 is a heterodimer formed by α and β subunits which are constitutively expressed. The β subunit is independent of O_2, whereas the protein stability of the α subunit depends on the cellular levels of O_2[15]. Under normoxic conditions, the α subunit is degraded by a complex process involving the prolyl-hydroxylases, the Von Hippel Landau protein and the 26 S proteasomes[15,16]. It is well established that NO impairs normoxic degradation of HIF-1α by inhibition of HIF-1 prolyl-hydroxylases and contributes to its stabilization[17].

HIF-1α confers protection against IRI by activating others genes such as heme oxygenase-1 (HO-1)[18-20] which plays an important cytoprotective role in liver graft preservation against cold IRI[21,22].

Recently, it has been demonstrated that anti ischemic drugs such as trimetazidine (TMZ) protect heart mesenchymal stem cells against hypoxic insult by increasing HIF-1α expression[23]. Similar results were reported by Jayle *et al*[14] in TMZ-treated pig kidneys subjected to warm IRI. In addition, the benefits of TMZ seem to be associated with its capacity to generate NO[24] and the activation of endothelial NO synthase (eNOS) when it was used as an additive in UW solution[25].

As HIF-1α is involved in cell survival during IRI, we examined how hypoxic adaptations can be exploited to protect fatty livers in preservation solutions. We hypothesized that the HIF-1α accumulation during cold ischemia would be hepato-protective in normoxic reperfusion as a result of the NO-stabilizing action induced by IGL-1.

To test this hypothesis, we added TMZ to IGL-1 solution, and examined the effects of various protocols on the preservation of livers excised from rats. We found that the addition of TMZ to IGL-1 solution has a synergistic effect on NO generation and thus favors HIF-1α accumulation during normothermic reperfusion. Preserved HIF-1α levels contribute to the increase in the over-expression of HO-1 in fatty liver grafts.

The results reported here reveal the importance of the HIF-1α/NO system in the prevention of liver cold IRI, especially when moderate steatosis is present.

MATERIALS AND METHODS

Homozygous obese (Ob) Zucker rats and heterozygous lean (Ln) Zucker rats (reference group), aged 16-18 wk, were obtained from Iffa-Credo (L'Abresle, France)[12,25].

An isolated perfused rat liver model was used to evaluate hepatic function separate from the influence of other organ systems, undefined plasma constituents, and neural/hormonal effects. Hepatic architecture, microcirculation, and bile production are preserved in this experimental model[26,27]. All procedures were performed under isoflurane inhalation anesthesia. This study adhered to European Union regulations (Directive 86/609/CEE) for animal experiments.

Liver procurement and experimental groups

The surgical technique was performed as described elsewhere[12,28]. After cannulation of the common bile duct, the portal vein was isolated and the splenic and gastroduodenal veins were ligated. All animals were randomly distributed into groups as described below.

Protocol Ⅰ: induction of HIF-1α in steatotic livers after 24 h of cold ischemia in IGL-1 preservation solution: In order to evaluate the potential generation of HIF-1α during liver graft storage (24 h, 4℃) in IGL-1 preservation solution, and the benefits of the addition of TMZ, the following experiments were carried out: (1) Cont 1: Control livers from 16 Zucker rats (8 Ln and

8 Ob) were flushed with Ringer's lactate solution immediately after laparatomy *via* the portal vein without cold storage; (2) UW: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in UW solution; (3) IGL-1: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in IGL-1 solution; and (4) IGL-1 + TMZ: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in IGL-1 with TMZ at a concentration of 10^{-6} mol/L, as previously described[25,28].

Aliquots of the effluent flush corresponding to groups 1, 2, 3 and 4 were sampled for measurements of cumulative transaminases. Liver tissue samples were used for HIF-1α activity determination after prolonged ischemia.

Protocol Ⅱ: effect of NO on HIF-1α stabilization and HO-1 induction after cold IRI: To examine the role of NO in stabilizing HIF-1α formed during cold ischemia, and the subsequent effect on HO-1 generation, fatty livers were subjected to 2 h-normoxic reperfusion, in the following groups: (5) Cont 2: Control livers from 16 Zucker rats (8 Ln and 8 Ob) were flushed with Ringer's lactate and immediately perfused *ex vivo* without ischemic preservation; (6) UW: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in UW solution and then inserted into an isolated perfused rat liver system for 2 h; (7) IGL-1: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved in IGL-1 solution and then inserted into an isolated perfused rat liver system for 2 h; (8) IGL-1 + TMZ: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in IGL-1 with the addition of 10^{-6} mol/L TMZ and then inserted into an isolated perfused rat liver system for 2 h[25,28]; and (9) IGL-1 + TMZ + NAME: Livers from 16 Zucker rats (8 Ln and 8 Ob) were preserved for 24 h in IGL-1 with the addition of 10^{-6} mol/L TMZ and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of NO synthesis, at a dose of 60 μg/g liver before cold ischemia, and then inserted into an isolated perfused rat liver system for 2 h[28].

In order to account for the period of rewarming during surgical implantation *in vivo*[28], after 24 h of cold preservation livers from groups 5, 6, 7, 8 and 9 (Protocol Ⅱ) were exposed to 22℃ for 30 min prior to reperfusion. Livers were then connected *via* the portal vein to a recirculating perfusion system for 120 min at 37℃[12,25]. Time 0 was the point at which the portal catheter was connected to the circuit. As previously reported[12,28], during the first 15 min of perfusion (initial equilibration period), the flow was progressively increased in order to stabilize the portal pressure at 12 mmHg (Pression Monitor BP-1; Pression Instruments, Sarasota, FL, USA). The flow was controlled using a peristaltic pump (Minipuls 3, Gilson, France)[12,25]. The reperfusion liquid consisted of a cell culture medium (William's medium E; Bio Whitaker, Barcelona, Spain) with a Krebs-Heinseleit-like electrolyte composition enriched with 5% albumin osmotic support. The buffer was continuously ventilated with 95% O_2 and 5% CO_2 gas mixture. The buffer was subsequently passed through a heat exchanger (37℃) and a bubble trap prior to entering the liver[25]. During 120 min of normothermic reperfusion, the effluent was collected at 30-min intervals to measure liver transaminases. After the initial equilibration period of 15 min, flow rate and vascular resistance were assessed continuously throughout the reperfusion. Bile output, hepatic clearance [expressed as percentage of sulfobromophthalein (BSP) in bile samples], HIF-1α activity, and NOS proteins were evaluated at 120 min of reperfusion.

Biochemical determinations

Liver injury: Hepatic injury was evaluated according to transaminase levels using a commercial kit from Boehringer Mannheim (Munich, Germany).

Liver function: Liver function was assessed by measuring bile production[12,25]. Bile was collected through the cannulated bile duct and output is reported as μL/g liver.

Hepatic clearance: As with bile output, hepatic clearance was considered as another parameter of hepatic function[27,28]. Thirty minutes after the onset of the perfusion (t_{30}), 1 mg of BSP (Sigma, Spain) was added to the perfusate. The concentration of BSP in bile samples (t_{120}) was measured at 580 nm with a UV-visible spectrometer. Bile BSP excretion was expressed as a percentage of perfusate content (t_{120} bile/t_{30} perfusate × 100)[28,29].

Vascular resistance: Liver circulation was assessed by measuring perfusion flow rate and vascular resistance[26,28]. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min × g. Vascular resistance was defined as the ratio of portal venous pressure to flow rate and expressed in mmHg × min × g/mL[26,28].

Glutamate dehydrogenase activity: Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme and was used as an indirect measure of mitochondrial damage[25,28]. GLDH activity was determined in perfusates as described elsewhere[25,28].

Determination of nitrite and nitrate: NO production in liver was determined by tissue accumulation of nitrite and nitrate, as previously reported[25,28].

eNOS immunohistochemistry: Frozen liver sections (18 μm thick) were fixed in pre-cooled acetone for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), permeabilization was performed with 0.5% Triton X-100 for 15 min each. Antibody non-specific binding was blocked for 1 h in PBS with 10% normal goat serum, 3% bovine serum albumin, 1.5% NaCl and 0.5% Triton X-100 for 1 h. The primary eNOS antibody (Polyclonal IgG anti-NOS-3, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied at a dilution of 1:100 in the blocking solution and incubated for 1 h at room

temperature in a moist chamber. After rinsing with PBS, permeabilization was performed again with 0.5% Triton X-100, 3 times for 10 min each. The secondary antibody goat anti-rabbit Alexa488 Fluor labeled (Invitrogen) was applied at a dilution of 1:400 in the blocking solution for 50 min at room temperature. After washing with PBS, the slides were mounted with ProLong Gold antifade (with DAPI) mounting medium, and eNOS immunohistochemistry images were obtained by fluorescence microscope.

Western blottings of eNOS and HO-1

Liver tissue was homogenized as previously described[28] and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were immunoblotted with antibodies against anti-eNOS (transduction laboratories, Lexington, KY, USA), against anti-HO-1 (Sigma Chemical, St Louis, MO, USA) and β-actin (Sigma Chemical, St. Louis, MO, USA). Signals were detected by enhanced chemiluminescence and quantified by scanning densitometry.

Preparation of nuclear fraction: Nuclear fractions were prepared from frozen liver tissues which were suspended in a hypotonic buffer that contained 10 mmol/L HEPES (pH 7.6), 15 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.2% Nonidet P-40 with protease inhibitor (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin) and homogenized for 40 s with a polytron homogenizer. After centrifugation at 850 *g* the supernatant that contained cytoplasmic and membrane protein was collected. Nuclear proteins were extracted at 4℃ by gently resuspending the nuclear pellet in hypertonic buffer that contained 25 mmol/L HEPES (pH 7.8), 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, 0.4 mol/L NaCl and protease inhibitor, followed by 30 min incubation at 4℃ with occasional vortexing. After centrifugation at 18 000 *g* for 15 min at 4℃, the supernatant that contained nuclear protein was collected and protein concentration was measured by the Bradford Protein method (Bio-Rad, Hercules, CA, USA).

HIF-1α measurement: HIF-1α translocation to nuclei was assessed by Western blotting and was also quantified by specific binding of HIF-1α to its specific oligonucleotide recognition hypoxia responsive element, using the Trans AM HIF-1α kit (Active Motiv, Carlsbad, CA, USA). Membranes were incubated with an antibody against anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were detected by enhanced chemiluminescence and quantified by scanning densitometry. For the quantitative method, samples were run in duplicate in microtiter plates coated with an oligonucleotide for HIF-1α following manufacturer's instructions. Results are expressed as μg HIF-1α/mg protein of nuclei extract[30,31].

Statistical analysis

Data are expressed as mean ± SE, and were compared statistically by variance analysis, followed by the Student-Newman-Keuls test (Graph Pad Prism software). $P < 0.05$ was considered significant.

RESULTS

Induction of HIF-1α in non-steatotic and steatotic livers after 24 h of cold ischemia

As indicated in Figure 1A and B, cumulative HIF-1α levels were determined in non-steatotic and steatotic livers preserved in IGL-1 + TMZ solutions and compared to levels observed in IGL-1 and UW alone. HIF-1α accumulation was significantly increased by the addition of TMZ to IGL-1 solution in both types of liver. Although steatotic livers showed slightly lower HIF-1α levels than non-steatotic livers, these differences were not significant (Figure 1A and B).

Liver ischemic damage was assessed by alanine transaminase (ALT) and aspartate transaminase (AST) levels after 24 h of cold storage as shown in Figure 1C and D. A significant reduction in release of ALT and AST occurred in non-steatotic and steatotic livers preserved in IGL-1 + TMZ when compared to IGL-1 and UW, respectively. This reduction correlated with the significant accumulation of HIF-1α observed for the IGL-1 + TMZ and IGL-1 solutions (Figure 1A and B).

Stabilization of HIF-1α in non-steatotic and steatotic livers after cold ischemia-reperfusion: role of NO

In order to evaluate whether HIF-1α could be stabilized by NO after hepatic reperfusion, we measured HIF-1α accumulation in steatotic and non-steatotic livers preserved in UW, IGL-1 and IGL-1 + TMZ solutions (24 h, 4℃) and then subjected to 2 h normoxic reperfusion (37℃). NO generation was inhibited by the addition of L-NAME to IGL-1 + TMZ solution (IGL-1 + TMZ + NAME).

Figure 2 shows the HIF-1α levels after 2 h of normothermic reperfusion in steatotic and non-steatotic livers preserved in IGL-1, IGL-1 + TMZ and UW solutions, respectively. HIF-1α levels were higher in the non-steatotic and steatotic livers stored in IGL-1 + TMZ solution than in those stored in IGL-1 and UW respectively. In all cases, the greatest increases were for the non-steatotic and steatotic livers preserved in IGL-1 enriched with TMZ (IGL + TMZ), and were significantly prevented by L-NAME (Figure 2A and B).

eNOS activity and nitrite/nitrate levels were higher in the steatotic and non-steatotic livers preserved in IGL-1 + TMZ when compared to UW and IGL-1 alone (Figure 2C, D and Figure 3). This enhanced eNOS activity and nitrite/nitrate levels were more evident when TMZ was added to IGL-1. In contrast, the addition of L-NAME to IGL-1 + TMZ solution reduced both.

In order to evaluate the benefits of HIF-1α and NO in livers preserved in IGL-1 and IGL-1 + TMZ after reperfusion, we evaluated liver injury (AST/ALT) and function (bile production, vascular resistance), as indicated in Figure 4.

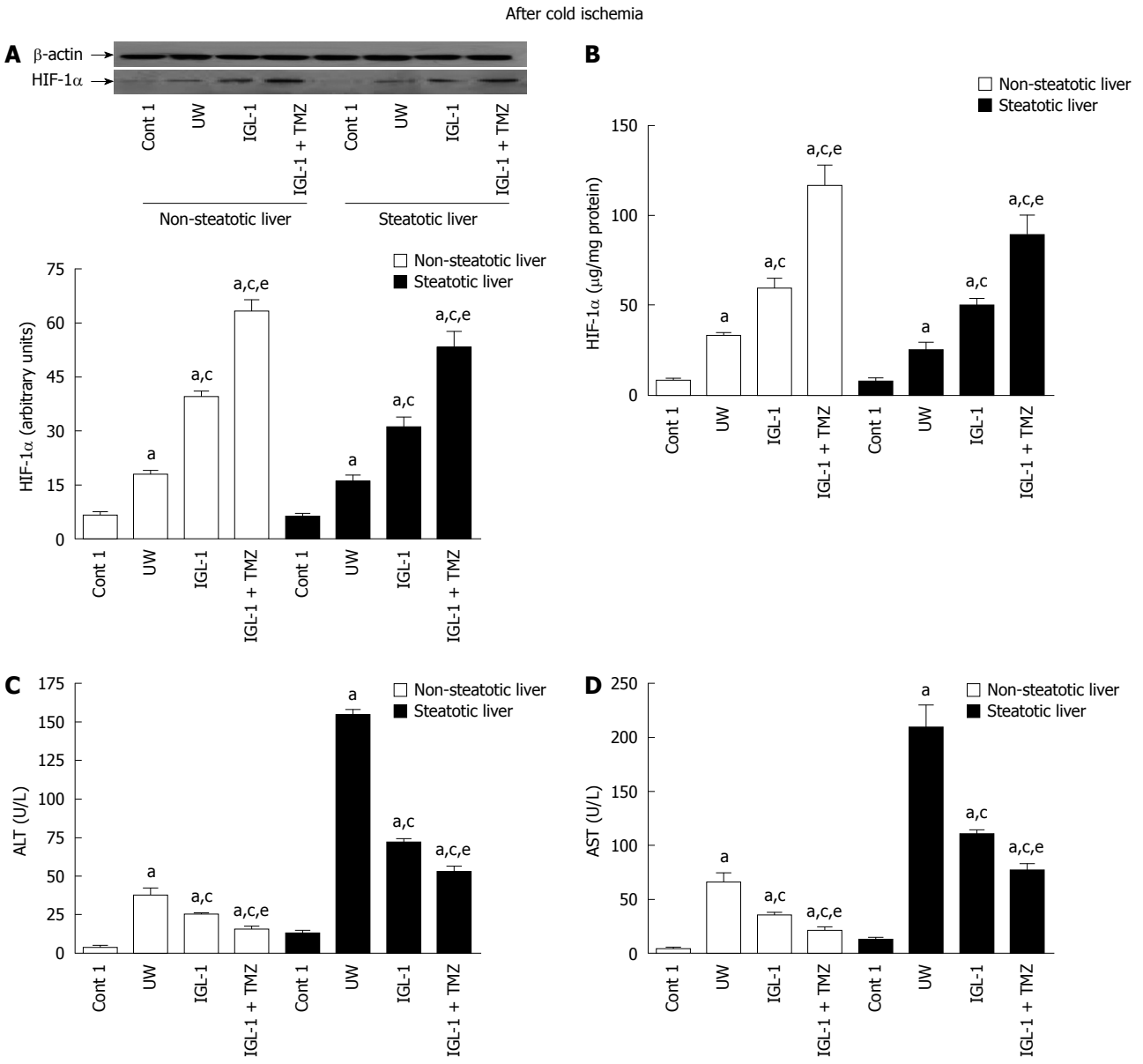

Figure 1 Hypoxia-inducible factor-1α protein levels in liver after 24 h of cold storage. A: Representative Western (top) and densitometric analysis (bottom); B: Hypoxia-induced factor-1α (HIF-1α) protein levels quantified by Trans AM HIF-1α kit; Alanine transaminase (ALT) (C) and aspartate transaminase (AST) (D) levels in flushing effluent after 24 h of cold storage. University Wisconsin (UW): Liver preserved in UW solution; IGL-1: Liver preserved in IGL-1 solution; IGL-1 + trimetazidine (TMZ) (trimetazidine): Liver preserved in IGL-1 solution with TMZ; Cont 1: Liver flushed without cold preservation. $^{a}P < 0.05$ *vs* Cont 1; $^{c}P < 0.05$ *vs* UW; $^{e}P < 0.05$ *vs* IGL-1.

At 2 h reperfusion release of ALT and AST was significantly lower in non-steatotic and steatotic livers preserved in IGL-1 + TMZ solution than in IGL-1 and UW solutions (Figure 4A and B). In contrast, the inhibition of NO by L-NAME showed significantly increased in AST/ALT release, in both kinds of liver. In all cases, the AST/ALT profiles were consistent with those obtained for HIF-1α accumulation during reperfusion (Figure 2).

TMZ also had beneficial effects on bile production and %BSP clearance in bile (Figure 4C and D). It also promoted a significant reduction in vascular resistance (Figure 4E), when compared to IGL-1 and UW solutions. The greatest reduction in vascular resistance occurred when TMZ was added to IGL-1. This finding is consistent with highest increases in eNOS activity and nitrite/nitrate levels as shown in Figures 2 and 3.

Mitochondrial damage was evaluated by measuring GLDH activity levels in perfusate at the end of the reperfusion period. Steatotic livers preserved in UW solution showed greater GLDH activity than did non-steatotic livers (Figure 5A). Livers stored in IGL-1 as well as in IGL-1 + TMZ solution reduced GLDH levels in both type of livers (Figure 5).

Finally, we examined the effect of HIF-1α accumulation during liver reperfusion on the induction of other cytoprotective agents, such as HO-1. HO-1 expression was exacerbated in both kinds of liver preserved in IGL-1 + TMZ solution when compared to IGL-1 and UW alone (Figure 5B). As with HIF-1α, HO-1 was inhibited by the presence of L-NAME in both livers. The HO-1 pattern profiles were similar to those obtained for HIF-1α (Figure 2).

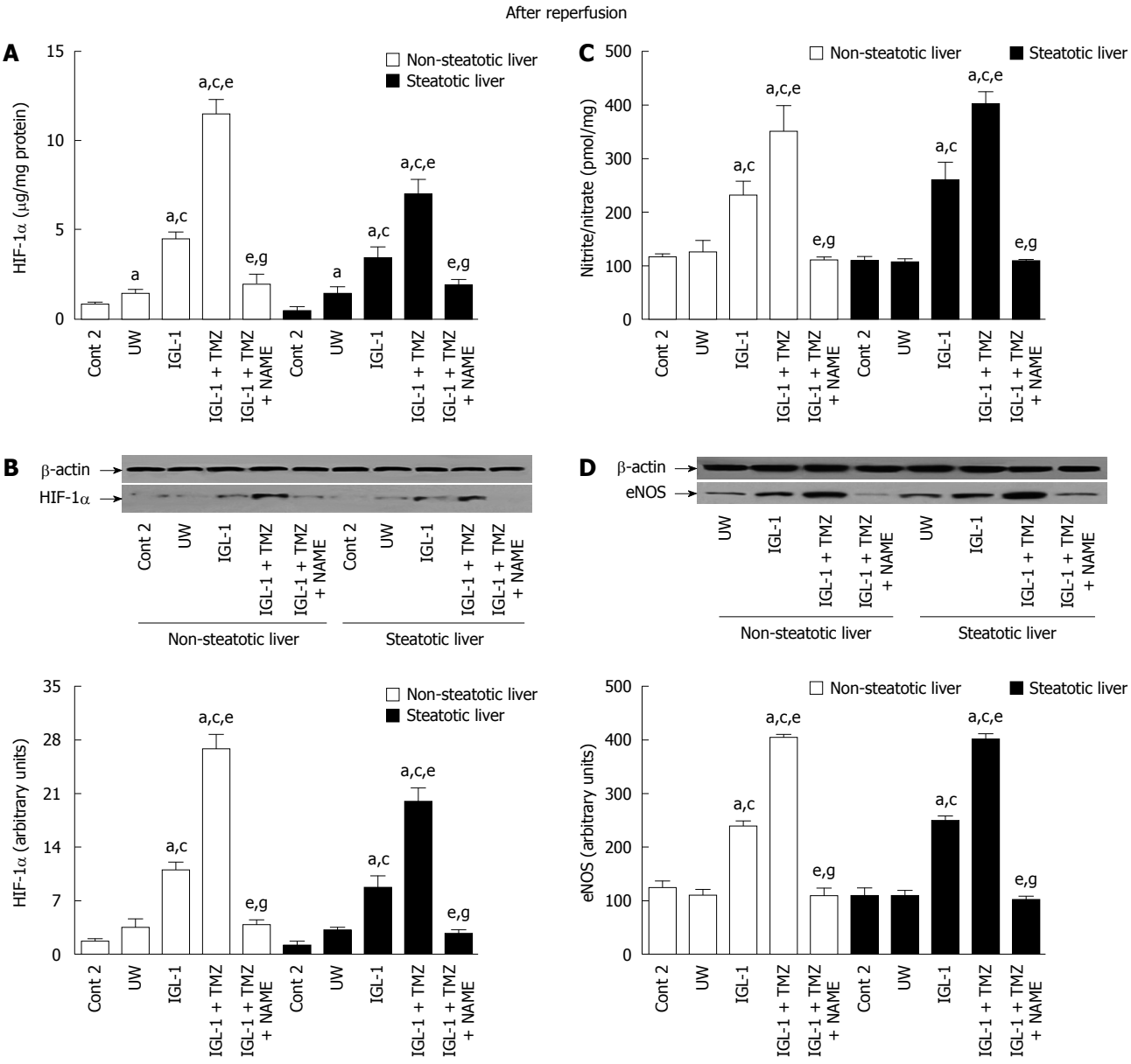

Figure 2 Hypoxia-inducible factor-1α, nitrite/nitrate and endothelial nitric oxide synthase. A: Hypoxia-induced factor-1α (HIF-1α) protein levels quantified by Trans AM HIF-1α kit after 24 h of cold storage and 120 min of normothermic reperfusion; B: Representative Western blotting of HIF-1α (top) and densitometric analysis (bottom) after 24 h of cold storage and 120 min of normothermic reperfusion; C: Nitrite/nitrate levels after 120 min of normothermic reperfusion; D: Endothelial nitric oxide synthase (eNOS) protein levels in liver after 120 min of normothermic reperfusion. Representative Western blotting (top) and densitometric analysis (bottom). Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; University Wisconsin (UW): Liver preserved in UW solution; IGL-1 + trimetazidine (TMZ) + NAME: Liver preserved in IGL-1 solution with TMZ and an nitric oxide synthesis inhibitor, L-NAME. $^{a}P < 0.05$ *vs* Cont 2; $^{c}P < 0.05$ *vs* UW; $^{e}P < 0.05$ *vs* IGL-1; $^{g}P < 0.05$ *vs* IGL-1 + TMZ.

DISCUSSION

HIF-1 is the main factor involved in the regulation of transcriptional responses to hypoxia[32,33]. HIF-1α levels accumulate and trigger an increase in expression of genes involved in glycolysis, glucose metabolism, mitochondrial function, cell survival and resistance to oxidative stress[20], in response to oxygen deprivation, and during organ cold storage. For this reason, we have explored new pharmacological strategies to promote the highest accumulation of cytoprotective factors such as HIF-1α to protect fatty livers against cold ischemia and reperfusion injury.

Here, we demonstrate for the first time that significant activation of HIF-1α occurs after 24 h of cold storage in both types of liver when TMZ is added to IGL-1 solution. This improves liver protection, as evidenced by the reduction in transaminase levels. This is of particular interest in clinical practice, since numerous pharmacological strategies that are effective in non-steatotic livers may not be useful in the presence of steatosis[34,35]. These results agree with the protective effects observed when TMZ was added to UW preservation solution[14,25].

Significant HIF-1α accumulation was also found after 2 h of liver reperfusion. This is consistent with the finding that mesenchymal stem cells pre-treated with TMZ were

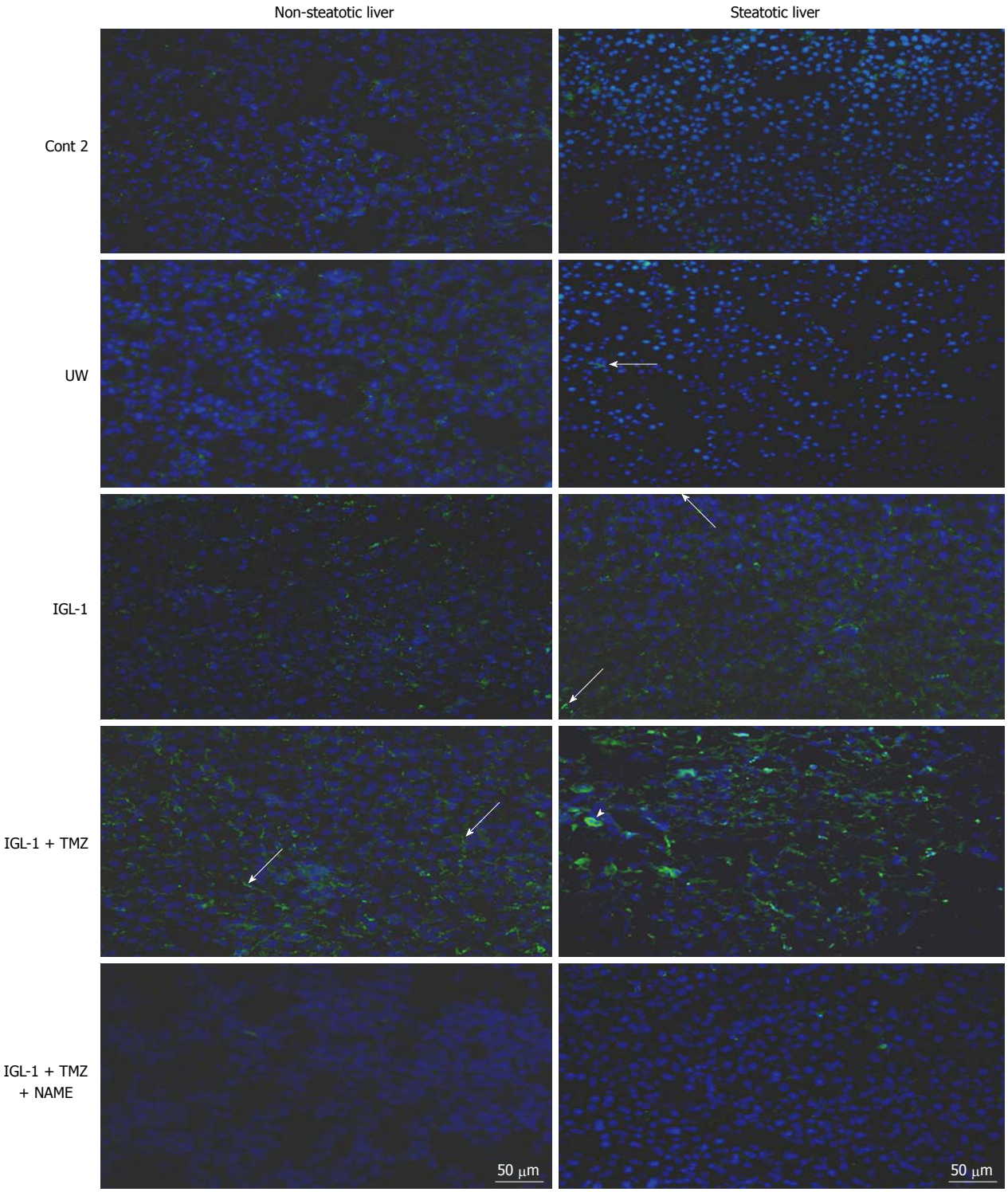

Figure 3 Endothelial nitric oxide synthase immunohistochemistry in steatotic and non-steatotic livers. Control livers and livers stored in University Wisconsin (UW) solution show only a little positivity for endothelial nitric oxide synthase (eNOS), localized typically in a few sinusoidal endothelial cells (arrows). The positivity increases when the livers are submitted to cold storage with IGL-1 solution and even more when trimetazidine (TMZ) is added to IGL-1 solution. When TMZ is added to IGL solution, some positive Kupffer cells (arrowhead) can be observed also. The addition of L-NAME to IGL-1 + TMZ solution reduced eNOS activity.

protected against H_2O_2 induced loss of cellular viability and membrane damage, with a concomitant increases in HIF-1α[23]. Similar observations were reported by Jayle *et al*[14] when pig kidneys were subjected to warm ischemia reperfusion.

Cumulative HIF-1α levels contributed to liver protection but they were abolished by the addition of L-NAME, an inhibitor of NO synthesis. This demonstrates by the first time in fatty liver preservation, the relevance of NO in HIF-1α accumulation during normoxic liver reperfusion.

NO is an intracellular and/or intercellular and dif-

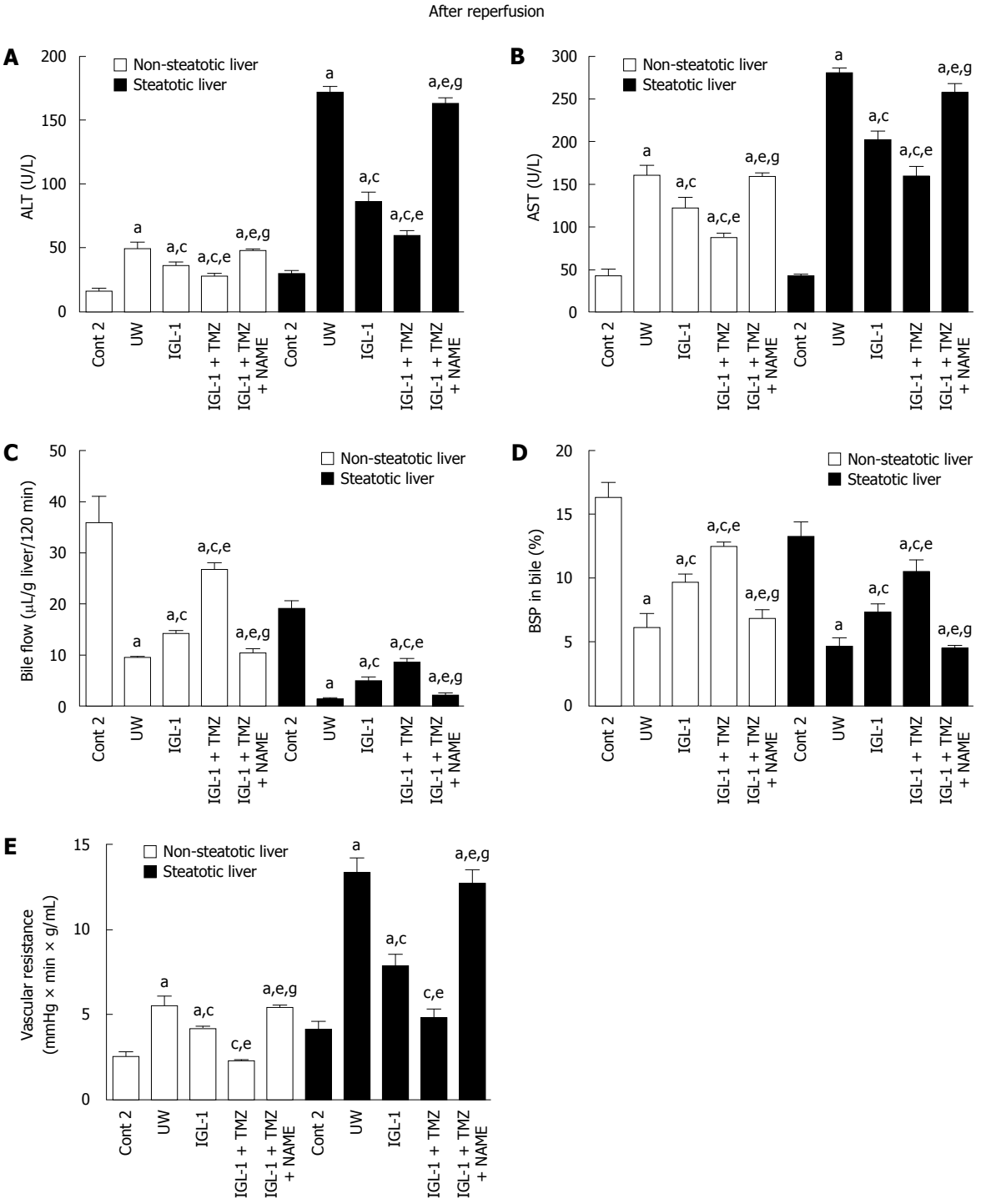

Figure 4 Alanine transaminase (A), Aspartate transaminase (B) levels in perfusate, bile flow (C), percentage of sulfobromophthalein in bile (D), and vascular resistance (E) of steatotic and non-steatotic livers after 120 min of normothermic reperfusion. [a]*P* < 0.05 *vs* Cont 2; [c]*P* < 0.05 *vs* University Wisconsin (UW); [e]*P* < 0.05 *vs* IGL-1; [g]*P* < 0.05 *vs* IGL-1 + trimetazidine (TMZ). BSP: Sulfobromophthalein; ALT: Alanine transaminase; AST: Aspartate transaminase.

fusible mediator whose role on hepatic IRI is controversial[28,36-38]. Overall, the important factors in determining the beneficial *vs* harmful effects of NO are the amount, duration, and site of NO production[39,40]. A low concentration eNOS-derived NO serves to maximize blood perfusion, promote cell survival and protect the liver against IRI[41]. However, a sustained presence of iNOS-derived NO might become detrimental by increasing toxic reactive oxygen species leading to liver injury[37,40-42]. The results reported here show that the NO benefits were derived mainly from eNOS and that they did not originate from iNOS activation (data not shown).

NO vasodilator properties prevent the alterations of microcirculation observed during liver reperfusion, which are exacerbated in the presence of steatosis[12,28]. The addition of NO donors to UW solution preserves livers against cold ischemic injury[13]. Thus, the benefits of the use of IGL-1 solution for fatty liver preservation are as-

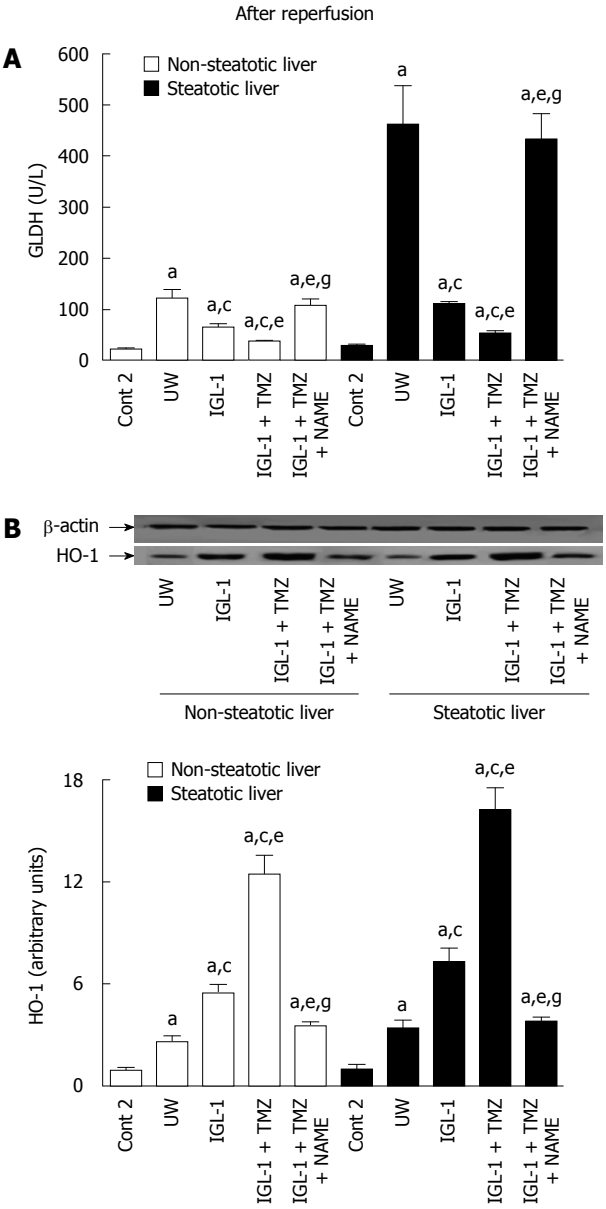

Figure 5 Glutamate dehydrogenase and heme oxygenase-1. A: Glutamate dehydrogenase (GLDH) levels after 120 min of reperfusion; B: Heme oxygenase-1 (HO-1) protein levels in liver after 120 min of normothermic reperfusion. Representative Western blotting at (top) and densitometric analysis at (bottom). [a]*P* < 0.05 *vs* Cont 2; [c]*P* < 0.05 *vs* University Wisconsin (UW); [e]*P* < 0.05 *vs* IGL-1; [g]*P* < 0.05 *vs* IGL-1 + trimetazidine (TMZ).

sociated with the capacity of generating NO[12]. However, the relevance of NO and it relationship with HIF-1α accumulation for increasing fatty liver graft protection against cold IRI is demonstrated here for the first time.

In recent years, there has been increasing interest in the relationship between HIF-1α and NO. NO profoundly affects the HIF-1α signaling pathway[43,44]. In normoxia, NO inhibits the prolyl-hydroxylases responsible for HIF-1α degradation, thus contributing to its stability[17].

Our results demonstrate that HIF-1α also accumulate during reperfusion in the presence of NO in liver grafts preserved in IGL-1 + TMZ or IGL-1 alone. Beneficial effects on HIF-1α accumulation are confirmed by the increased constitutive eNOS activity and nitrite/nitrate levels in liver grafts preserved in both solutions. In addition, these data confirm the synergism between IGL-1 solution and TMZ, responsible for the increased NO production by the activation of constitutive eNOS[24,28]. NO levels generated from the synergistic action of TMZ in IGL-1 solution are determinant of effective HIF-1α accumulation and subsequent protection of the liver graft against IRI. This differs from the effects observed when TMZ was added to UW, where the induction of eNOS activity was lower (data not shown).

Previous studies from our group showed that bile production is higher when livers are preserved in IGL-1than when they are preserved in UW[12]. Similarly, TMZ increased bile secretion when added to UW solution[25]. Our results show a synergistic action when TMZ is added to IGL-1 solution, which enhances the increase in bile production.

Fat accumulation in the cytoplasm of the hepatocytes is associated with an increase in cell volume, which may result in the partial or complete obstruction of the hepatic sinusoidal space[45,46]. Our results demonstrate that NO generated from the synergistic action mentioned above contributes to the reduction in vascular resistance. In fact NO inhibition by L-NAME increased vascular resistance, confirming the role of NO in fatty liver preservation.

Fatty degeneration, which induces a series of ultrastructural and biochemical alterations in mitochondria[47,48] may render these organelles intrinsically more susceptible to I/R injury. Mitochondrial injury is a common pathway of cell necrosis and apoptosis in IRI. Recent studies have shown that activation of HIF-1α prevents mitochondrial injury after mouse liver ischemia reperfusion[49]. Our results indicate that the HIF-1α accumulation after normoxic reperfusion prevented mitochondrial injury.

Finally, we measured the expression of HO-1 after 2 h of normothermic reperfusion, as a potential gene target induced by HIF-1α activation[18,19]. HO-1 is activated during cellular stress: its over-expression is a requisite for increasing organ cytoprotection against IRI, especially when steatosis is present[20,21,45,50].

We confirmed increased HO-1 expression in livers preserved in IGL-1 and IGL-1 + TMZ solutions. This is in line with the better protection against reperfusion injury in livers stored in IGL-1 + TMZ solution than in IGL-1 or UW alone. The abolition of HIF-1α stabilization by NO inhibition with L-NAME reversed HO-1 levels and increased liver injury after reperfusion.

In conclusion, we report by the first time the importance of NO in fatty liver reperfusion in stabilizing HIF-1α which is generated during liver cold storage.HIF-1α accumulation determines, in part, the increase in HO-1, a cytoprotective factor which improves the outcome of grafts after transplantation.

ACKNOWLEDGMENTS

We are grateful to Robin Rycroft at the Language Advisory Service of the University of Barcelona for revising the English text.

COMMENTS

Background

Steatosis is a major risk against cold ischemia-reperfusion injury (IRI). Hypoxia inducible factor-1α (HIF-1α) is a cytoprotective factor generated in response to lack of oxygen, as occurs during cold storage of liver grafts in preservation solutions. IGL-1 solution has been proposed as an effective alternative to University Wisconsin for steatotic liver preservation. The benefits of IGL-1 solution are due in part to its capacity to increase the levels of nitric oxide (NO).

Research frontiers

NO impairs normoxic degradation of HIF-1α by inhibition of prolyl-hydroxylases and contributes to its stabilization. HIF-1α confers protection against IRI by activating genes such as heme oxygenase-1 which plays an important cytoprotective role in liver graft preservation against cold IRI. In this study, the authors focused on the importance of HIF-1α and NO in fatty liver preservation.

Innovations and breakthroughs

The authors provide evidence that the enrichment of IGL-1 solution with trimetazidine (an anti-ischemic drug) increases NO generation and prevents HIF-1α degradation during steatotic liver graft reperfusion.

Applications

The use of modified IGL-1 solutions should be a useful strategy for increasing steatotic liver graft preservation through HIF-1α accumulation in normothermic reperfusion.

Peer review

In essence, the paper demonstrates that the HIF/NO system is important in fatty liver preservation following ischemia reperfusion injury. The study is well designed and the interpretation of the data is adequate.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3510

World J Gastroenterol 2010 July 28; 16(28): 3510-3520
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Accuracy of ultrasound to identify chronic liver disease

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Received: March 24, 2010 Revised: April 21, 2010
Accepted: April 28, 2010
Published online: July 28, 2010

Abstract

AIM: To identify and assess studies reporting the diagnostic performance of ultrasound imaging for identifying chronic liver disease (CLD) in a high risk population.

METHODS: A search was performed to identify studies investigating the diagnostic accuracy of ultrasound imaging for CLD. Two authors independently used the quality assessment of diagnostic accuracy studies (QUADAS) checklist to assess the methodological quality of the selected studies. Inter-observer reliability of the QUADAS tool was assessed by measuring the degree of agreement (percent agreement, κ statistic) between the reviewers for each assessment prior to a consensus meeting. The characteristics of each study population, sensitivity and specificity results for the index tests, and results of any testing for observer agreement were extracted from the reports. Receiver Operator Characteristic plots were generated using Microsoft Excel 2003 software and used to graphically display the diagnostic performance data and to explore the relationships between the reported ultrasound techniques and study characteristics, and methodology quality.

RESULTS: Twenty-one studies published between 1991 and 2009 were retained for data extraction, analysis and assessment for methodological quality. Assessment of methodology quality was performed on the 21 selected studies by two independent reviewers (RA & KT) using the QUADAS assessment tool. Across all studies the mean number of responses within the QUADAS assessment tool was 10 (range 7-13) for "Yes", 1 (range 0-3) for "No" and 3 (range 0-6) for "unclear". Inter-rater agreement for assessment of methodology quality was significantly greater than chance when assessing for representative spectrum, clear selection criteria, appropriate delay between reference and index tests, adequate descriptions of the index and reference tests, reference and index test blinding, and if relevant clinical information was provided. Seven studies reported moderate to high observer agreement for ultrasound techniques. Studies which clearly reported blinding performed better than the other studies for diagnostic accuracy, and lower diagnostic accuracy was evident for populations with lower prevalence of disease. Assessment of the liver surface using ultrasound consistently had moderate diagnostic accuracy across studies which demonstrated good research methodology. Other techniques demonstrated variable or poor to fair diagnostic accuracy.

CONCLUSION: Ultrasound of the liver surface is a useful diagnostic tool in patients at risk of CLD when assessing whether they should undergo a liver biopsy.

Key words: Chronic liver disease; Liver surface; Systematic review; Ultrasonography

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Allan R, Thoirs K, Phillips M. Accuracy of ultrasound to identify chronic liver disease. *World J Gastroenterol* 2010; 16(28): 3510-3520 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3510.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3510

INTRODUCTION

Chronic liver disease (CLD) is a significant cause of morbidity and mortality in developed nations. It is commonly caused by viral hepatitis and alcohol abuse with significant contributions from metabolic disorders[1]. Accurate diagnostic testing for CLD to identify asymptomatic patients in a high risk population has become more important due to recent advances in management and treatment options that provide better patient outcomes if the diagnosis of fibrosis or cirrhosis can be made before cirrhosis becomes clinically apparent[2]. In some cases, liver fibrosis has been demonstrated to be reversible[3], a phenomenon that was previously not considered possible.

The standard method for determining, staging and grading CLD is liver biopsy[4]. The invasiveness of this method, and its associated morbidity and mortality has led to the emergence of less invasive methods which include medical imaging techniques (computed tomography, magnetic resonance imaging and ultrasound), serum markers (both direct and indirect markers of fibrosis) and transient elastography[2]. All of these techniques have the potential to reduce the number of biopsies performed in a high risk population.

Ultrasound can identify the manifestations of CLD such as liver fibrosis and cirrhosis which are characterized by the presence of vascularized fibrotic septa and regenerating nodules[1,5-7]. Ultrasound is an attractive diagnostic tool because it is readily available, inexpensive, well tolerated and is already extensively used in the diagnostic work-up of patients with CLD. The diagnostic accuracy of ultrasound needs to be established to inform clinicians of its role in patients at high risk of CLD.

The aim of the following systematic review was to identify and assess studies reporting the diagnostic performance of ultrasound imaging for identifying CLD in a high risk population.

MATERIALS AND METHODS

Search strategy

A search of electronic databases in November 2009 was performed by one author (RA) to identify studies reported in English, investigating the diagnostic accuracy of ultrasound imaging for CLD. MEDLINE, EMBASE, CINAHL and Science Citation Index databases were searched using the terms "chronic liver disease", "cirrhosis", "fibrosis", "liver biopsy". The truncated terms "sonograph*" and "ultraso*" were also used in the search for alternate terms used for ultrasound such as sonography, sonographic, ultrasonic, ultrasound and ultrasonography. A Boolean search strategy was employed for the above terms in the following form: (sonograph* OR ultraso*) AND (chronic liver disease OR cirrhosis OR fibrosis) AND liver biopsy. No search filters were used. "Pearling" of the reference lists of all selected studies was also performed.

Eligibility and study selection

One author (RA) determined the eligibility of studies for inclusion in this review. Inclusion and exclusion criteria were created to identify studies that were likely to conform to the highest level of evidence for studies of diagnostic tests using the National Health and Medical Research Council of the Australian Government Level Ⅱ criteria[8].

The inclusion and exclusion criteria for the systematic review are described in Table 1. Initially, abstracts of all identified studies were assessed to determine if the study met the inclusion and exclusion criteria. Studies were retained if they clearly met the inclusion criteria, did not meet the exclusion criteria, or if it was unclear from the abstract if the study met the exclusion and inclusion criteria. The full text reports of all retained studies were then re-assessed for inclusion. All studies clearly meeting any of the exclusion criteria were excluded, and all studies meeting all the inclusion criteria were retained for assessment of methodological quality, data extraction and analysis.

Assessment of methodological quality

Two authors (RA, KT) independently used the quality assessment of diagnostic accuracy studies (QUADAS)[9] checklist to assess the methodological quality of the selected studies. The QUADAS checklist (Table 2) contains 14 assessment items, each assessing an aspect of the study that impacts on methodological quality. Each author assessed the selected studies by rating each assessment item for each study as "yes", "no" or "unclear". The studies were not given an overall score, nor were they stratified into high or low quality groups. Inter-observer reliability of the QUADAS tool was assessed by measuring the degree of agreement (percent agreement, κ statistic) between the reviewers for each assessment prior to a consensus meeting. A consensus meeting was held to resolve any discrepant scores between the two assessors. A third independent assessor (MP) reviewed the discrepant scores and acted as a final adjudicator if a consensus could not be reached.

Data extraction

The characteristics of each study population were extracted from the reports and included country of origin, sample size, gender, aetiology, age (mean, range and SD),

Table 1 Inclusion and exclusion criteria for studies

Inclusion criteria	Exclusion criteria
Evaluated diagnostic accuracy	Did not evaluate diagnostic accuracy
Quantitative results of diagnostic performance presented in a format that enabled a 2 × 2 contingency table to be extracted OR results presented as sensitivity, specificity and prevalence	2 × 2 contingency table could not be extracted from results of diagnostic performance OR sensitivity, specificity and prevalence results not presented
Index test of study was an ultrasound imaging technique	Index test included was not an ultrasound imaging technique OR included a non-ultrasound imaging technique as part of the index test
Studies were conducted prospectively	Studies were not conducted prospectively
The reference test for all subjects in the study was liver biopsy	The reference test for the study was not liver biopsy OR liver biopsy was not used for all subjects
The sample population described were adults at risk of chronic liver disease	The sample population described included children OR sample population included adults not at risk of chronic liver disease
	The study was published as a case study, review or editorial

Table 2 Quality assessment of diagnostic accuracy studies assessment items

Item	Question	Guidelines for assessment	Aspect of study assessed
1	Was the spectrum of patients representative of the patients who will receive the test in practice?	Patients who receive the test in clinical practice will be suspected of having chronic liver disease but not yet have decompensated cirrhosis Sample populations should fit this general characteristic. Samples may be a mixed population or may be restricted to one disease type if this is a common and clinically important disease, in this case alcohol abusers or viral hepatitis Score "yes" if clearly stated and meet the above definitions, "no" if the spectrum is clearly outside this definition and "unclear" if there is insufficient information	Generalisability
2	Were selection criteria clearly described?	Clear definitions of the inclusion and exclusion criteria should be included. "Yes" if clearly stated, "no" if not stated and 'unclear" if only partially stated	Quality of reporting
3	Is the reference standard likely to correctly classify the target condition?	Liver biopsy must be used as the reference standard. "Yes" if biopsy used, "no" if not and "unclear' if not stated	Presence of bias
4	Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests?	The time period must be no more than one month for all cases to avoid discrepancies between the index and reference test due to disease progression. The order in which the tests are done is not relevant. Score "yes" if one month or less, "no" if more than one month and "unclear" if not clearly stated	Presence of bias
5	Did the whole sample or a random selection of the sample, receive verification using a reference standard?	All patients should receive a biopsy unless some form of randomisation was used. Score "no" if some patients were excluded. Score "unclear" if this information is not reported by the study	Presence of bias
6	Did patients receive the same reference standard regardless of the index test result?	If it is clear all patients received a liver biopsy, score "yes". If some received laparoscopy (or other test), score "no". If it is not stated, score "unclear"	Presence of bias
7	Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)?	Score 'yes" if the index test did not form part of the reference test, "no" if it did and "unclear" if not stated or there is doubt	Presence of bias
8	Was the execution of the index test described in sufficient detail to permit replication of the test?	Studies should describe equipment and techniques in sufficient detail to enable replication. Ultrasound criteria for identifying fibrosis or cirrhosis must be clearly stated and be able to be replicated (e.g. clear and easily reproducible system for assessing grey scale appearances or Doppler measurements or indices) Score "yes" if the above is true, "no" if these details are not stated or if the technique described is not able to be replicated and "unclear" if an incomplete description is given	Quality of reporting
9	Was the execution of the reference standard described in sufficient detail to permit its replication?	A clear description of the biopsy technique sufficient to enable replication. Ideally this should include information about the needle technique used and the minimum size of the sample. A recognised staging system for fibrosis or a description with sufficient detail to enable replication must be provided Score "yes" if the above are true, "no" if no description of technique is given OR no staging system used and "unclear" if a partial description is given from which conclusions cannot be reached	Quality of reporting
10	Were the index test results interpreted without knowledge of the results of the index test?	Score "yes" if the ultrasound was performed and reported without knowledge of the biopsy. Score "no" if this is not the case and "unclear" if it is not stated	Presence of bias
11	Were the reference standard results interpreted without knowledge of the results of the reference test?	Score "yes" if the biopsy was performed and reported without knowledge of the ultrasound. Score "no" if this is not the case and "unclear" if it is not stated	Presence of bias

12	Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?	Score "yes' if pre-test clinical data was available for the ultrasound and biopsy. Score "no" if it was not available. Score "unclear" if it is not stated	Presence of bias
13	Were uninterpretable/intermediate results reported?	Score "yes" if all test results, including uninterpretable or indeterminate results, are accounted for. Score "no" if some data is missing and not explained or has been excluded from analysis. Score "unclear" if it is not clear whether all results have been included	Quality of reporting
14	Were withdrawals from the study explained?	A flow chart or matching numbers in a 2 × 2 table can help assess this item If it is clear what happened to all participants, score "yes". If some patients are not accounted for, score "no". Score "unclear" if interpretation is difficult	Quality of reporting

Table 3 Inter-rater reliability for quality assessment of diagnostic accuracy studies items

QUADAS item	Agreement (%)	κ
Representative spectrum?	90	0.462[1]
Selection criteria clear?	81	0.632[1]
Appropriate reference standard?	100	1.000[1]
Appropriate delay between tests?	100	1.000[1]
Partial verification avoided?	95	-[2]
Differential verification avoided?	95	-[2]
Incorporation avoided?	100	1.000[1]
Adequate index test description?	86	0.468[1]
Adequate reference test description?	76	-[2]
Index test blinded?	86	0.704[1]
Reference test blinded?	95	0.901[1]
Relevant clinical information available?	86	0.712[1]
Uninterpretable results reported?	29	0.022
Withdrawals explained?	33	0.033

[1]Agreement significantly greater than chance ($P < 0.05$); [2]A κ statistic could not be calculated because one reviewer responded "yes" for all studies on this item. QUADAS: Quality assessment of diagnostic accuracy studies.

exclusion and inclusion criteria, severity of disease, prevalence, staging system of liver biopsy, and the ultrasound technique(s) used. Sensitivity and specificity results for the index tests were extracted from the reports or from constructed contingency tables. The results of any testing for observer agreement were also extracted.

Statistical analysis

Receiver Operator Characteristic (ROC) plots were generated using Microsoft Excel 2003 software and used to graphically display the diagnostic performance data and to explore the relationships between the reported ultrasound techniques and study characteristics[10]. To demonstrate any patterns and relationships between methodology quality and diagnostic quality, plots were also produced for items on the QUADAS checklist.

RESULTS

Search results

No previous systematic reviews addressing the diagnostic accuracy of ultrasound in liver fibrosis or cirrhosis were identified. A total of 1355 separate studies were revealed from the following databases: MEDLINE (n = 464), EMBASE (n = 1155), CINAHL (n = 18) and Science Citation Index searches (n = 639). Attrition of studies after an initial assessment of the abstracts against the inclusion and exclusion criteria resulted in a residual of 38 studies [MEDLINE (n = 33), EMBASE (n = 3), Science Citation Index (n = 2)]. An additional 8 studies were revealed after pearling of the residual 38 studies (n = 46). After assessment of the full text reports of these 46 studies against the selection criteria, there was further attrition of 25 studies resulting in a total of 21 studies retained for data extraction, analysis and assessment for methodological quality.

Methodology quality assessment results

Assessment of methodology quality was performed on the 21 selected studies by two independent reviewers (RA & KT) using the QUADAS assessment tool. Inter-rater agreement for each item, across all studies, was assessed by calculating the percentage agreement and kappa value (κ) (Table 3). For items where there was disagreement between the reviewers, consensus was achieved without the need for an independent adjudicator.

Across all studies the mean number of responses within the QUADAS assessment tool was 10 (range 7-13) for "Yes", 1 (range 0-3) for "No" and 3 (range 0-6) for "unclear".

Characteristics of study populations

The studies included in this review were published between 1991 and 2009. The characteristics of the study populations are reported in Table 4.

The method for staging the histology obtained at liver biopsy was either not reported or unclear in 5 studies, all of which were published prior to the year 2000. Across the other 16 studies a total of seven staging systems were used. METAVIR[11] (n = 7), Ishak[12] (n = 3), Desmet[13] (n = 2) and four other systems which were each used once[14-17].

Measurements of observer agreement

Seven studies reported observer agreement assessment of the ultrasound technique[18-24]. When reported, results for observer agreement were acceptable, with κ values ranging from 0.51-0.93, coefficient of variation values ranging from 2%-8%, and correlation coefficients ranging from 0.82-0.9.

Ultrasound techniques

Diagnostic accuracy was determined for a range of ultra-

Table 4 Characteristics of included studies

Author	Country	Sample	Males (%)	Mean age in years (range)	Prevalence of disease (%)	Aetiology (largest disease type)	Inclusion criteria	Exclusion criteria	Severity of disease
Joseph *et al*[17]	UK	50	NR	NR (NR)	62	Mixed (alcohol)	Abnormal LFT, clinical suspicion	NR	NR
Cioni *et al*[32]	Italy	117	77 (66)	47 (NR)	50	NR	Raised ALT	Decompensation, refused biopsy	Mild
Ladenheim *et al*[26]	USA	50	NR	NR (NR)	16	NR	NR	NR	NR
Ferral *et al*[35]	Mexico	70	28 (40)	49 (18-84)	46	Unclear	Abnormal LFT, non-specific clinically	Did not have biopsy (reasons not specified)	NR
Hultcrantz *et al*[28]	Sweden	83	47 (57)	41 (NR)	17	Mixed ("fatty" 54%)	Asymptomatic, raised AST/ALT	Signs of liver disease	Mild
Colli *et al*[29]	Italy	52	30 (58)	52 (22-65)	31	Viral	HCV, Child-Pugh class "A"	Decompensation, PHT	Mild
Gaiani *et al*[20]	Italy	212	128 (60)	49 (15-71)	22	Mixed (HCV 57%)	Raised AST, no prev. cirrhosis	Decompensation, PHT, previous history cirrhosis	Mild
Xu *et al*[22]	China	66	42 (64)	39 (NR)	36	Viral	HBV	NR	NR
Mathiesen *et al*[27]	Sweden	165	110 (67)	48 (22-77)	9	Mixed ("fatty" 40%)	Asymptomatic, raised AST/ALT	Decompensation	Mild
Colli *et al*[18]	Italy	300	234 (78)	49 (17-78)	36	Mixed (HCV 41%)	Asymptomatic, raised AST/ALT	Heart failure, atrial fibrillation	Mild
Nishiura *et al*[25]	Japan	103	60 (58)	51 (38-75)	21	Mixed (viral 88%)	Raised AST, no prev. cirrhosis	Decompensation, previous history cirrhosis	Mild
Colli *et al*[19]	Italy	176	96 (55)	54 (NR)	38	Viral	HCV, raised AST, Child-Pugh "A"	Decompensation, biopsy contra-indicated	Mild
Vigano *et al*[33]	Italy	108	55 (51)	53 (NR)	34	Viral	HCV	NR	NR
D'Onofrio *et al*[31]	Italy	105	73 (70)	47 (NR)	27	Viral	Asymptomatic viral hepatitis, raised AST/ALT	NR	Mild
Schneider *et al*[30]	Germany	119	66 (55)	45 (20-78)	14	Viral	HCV	NR	NR
Shen *et al*[16]	China	324	272 (84)	36 (18-60)	9	Viral	HCV,HBV, raised ALT	Decompensation, HIV, other causes of CLD	Mild
Liu *et al*[21]	Taiwan	503	271 (54)	52 (NR)	33	Viral	HCV	HBV, HIV, NASH, alcohol abuse, refused biopsy or contra-indicated	NR
Iliopoulos *et al*[23]	Greece	72	45 (63)	57 (NR)	39	Viral	Unclear	Unclear	NR
Paggi *et al*[24]	Italy	430	237 (55)	53 (25-71)	37	Viral	HCV	HBV, HIV, decompensation	Mild
Wang *et al*[39]	Taiwan	320	199 (62)	51 (NR)	33	Viral	HBV, HCV	HCC	NR
Gaia *et al*[34]	Italy	61	41 (67)	NR	36	Viral (62%)/ NASH (38%)	NR	NR	NR

LFT: Liver function test; NR: Not reported; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PHT: Portal hypertension; CLD: Chronic liver disease; HIV: Human immunodeficiency virus; NASH: Non-alcoholic steato-hepatitis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

sound techniques across all studies. There were 48 reports of diagnostic accuracy for specific ultrasound techniques within the 21 included studies. Thirty different ultrasound techniques were reported of which 23 were reported once. Seven techniques were reported multiple times. The ultrasound techniques could be broadly described according to four main categories: (1) low frequency grey scale imaging, where an assessment of the liver parenchyma, liver shape and size, spleen size and hepatic vessel appearance or calibre was made from an ultrasound examination using a low frequency (≤ 5 MHz) convex or sector transducer (n = 14 reports); (2) high frequency grey scale imaging, where the liver surface was assessed using a high frequency linear (> 5 MHz) array transducer (n = 8 reports); (3) Doppler techniques, where a Pulsed Wave (PW) Doppler study of the portal, hepatic and splenic veins and/or the hepatic artery was performed to determine measurements of maximum or mean velocities, ratios and/or indices of resistance and/or pulsatility, and/or subjective assessments of haemodynamic waveforms (n = 19 reports); and (4) Scoring system using a combination of techniques, where more than one technique and/or parameter described in categories 1-3 provided a quantitative or qualitative assessment (n = 7 reports).

The diagnostic accuracy of the ultrasound techniques by group are demonstrated in Table 5.

Statistical analysis

A ROC plot (Figure 1A) was generated for all 48 reports of diagnostic accuracy according to the predetermined broad group categories. One scoring system achieved perfect results[25], while one report of high frequency liver surface technique[26] indicated a performance no better than chance.

A ROC plot (Figure 1B) was generated for ultrasound techniques that were reported more than once.

Table 5 Diagnostic accuracy of all ultrasound techniques

Study	Specific technique	Sensitivity	Specificity
Low frequency grey scale techniques			
Schneider *et al*[30]	Spleen width	86.3	35.3
Schneider *et al*[30]	Spleen length	77.5	53.0
Joseph *et al*[17]	Liver parenchyma heterogeneity	77.0	89.0
Shen *et al*[16]	PV diameter	76.7	45.0
Iliopoulos *et al*[23]	Spleen volume	75.0	70.0
Shen *et al*[16]	Spleen length	60.0	75.0
Shen *et al*[16]	Splenic vein diameter	60.0	78.0
Hultcrantz *et al*[28]	Liver parenchyma echogenicity	43.0	42.0
Iliopoulos *et al*[23]	Liver parenchyma heterogeneity	43.0	77.0
Colli *et al*[18]	Caudate/Right lobe ratio	41.0	91.0
Mathiesen *et al*[27]	Liver parenchyma echogenicity	40.0	38.6
D'Onofrio *et al*[31]	Collateral vessels	39.0	84.0
D'Onofrio *et al*[31]	Caudate/Right lobe ratio	32.0	99.0
D'Onofrio *et al*[31]	Liver parenchyma heterogeneity	29.0	99.0
High frequency grey scale techniques			
Ferral *et al*[35]	Surface	87.5	81.6
Colli *et al*[19]	Surface	60.0	92.0
Colli *et al*[18]	Surface	54.0	95.0
D'Onofrio *et al*[31]	Surface	54.0	78.0
Vigano *et al*[11]	Surface	51.0	90.0
Ladenheim *et al*[12]	Surface	12.5	88.0
Gaia *et al*[34]	Surface	63.0	86.0
Paggi *et al*[24]	Surface	73.0	90.0
Doppler techniques			
Liu *et al*[21]	SA PI = 0.85	94.0	39
Liu *et al*[21]	SA PI = 1.20	88.0	82
Iliopoulos *et al*[23]	PV congestion index (PV cross-sectional area/PV Vtam)	86.0	66
Iliopoulos *et al*[23]	PV Diameter/PV Vmax	86.0	59.0
Iliopoulos *et al*[23]	PV Diameter/Vtam	86.0	68.0
Iliopoulos *et al*[23]	HA Vtam/PV Vtam	86.0	61.0
Iliopoulos *et al*[23]	PV Vmax	77.0	71.0
Schneider *et al*[30]	PV undulations	76.5	100.0
Colli *et al*[29]	HV pulsatility	75.0	78.0
Iliopoulos *et al*[23]	PV Vtam	75.0	71.0
Schneider *et al*[30]	PV Vmax	74.5	53.0
Iliopoulos *et al*[23]	HA RI	71.0	55.0
Cioni *et al*[32]	PV Vmax	66.0	98.0
Liu *et al*[21]	SA PI = 1.10	61.0	98.0
Iliopoulos *et al*[23]	PV blood flow (BF) (mL/min)	59.0	75.0
Colli *et al*[18]	HV pulsatility	57.0	76.0
Liu *et al*[21]	SA PI = 1.40	45.0	99.0
Iliopoulos *et al*[23]	Doppler perfusion index HA BF/(HA BF + PV BF)	43.0	91.0
Schneider *et al*[30]	HV pulsatility	31.4	47.1
Scoring systems			
Nishiura *et al*[25]	Sequential score (high and low frequency techniques)	100.0	100.0
Xu *et al*[22]	4 parameter score (low frequency techniques)	87.8	97.6
Gaiani *et al*[20]	Score of low frequency and PV Vtam	82.2	79.9
Gaiani *et al*[20]	Score of 5-7 techniques (low frequency and PV Vtam)	78.7	80.6
D'Onofrio *et al*[31]	Any of 4 techniques (low frequency and liver surface)	68.0	68.0
D'Onofrio *et al*[31]	All of 4 techniques (low frequency and liver surface)	25.0	100.0
Wang *et al*[39]	Score of 4 parameters (low frequency techniques)	74.0	86.0

PV: Portal vein; SA: Splenic artery; PI: Pulsatility index; Vtam: Time averaged mean velocity; Vmax: Maximum velocity; HA: Hepatic artery; HV: Hepatic vein; RI: Resistive artery; BF: Blood flow.

The ROC plots demonstrate that results for liver echogenicity were consistent but had poor diagnostic accuracy[27,28], results for hepatic vein pulsatility were highly variable[18,29,30], results for liver parenchyma[17,23,31], portal vein maximum velocity[23,30,32], and spleen size[16,23,30] were variable, results for caudate to right lobe ratio were consistent but fair in diagnostic accuracy, and results for liver surface consistently had moderate diagnostic accuracy[18,19,23,31,33,34] except for two outlying reports[26,35].

Reference test blinding (QUADAS item 11) was the only item of methodology quality which demonstrated an obvious trend when plotted on a ROC for diagnostic accuracy; most studies which clearly reported blinding performed better than the other studies (Figure 1C).

ROC plots of diagnostic accuracy across disease characteristics (histology staging definition, prevalence, disease aetiology and severity of disease) demonstrated no obvious patterns except that diagnostic accuracy was generally lower for populations with lower prevalence of disease (Figure 2).

DISCUSSION

The aim of this review was to assess the results and quality of studies reporting the diagnostic accuracy of ultrasound imaging techniques used to identify patients with CLD in a high risk population. The search was restricted to techniques that used ultrasound imaging techniques. Transient elastography, which has demonstrated good diagnostic performance[36] and is becoming more widely used in hepatology practice, was not included because it is a non-imaging technique and currently is not an option on standard ultrasound equipment. A review to establish the performance of stand alone ultrasound is useful because ultrasound scans are often provided by medical imaging departments that do not have access to elastography.

The search strategy was optimized for sensitivity rather than precision, as recommended by the Cochrane Collaboration[37] with no filters used which could potentially restrict the search. Efforts to identify as many relevant studies as possible included expanding the search to databases beyond MEDLINE and EMBASE, reading the abstracts of all identified studies and "pearling" of reference lists. Pearling was particular valuable with an additional eight studies identified, however, it is possible that relevant studies may have been missed because the search strategy did not include the grey literature and was restricted to English. Across the studies in this review there was a wide

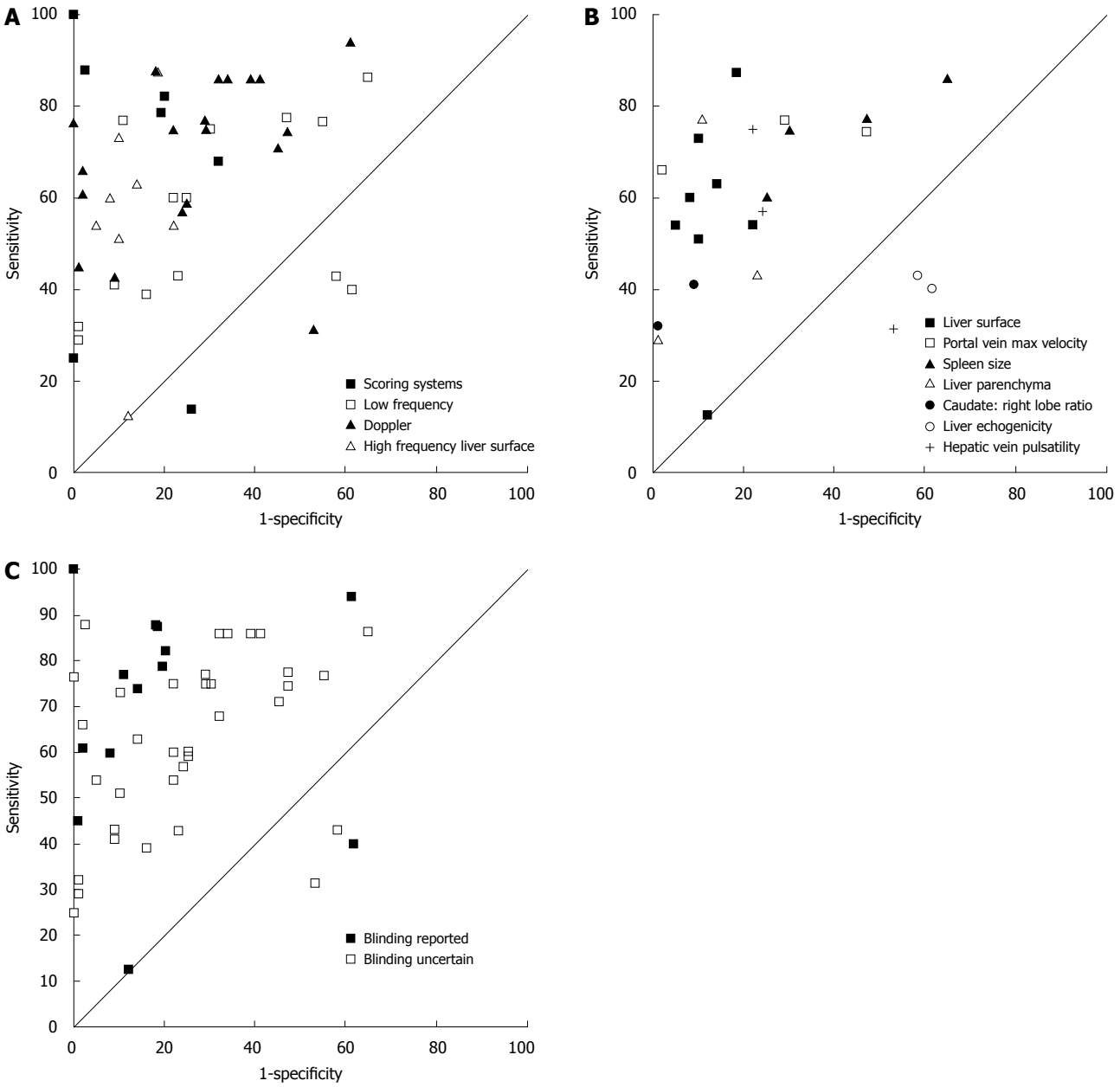

Figure 1 Receiver operator characteristic plot. Diagnostic performance data for categories of ultrasound techniques (A), ultrasound techniques reported multiple times (B) and relating to reference test blinding (C).

range of complexity and clarity of the described ultrasound techniques.

Methodology quality of the included studies was assessed with the QUADAS quality assessment tool, an independently validated method recommended by the Cochrane Collaboration[37]. As recommended[9] the QUADAS tool was modified for the specific needs of the review. Inter-rater variability testing of QUADAS showed good agreement over most of the QUADAS items with nine of 14 having substantial or almost perfect agreement. At the consensus meeting addressing differences in QUADAS ratings it was found that differences tended to relate to differing interpretations of item guidelines. Involving both reviewers in the formulation of the guidelines may have resulted in clearer guidelines and more consistent interpretations.

There was no identifiable group of studies that were clearly superior to the rest nor was there a group of studies that was markedly inferior; therefore all studies in the review were assessed for diagnostic accuracy. Blinding was the only item of methodology quality which demonstrated a relationship with diagnostic accuracy results. Studies reporting blinding for the reference test also reported higher diagnostic accuracy than studies which did not report reference test blinding. This finding further endorses the studies reporting higher diagnostic accuracy, because the chance of bias in these reports is reduced.

The only study characteristic that showed a relationship to diagnostic accuracy was prevalence, with studies reporting low prevalence also tending to have lower diagnostic accuracy. Whilst this may seem surprising, as sensitivity and specificity should be independent of prev-

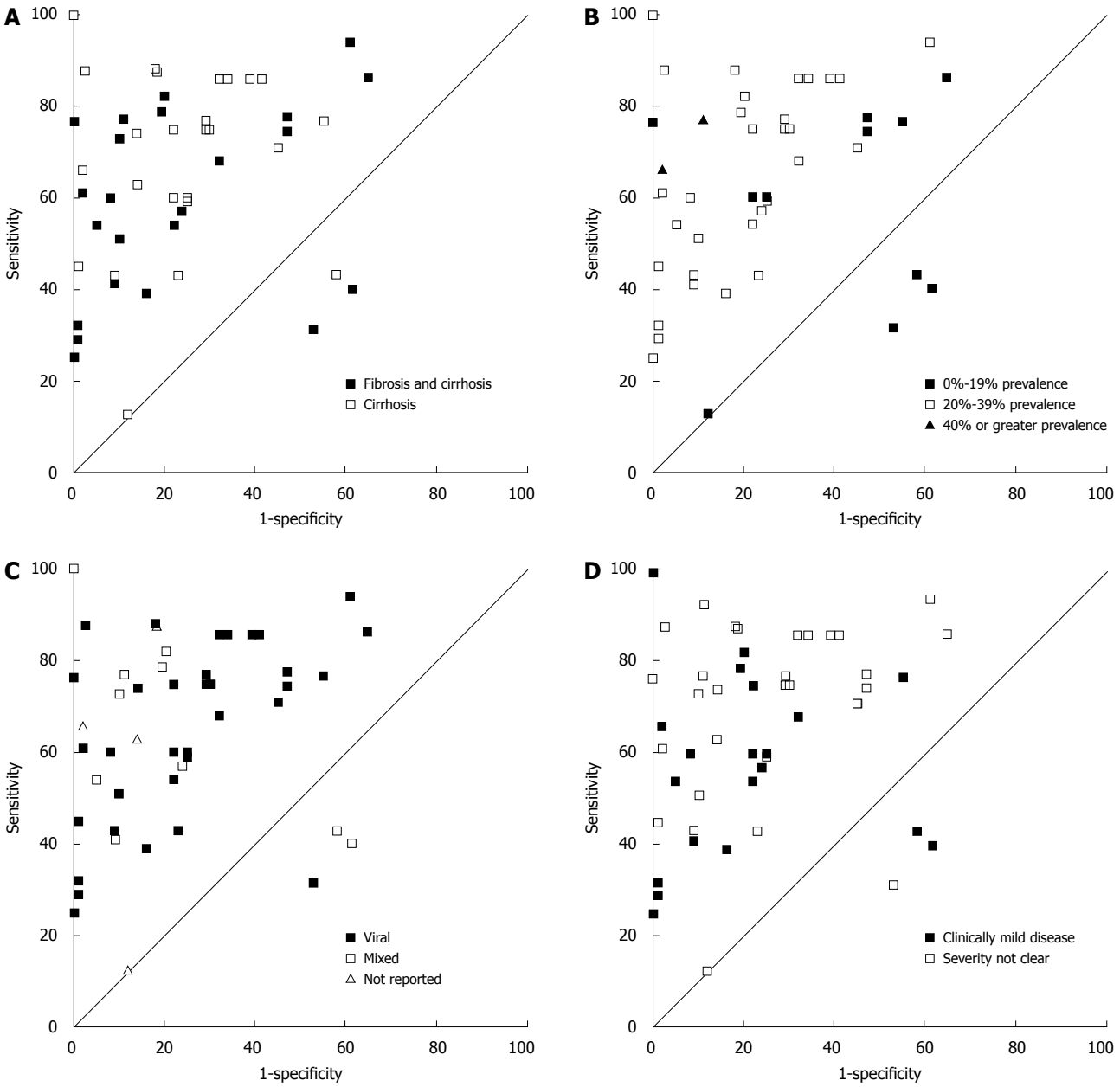

Figure 2 Receiver operator characteristic plot displaying diagnostic accuracy across disease characteristics. A: Histology staging definition; B: Prevalence; C: Disease aetiology; D: Severity of disease.

alence, it has recently been shown that prevalence can affect diagnostic accuracy due to clinical or artefactual variability in studies[38].

Liver biopsy was chosen as the reference test in this review although it has a significant false negative rate due to difficulties with the biopsy technique and sampling error which make it a less than ideal reference test. We justify our choice because it is the test used in clinical practice and is the only practical choice for a reference test. Whilst laparoscopy may be more accurate, it is much more invasive, with significantly more risk, and generally not used in normal clinical practice. Studies using laparoscopy as the reference test were excluded as including more than one reference test has the potential to introduce differential verification bias[9].

Studies were included if the diagnostic accuracy results were either given as true positive (TP), false positive (FP), true negative (TN) and false negative (FN) data or simply in the form of sensitivity and specificity. Restricting studies to those that expressed results in full (TP, FP, TN, FN) would have reduced the range of studies included. Whilst potentially this would have enabled the use of forest plots and meta-analysis to assess the diagnostic accuracy, this was not performed because the numbers of studies of techniques similar enough to enable comparison was too small to provide meaningful results. Instead all studies included in this review were analysed visually using the ROC plot technique. This provided an effective method for comparing data and exploring the relationship between diagnostic accuracy and the quality and characteristics of

the studies[10]. The area under the ROC for the various ultrasound techniques was not calculated due to the lack of reported raw data to make this possible.

Across all studies there was wide variation in both the ultrasound techniques used and in the reported diagnostic sensitivities and specificities for liver fibrosis and cirrhosis. For ultrasound to be clinically useful as a test that can reduce the number of patients requiring liver biopsy it needs to accurately confirm chronic liver disease. To be effective it should have a low false positive rate resulting in high specificity and a high positive predictive value. In this way patients with positive ultrasound results may be able to avoid the risks of liver biopsy. Two studies[22,25] stand out as having very high specificity (100% and 97.6%, respectively) and very high sensitivity (100% and 87.8%, respectively). Both of these studies used scoring systems and this suggests that this may be the best method of identifying severe fibrosis and cirrhosis; however, these results need to be treated with caution. The scoring systems used in both studies were complex, subjective and relied on the compounding of several ultrasound techniques. The use of multiple techniques[20,22,25,31,39] raises concerns regarding reproducibility, as variations may occur with each of the methods used and become magnified with compounding of methods. It is also a concern that in one of these studies[22] it was unclear if blinding had been used, if there were any subject withdrawals, how the selection criteria were applied, how the reference test was applied and how the scoring system was applied. In contrast, the other study[25] scored very well for methodological quality excepting that observer agreement was not reported.

The reporting of observer agreement was poor in many of the reviewed studies despite it being an important consideration when assessing the usefulness of a diagnostic test. We made an assessment of consistency of results across studies which reported similar techniques as a proxy method to determine the reproducibility of a technique in the absence of agreement reporting. Confidence in the results of a study's results can be increased if the technique has been reported over multiple studies with consistent results. We could make this assessment for the following ultrasound techniques; liver echogenicity, caudate lobe to right lobe ratio, portal vein maximum velocity, hepatic vein pulsatility, liver parenchyma echo-pattern, spleen size and liver surface.

The results for portal vein maximum velocity, hepatic vein pulsatility, liver parenchyma echo-pattern and spleen size were inconsistent between studies.

Consistently poor results of diagnostic accuracy were demonstrated between the two studies which tested measurements of liver echogenicity[27,28]. Liver echogenicity is known to be associated with liver steatosis but not with fibrosis[40] so this result is not surprising. Consistent results of diagnostic accuracy were demonstrated for the caudate lobe to right lobe ratio across two studies[18,31] with high specificity (> 90%) and low sensitivity (41% and 32%, respectively). The liver surface technique was the most frequently reported technique (n = 8 reports). Diagnostic accuracy was consistent across six of these studies, with high specificities (78%-95%) and moderate sensitivities (51%-73%)[18,19,23,30,32,34]. These studies were also of reasonable or good methodological quality. There were two studies reporting the liver surface technique[26,34] which produced results that were outliers compared to the other six and contained methodological flaws that were serious enough to not accept their findings. The flaws included an unclear description of patient spectrum or selection criteria in one study[26] together with a reported low prevalence of CLD which does not represent a high risk population which was the population of interest in this review. The other study[35] scored poorly for verification and differential bias and had a significant number of unexplained withdrawals.

The findings of consistent results of diagnostic studies that are methodologically sound make the assessment of liver surface appealing to apply in the clinical environment. This technique also appeared simple to implement, was defined clearly in the reports, and used a simple dichotomous categorical classification technique to interpret definitions of normal and abnormal. Three of these studies[18,19,23] also reported substantial inter and/or intra-observer agreement. Although these studies did not demonstrate high sensitivities, the high specificity and therefore high positive predictive value indicate this technique should be accurate for identifying patients who have a high likelihood of severe fibrosis or cirrhosis and who may benefit by avoiding the risks associated with liver biopsy.

In conclusion, a wide range of ultrasound techniques have been reported in the literature and investigated for their diagnostic accuracy to identify CLD in a high risk population. The most robust ultrasound technique for assessment of CLD appears to be the assessment of liver surface. The studies investigating the liver surface technique consistently demonstrated good observer agreement and high specificity. This review has revealed that an assessment of the liver surface is a useful screen for patients at risk of CLD to assist in determining who should undergo a liver biopsy.

COMMENTS

Background

Chronic liver disease (CLD) is a significant cause of morbidity and mortality. Accurate diagnostic testing to identify early CLD in asymptomatic patients at high risk is advantageous due to recent management and treatment advances. Biopsy, which is the current method of choice, is invasive and carries a significant risk. Less invasive techniques have the potential to reduce biopsy numbers. Ultrasound is one such technique which is readily available, inexpensive and well-tolerated. However, there are several ultrasound techniques in current practice. For an ultrasound study to be clinically useful it has to demonstrate accuracy in confirming CLD. This systematic review informs clinicians of the usefulness of ultrasound in early diagnosis of CLD in high risk patients, in particular, which method is shown to be the most specific and sensitive.

Research frontiers

There have been no identified published systematic reviews addressing diagnostic accuracy in ultrasound of CLD.

Innovations and breakthroughs

This rigorous systematic review identifies methodological and/or reporting flaws in several of the selected papers. It also highlights the variety and range of

diagnostic ultrasound techniques for liver examination in CLD in current usage. This review demonstrates that the most robust ultrasound technique for assessment of CLD appears to be high frequency ultrasound assessment of the liver surface.

Applications

The high specificity of ultrasound of the liver surface provides a clinician with confidence that if signs of CLD are evident then the condition is present. The moderate sensitivity means that if ultrasound signs of CLD are not present, a liver biopsy may be performed to confirm the presence of CLD. Performing high frequency ultrasound of the liver surface in high risk patients has the potential to reduce the number of biopsies in patients at high risk of CLD.

Terminology

Pulse-wave Doppler: A technique by which the ultrasound machine can determine the velocity of blood flowing in vessels. In addition, it allows evaluation of the direction and character of the blood flow. Pulse-wave Doppler is displayed as a spectral waveform on the screen. Maximum velocity: The velocity of blood cells flowing along a vessel will vary according to the position within the blood vessel. The maximum velocity is the greatest velocity detected in a particular vessel in a selected area; pulsatility and resistance indices and the spectral waveform allows quantification of the pulsatility of the blood flow by calculations using the maximum, minimum and mean velocities displayed. The indices are an indication of resistance to blood flow in the vessel and variation from normal may be an indication of disease, either in the vessel itself or the organ it supplies.

Peer review

This is a well written review on the quality and accuracy of ultrasound imaging techniques for identifying patients with chronic liver disease.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3521

World J Gastroenterol 2010 July 28; 16(28): 3521-3528
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma

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Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Yamamoto H, Imai K and Shinomura Y) and Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (to Yamamoto H)
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Received: January 25, 2010 Revised: April 6, 2010
Accepted: April 13, 2010
Published online: July 28, 2010

Abstract

AIM: To clarify the association of glypican-3 (GPC3) expression with Wnt and other growth signaling molecules in hepatocellular carcinoma (HCC).

METHODS: Expression of GPC3, Wnt, matrix metalloproteinases (MMPs), sulfatase (SULF)1, SULF2, and other growth signaling molecules was analyzed in HCC cell lines and tissue samples by real-time reverse transcription-polymerase chain reaction, immunoblotting, and/or immunostaining. Expression of various genes in *GPC3* siRNA-transfected HCC cells was analyzed.

RESULTS: GPC3 was overexpressed in most HCCs at mRNA and protein levels and its serum levels were significantly higher in patients with HCC than in non-HCC subjects ($P < 0.05$). Altered expressions of various *MMPs* and growth signaling molecules, some of which were correlated with *GPC3* expression, were observed in HCCs. Down-regulation of GPC3 expression by siRNA in GPC3-overexpressing HCC cell lines resulted in a significant decrease in expressions of MMP2, MMP14, fibroblast growth factor receptor 1, insulin-like growth factor 1 receptor. GPC3 expression was significantly correlated with nuclear/cytoplasmic localization of β-catenin.

CONCLUSION: These results suggest that GPC3, in conjunction with MMPs and growth signaling molecules, might play an important role in the progression of HCC.

Key words: Glypican-3; Wnt; Matrix metalloproteinases; Sulfatase; Hepatocellular carcinoma

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Akutsu N, Yamamoto H, Sasaki S, Taniguchi H, Arimura Y, Imai K, Shinomura Y. Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(28): 3521-3528 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3521.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3521

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most com-

mon cancers in the world[1]. HCC is associated with well-defined viral and non-viral etiological factors. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), chemical carcinogens (i.e. aflatoxins), and other environmental and host factors causing liver injury have etiologically been linked to HCC[2,3]. Various genetic and epigenetic abnormalities have been identified in HCC, suggesting a multi-step nature of hepatocarcinogenesis[3,4].

The pathogenesis of HCC has been known to involve *p53*[5], *β-catenin*[6], *TGFβ*[7] and the retinoblastoma gene[8]. *p53* gene mutation occurs in one-third of HCC[5,9]. Activating mutations in *β-catenin* have been reported in 18% of HCC patients and *axin* mutations in 6%[6,10]. HCCs are also known to express various Wnt family members[11] and the activation of the canonical Wnt signaling pathway occurs in 18% of HCC[10].

Glypican-3 (GPC3) is a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans. GPC3 is highly expressed in HCC cells and tissues[12-19]. It is thought that GPC3 stimulates the growth of HCC cells by upregulating autocrine/paracrine canonical Wnt signaling[20]. GPCs have been reported to stimulate both the canonical and non-canonical pathways[20]. GPC3 reportedly regulates migration, adhesion, and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation[21].

Matrix metalloproteinases (MMPs) also play an important role in HCC[22]. It has been reported that GPC3 may regulate MMP activity in breast cancer[23]. It has also been demonstrated that secreted MMP-9 associates with glypican-like proteoglycans through their heparan sulphate chains, and plays a crucial role in cell motility of murine colon cancer cell line LuM1 cells[24].

GPC3 has been shown to bind to fibroblast growth factor (FGF)2 and may function as a coreceptor for FGF2[25]. Two recently identified human heparin-degrading endosulfatases, named sulfatase 1 (SULF1) and SULF2, have been found to be involved in liver carcinogenesis[26,27]. Interestingly, SULF2 reportedly up-regulates GPC3, promotes FGF signaling, and decreases survival in HCC[27].

Moreover, GPC3 reportedly confers oncogenicity through the interaction between insulin-like growth factor (IGF)-Ⅱ and its receptor, and the subsequent activation of the IGF signaling pathway[28]. Specific interactions both between GPC3 and IGF-Ⅱ and between GPC3 and IGF 1 receptor (IGF1R) have been reported[28].

These results suggest that GPC3 joins a multiprotein complex, which is composed of the ligand, receptor, GPC3, and probably other proteins[28]. However, the association of GPC3 with Wnt, MMPs, SULF1, SULF2, and other growth signaling molecules has not been systematically analyzed in HCC. In this study, we analyzed expression of these molecules in HCC cell lines and tissue samples by real-time reverse transcription-polymerase chain reaction (RT-PCR), immunoblotting, and/or immunostaining. Expression of various genes in *GPC3* siRNA-transfected HCC cells was analyzed.

MATERIALS AND METHODS

Cell lines

HCC cell lines, HepG2, Hep3B, JHH-4, HuH-7, HLE, HLF, PLC/PRF/5, Li-7, huH-1, HT17, CHC4, and CHC32, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Rockville, MD, USA), and cultured as recommended. Cells were maintained at 37℃ in an atmosphere of humidified air with 5% CO_2.

Tissue samples

All tissue and serum samples were obtained from Japanese patients. Informed consent was obtained from each patient. Tissue microarray of HCC tissues was purchased from SuperBioChips Laboratories (Seoul, Korea). Each tissue specimen was divided into two pieces. One sample was used for total RNA extraction. The other sample was processed for pathological examination using hematoxylin and eosin staining for the evaluation of the tumor cell content. Only specimens containing more than 80% tumor cells were used for analysis. The tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer and the International Union against Cancer was used for the pathologic diagnosis and classification of variables.

Semiquantitative RT-PCR and real-time RT-PCR

Semiquantitative RT-PCR was performed as described previously[29]. The primer sequences used were 5'-AGGTAGCTGCGAGGAAAC-3' and 5'-AGGTCACGTCTTGCTCCTC-3' for *GPC3* and 5'-TGGACATCAATGAGTGCCTC-3' and 5'-CACATTCT GGTGAGCATTCG-3' for *GAPDH*. Real-time RT-PCR was performed by using TaqMan real-time PCR system as described previously[30]. The following genes were analyzed: *GPC3*, *WNT* (*WNT1*, *WNT2*, *WNT2b*, *WNT3a*, *WNT4*, *WNT5a*, and *WNT7b*), *MMP* (*MMP2*, *MMP3*, *MMP7*, *MMP9*, and *MMP14*), *SULF1*, *SULF2*, *FGF2*, *FGF receptor* (*FGFR*) *1*, *FGFR2*, *FGFR3*, *FGFR4*, *epidermal growth factor receptor* (*EGFR*), *erb-b2* (*ERBB2*), *IGF2* and *IGF1R*. A comparative threshold cycle was used to determine gene expression relative to the no-tissue control (calibrator).

Immunoblotting

Immunoblotting using total cell lysates was performed as previously described[29]. The antibodies used were MMP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP14 (Abcam, Cambridge, UK), FGFR1 (Abcam), and IGF1R (Santa Cruz Biotechnology). The protein was visualized using the enhanced chemiluminescence plus detection system (Amersham Biosciences, Piscataway, NJ, USA), and the membranes were stripped and reprobed with mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA, USA).

Gelatin zymography

Gelatinase activity of conditioned media of siRNA-trans-

fected HepG2 was measured using SDS-polyacrylamide gels containing 0.1% gelatin as described previously[22]. Gels were washed in 2.5% Triton X-100 for 30 min prior to incubation in 20 mmol/L glycine, 10 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$ (pH 8.3) for 48 h at 37℃, before staining with Commassie Brilliant Blue.

siRNA transfection

siRNAs for the *GPC3* gene were purchased from Ambion (Silencer® Pre-designed siRNA, Austin, TX, USA). Unrelated nonspecific siRNAs (Silencer® Negative Control siRNA) were used as a control. siRNA transfection was performed following the manufacturer's instructions[29]. After incubation for 72 h, siRNA-transfected HepG2 cells were analyzed by real-time RT-PCR and immunoblotting to validate knockdown effect on GPC3. These cells were used for real-time RT-PCR.

Immunohistochemistry

Immunohistochemistry with an anti-human GPC3 mouse monoclonal antibody (Clone 1G12 used at 5 mg/mL, BioMosaics, Burlington, VT, USA) and an anti-human β-catenin monoclonal antibody (Santa Cruz Biotechnology) was performed as described previously[29]. A case was considered negative for GPC3 if < 5% of the carcinoma cells exhibited immunoreactivity[31]. Cancer cases were categorized into the following 4 groups corresponding to immunostaining patterns of β-catenin as previously described: membranous, membranous staining pattern similar to that in normal; weak, no staining or weaker staining than normal; cytoplasmic, staining in the cytoplasm or cytoplasm/cell membrane; accumulated, staining in the nucleus or nucleus/cytoplasm.

Serum GPC3 levels by ELISA

Serum GPC3 levels were measured using a commercially available sandwich ELISA kit (Biomosaics) following the manufacturer's protocol[16]. The subjects were 6 healthy volunteers, 6 healthy HBV carriers, 8 patients with chronic hepatitis, 11 patients with cirrhosis, and 64 patients with HCC.

Statistical analysis

The results were assessed for associations with clinicopathological parameters using the following statistical tests: Student's *t* test for age, the Mann-Whitney test for lymph node metastasis and pTNM stage, and the χ^2 test or Fisher's exact test for the remaining parameters. To explore relationships between the variables selected for the models, Spearman's correlation coefficients were calculated. A *P* value of less than 0.05 was considered significant. A *P* value between 0.05 and 0.10 was considered as trend toward an association.

RESULTS

Expression of GPC3 mRNA in HCC cell lines

GPC3 expression was detected in 7 (58%) of 12 HCC cell lines (Figure 1). *GPC3* mRNA was detected in HT17, PLC/PRF/5, HuH-1, HepG2, Hep3B, and HuH-7 at a high level and HLE at a low level. *GPC3* mRNA was positive in well differentiated HuH7 and poorly differentiated HT17 cell lines. There was no correlation between *GPC3* positivity and histopathology. Concordant results were obtained by real-time RT-PCR (Figure 2). There was no correlation between quantitative *GPC3* mRNA levels and histopathology.

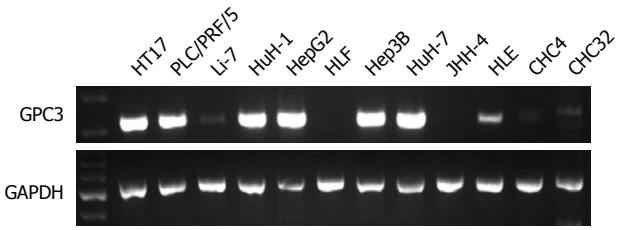

Figure 1 Semiquantitative reverse transcription-polymerase chain reaction analysis of glypican-3 in hepatocellular carcinoma cell lines. Expression of glypican-3 (GPC3) mRNA was analyzed in hepatocellular carcinoma cell lines by semiquantitative reverse transcription-polymerase chain reaction. GAPDH served as a control.

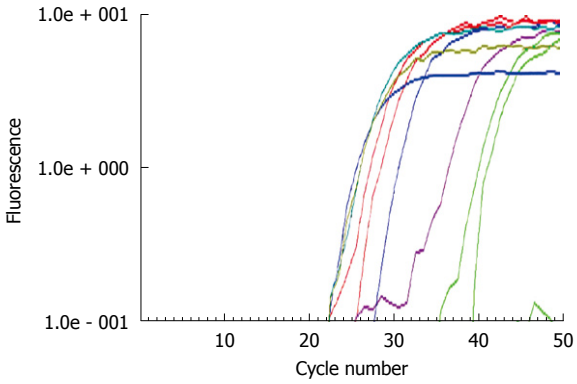

Figure 2 Real time reverse transcription-polymerase chain reaction analysis of glypican-3 in hepatocellular carcinoma cell lines. cDNA was used for quantitative polymerase chain reaction (PCR). The amount of PCR products was determined by reading the midpoint of the linear portion of the S-shaped real-time curves, called the Ct point or threshold cycle. The Ct refers to the number of cycles it takes a sample to reach a specific fluorescence threshold.

mRNA expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules in HCC cell lines

WNT1, *WNT2*, *WNT2b*, *WNT3a*, *WNT4*, *WNT5a*, *WNT7b* expression was detected in 0 (0%), 7 (58%), 0 (0%), 0 (0%), 5 (41%), 9 (75%), 4 (33%) of 12 HCC cell lines. Thus, *WNT2* and *WNT5a* were frequently detected compared with other *WNTs*. *MMP2*, *MMP3*, *MMP7*, *MMP9*, *MMP14* expression was detected in 7 (58%), 2 (16%), 8 (67%), 2 (16%), 11 (92%) of 12 HCC cell lines. Thus, the positivity was considerably different among *MMPs*. *SULF1* and *SULF2* expression was detected in all of the 12 HCC cell lines. *EGFR*, *ERBB2*, *FGF2*, *FGFR1*, *FGFR2*, *FGFR3*, *FGFR4* expression was detected in 12 (100%), 12 (100%), 12 (100%), 12 (100%), 6 (50%), 2

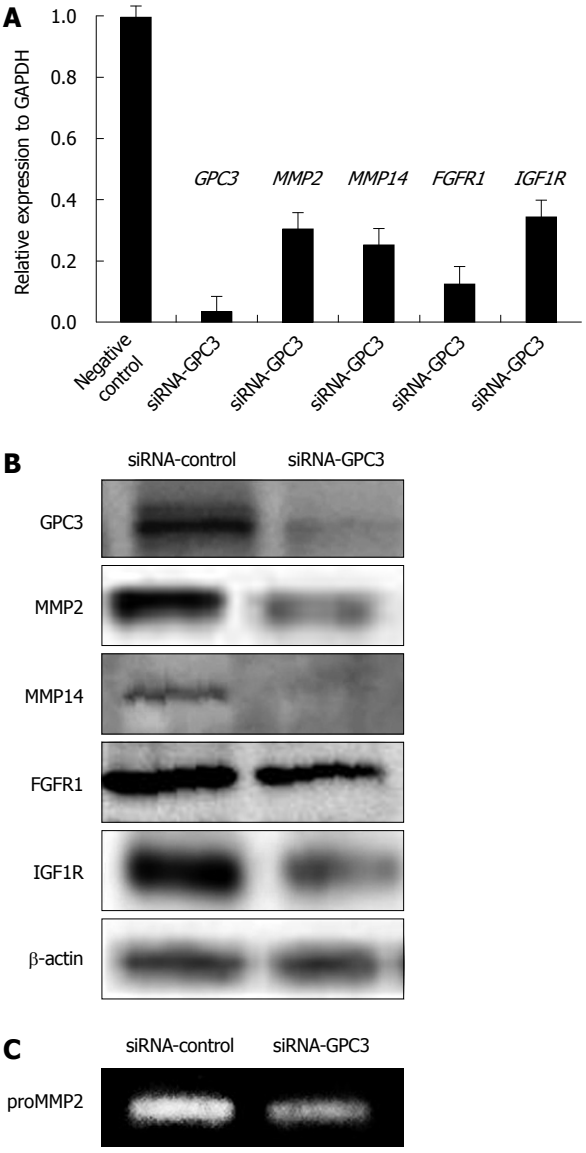

Figure 3 Down-expression of various molecules in glypican-3 siRNA-transfected hepatocellular carcinoma cell line. A: mRNA levels analyzed by real-time reverse transcription-polymerase chain reaction. The mRNA expressions of matrix metalloproteinase (MMP)2, MMP14, fibroblast growth factor receptor 1 (FGFR1), and insulin-like growth factor 1 receptor (IGF1R) were significantly down-regulated in HepG2 cells treated with glypican-3 (GPC3) specific siRNA ($P < 0.05$). Bar: SE; B: Protein levels analyzed by immunoblotting. The protein expressions of MMP2, MMP14, FGFR1, and IGF1R were significantly down-regulated in HepG2 cells treated with GPC3 specific siRNA; C: MMP2 activity analyzed by gelatin zymography. Conditioned media of siRNA-transfected HepG2 were used for gelatin zymography.

(16%), 12 (100%) of 12 HCC cell lines. Thus, growth factor receptors were frequently detected in HCC cell lines. *GPC3* expression was significantly correlated with expression of *MMP14*, *ERBB2*, *FGFR3*, and *FGFR4*.

Overexpression of GPC3 mRNA in HCC tissue

In 18 (78%) of 23 cases, mRNA expression of *GPC3* was upregulated in cancer tissue compared with non-tumor tissue samples. There was no significant difference in *GPC3* mRNA levels among non-tumor liver tissue, including from chronic hepatitis and liver cirrhosis cases. Overexpression of *GPC3* was not related to HBV or to HCV infection. Overexpression of *GPC3* was not significantly correlated with other clinicopathological characteristics (data not shown).

mRNA expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules in HCC tissue

mRNA expression of *SULF1* was downregulated in 17 (74%) and *SULF2* was upregulated in 7 (30%) of 23 HCC tissue samples compared with non-tumor tissue. Overexpression of *GPC3* was significantly correlated with *MMP2* ($P = 0.0460$), *FGF2* ($P = 0.0001$), *FGFR1* ($P = 0.0417$), *FGFR2* ($P = 0.0023$), *SULF1* ($P = 0.0202$), and *SULF2* ($P = 0.0081$).

Knockdown effect of GPC3 on expression of various molecules in HCC cells

To assess the effect of GPC3 expression on gene expression in HCC, mRNA expression of *WNT*, *MMPs*, *SULF1*, *SULF2*, and other growth signaling molecules was analyzed by real-time PCR after treatment with specific siRNA for the *GPC3* gene. Transfection with siRNA resulted in over 80% inhibition of mRNA and protein expression of GPC3 (Figure 3). Among the genes analyzed, mRNA expression of *MMP2*, *MMP14*, *FGFR1*, and *IGF1R* was downregulated in *GPC3* siRNA-transfected cells compared with control siRNA-transfected counterparts (Figure 3A). Down-expression of MMP2, MMP14, FGFR1, and IGF1R was confirmed at protein levels analyzed by immunoblotting (Figure 3B). Down-regulation of MMP2 activity was further confirmed by zymography (Figure 3C).

Immunohistochemical expression of GPC3 and β-catenin in HCC tissue

Figure 4 shows representative results of immunohistochemistry for GPC3 in HCC tissue samples. GPC3 protein was strongly expressed in the cytoplasm and/or membrane of carcinoma cells, when compared with adjacent non-tumor cells. GPC3 expression was positive in 54 (75%) of the 72 cases. GPC3 positivity was not significantly correlated with clinicopathological characteristics. Figure 5 shows representative results of immunohistochemistry for β-catenin in HCC tissues. Membranous, weak, cytoplasmic, and accumulated pattern was observed in 18 (64%), 0 (0%), 6 (22%), and 4 (14%), respectively. Nuclear/cytoplasmic localization of β-catenin was observed in a significantly higher percentage of carcinomas with GPC3 expression (9 of 18, 50%) than in those without (1 of 10, 10%, $P = 0.040$).

Serum GPC3 levels

Serum GPC3 levels were under cut-off levels in healthy volunteers, HBV carriers and chronic hepatitis patients (Figure 6). One patient with liver cirrhosis and 32 (50%) of the 64 patients with HCC showed elevated serum GPC3 levels. Serum GPC3 levels in patients with HCC were significantly higher than in non-HCC subjects ($P < 0.05$). There was no correlation in positivity between

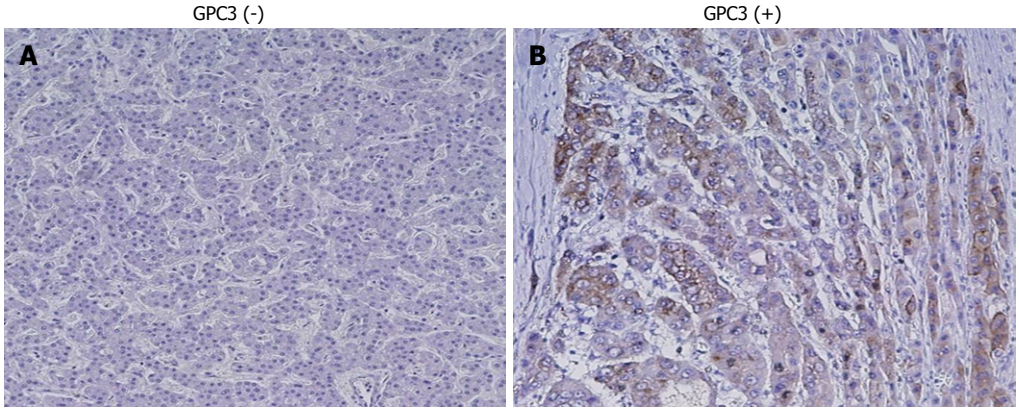

Figure 4 Immunohistochemistry for glypican-3 in liver tissue. A: Normal liver negative for glypican-3 (GPC3); B: Hepatocellular carcinoma positive for GPC3. Original magnification, × 200. Expression of GPC3 was immunohistochemically analyzed with an anti-human GPC3 mouse monoclonal antibody. GPC3 protein was expressed in the cytoplasm and/or membrane of carcinoma cells.

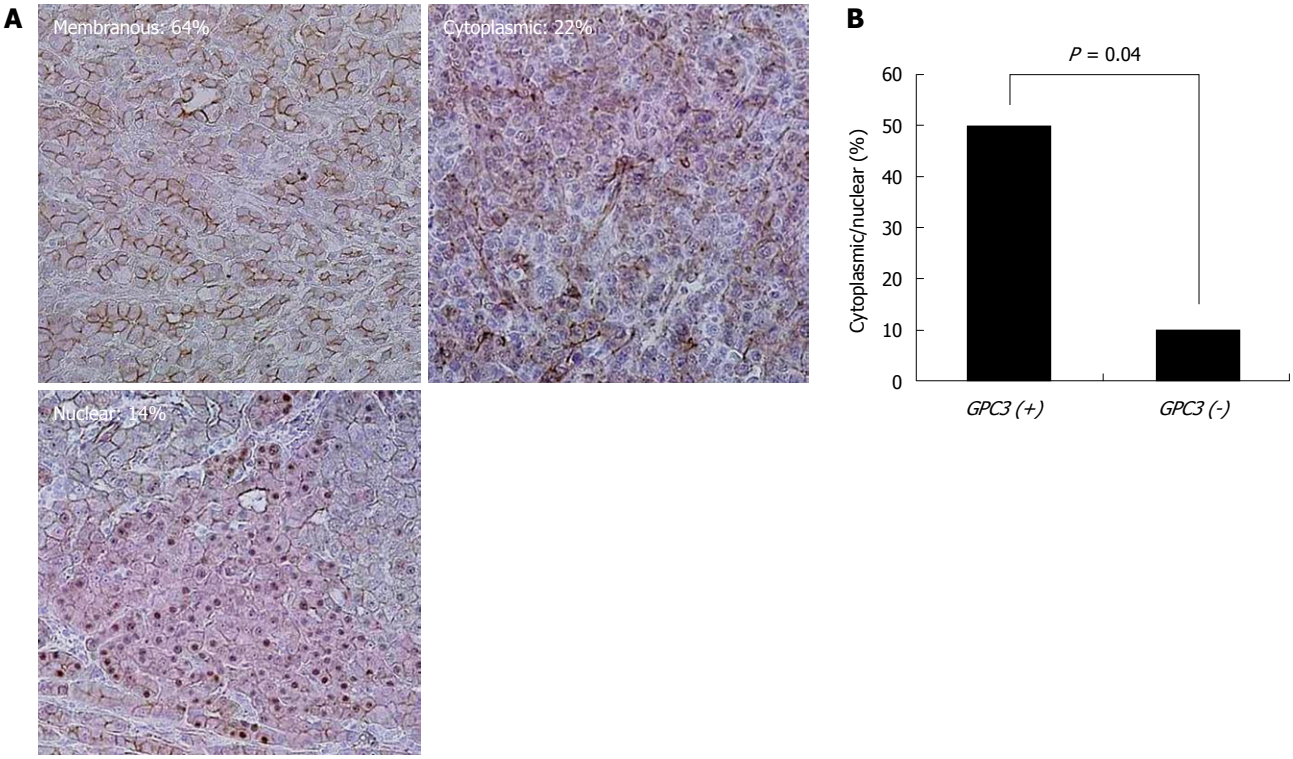

Figure 5 Immunohistochemistry for β-catenin in hepatocellular carcinoma tissue. A: Membrane staining, cytoplasmic staining, nuclear staining of β-catenin. Expression of β-catenin was immunohistochemically analyzed with an anti-human β-catenin monoclonal antibody. Original magnification, × 200; B: Association of nuclear/cytoplasmic localization of β-catenin with glypican-3 (GPC3) expression.

GPC3, α-fetoprotein (AFP) levels and vitamin K absence or antagonist-Ⅱ (PIVKA-Ⅱ) levels (data not shown).

DISCUSSION

In this study, we found overexpression of *GPC3* mRNA in HCC cell lines and tissue samples. The overexpression of GPC3 in HCC was also observed at protein level analyzed by immunohistochemistry. These results further support the notion that GPC3 plays an important role in hepatocarcinogenesis.

We analyzed the association of GPC3 with WNT, MMPs, SULF1, SULF2, and other growth signaling molecules, in HCC cell lines and tissue samples. *GPC3* expression was correlated with expression of *MMP14*, *ERBB2*, *FGFR3*, and *FGFR4* in HCC cell lines. Overexpression of *GPC3* was significantly correlated with *MMP2*, *FGF2*, *FGFR1*, *FGFR2*, *SULF1*, and *SULF2* in HCC tissue. To assess the effect of GPC3 expression on gene expression in HCC cells, expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules was analyzed in HCC cell lines after treatment with specific siRNA for the *GPC3* gene. Down-expression of MMP2, MMP14, FGFR1, and IGF1R was observed at mRNA and

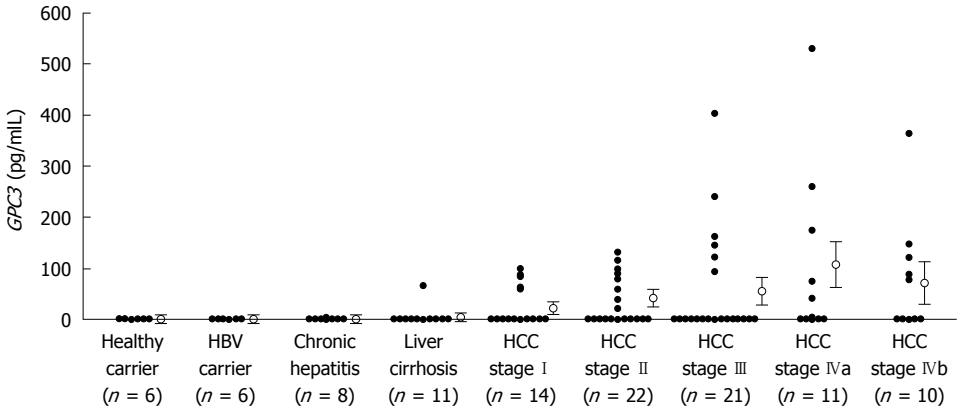

Figure 6 Serum glypican-3 levels. Serum glypican-3 (GPC3) levels were measured using a commercially available sandwich ELISA kit. Bar: Standard error. Serum GPC3 levels were significantly higher in patients with hepatocellular carcinoma (HCC) than in non-HCC subjects ($P < 0.05$). HBV: Hepatitis B virus.

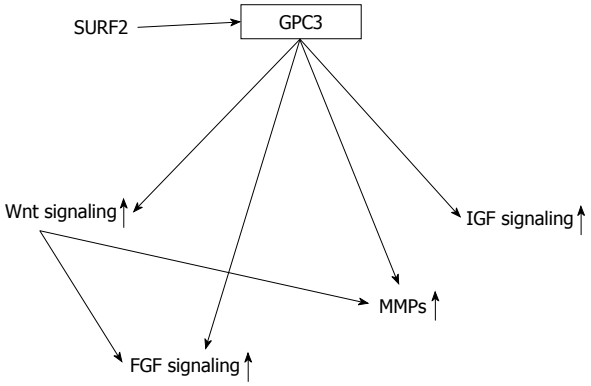

Figure 7 Associations of glypican-3 expression with matrix metalloproteinases and growth signaling molecules in hepatocellular carcinoma. A summary diagram is shown. MMPs: Matrix metalloproteinases; SULF2: Sulfatase 2; GPC3: Glypican-3; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor.

protein levels, and that of MMP2 was also observed at gelatinolytic activity levels.

MMP2 and its activator MMP14 play an important role in HCC progression[22]. Therefore, down-regulation of both MMP2 and MMP14 by GPC3 suppression is interesting. It has been reported that GPC3 may regulate MMP activity[23]. FGF-FGFR signaling plays an important role in hepatocarcinogenesis[27,32]. GPC3 has been shown to bind to FGF2 and may function as a coreceptor for FGF2[25]. SULF2 reportedly up-regulates GPC3, and promotes FGF signaling in HCC[27]. Moreover, GPC3 reportedly confers oncogenicity through the interaction between IGF-Ⅱ and IGF1R, and the subsequent activation of the IGF signaling pathway[28]. Specific interactions both between GPC3 and IGF-Ⅱ and between GPC3 and IGF-IR have been reported[28]. Therefore, the association of GPC3 expression with expressions of these molecules found in this study is interesting (Figure 7).

Nuclear/cytoplasmic localization of β-catenin was observed in a significantly higher percentage of HCCs with GPC3 expression than in those without. GPC3-induced accumulation of cytoplasmic β-catenin has been reported in HCC cells[20]. Therefore, in addition to *β-catenin* mutation or deletion, *axin* mutations, and other alterations of molecules in the canonical WNT signaling pathway, GPC3 may play a role in nuclear/cytoplasmic localization of β-catenin in HCC. These results may explain, in part, the association between GPC3 and growth signaling molecules in HCC. Further studies are necessary to clarify the direct and/or indirect interactions between GPC3 and growth signaling molecules in HCC.

The implication of a part played by GPC3 in HCC was further substantiated by the fact that serum GPC3 levels were significantly higher in patients with HCC than non-HCC subjects. However, there was a discrepancy between GPC3 overexpression in HCC tissues (75%) and GPC secretion (50%). The discrepancy was also reported in previous studies[14,16]. The sandwich ELISA kit used in this study used a polyclonal antibody and a monoclonal antibody, both raised against the last 70 amino acids of the COOH-terminal portion of GPC3, to detect glycanated GPC3[16,19]. This may be one of the reasons why serum GPC3 positivity was lower than GPC3 positivity in HCC tissue[17]. Comparison of our results with those analyzed by a kit using antibody against the NH_2-terminal portion of GPC3 will further strengthen the notion that GPC3 is a useful serum marker for HCC[17]. There was no correlation between GPC and AFP levels. Therefore, detection of both glycanated and NH_2-terminal truncated GPC3, as well as AFP, may provide additional useful markers for HCC.

Taken together, our results suggest that GPC3 overexpression plays an important role in HCC. As a target gene for molecular therapy, its expression in normal adult tissues is important. Considering the expression pattern of GPC3 together with its oncogenic function, GPC3 could be an attractive target for molecular therapy. Antitumor effects of the anti-GPC3 antibody have been reported[33]. Interestingly, we have recently reported the tumor suppressive effect of tyrosine kinase inhibitor of IGF1R, NVP-AEW541, on GPC-3-expressing HCC cell line PLC/PRF/5[34]. Combination of the anti-GPC3 antibody and molecular therapy targeting GPC3-related molecules, such as FGFR, found in this study will be a promising new cancer therapy in the future.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Understanding the molecular biological features of HCC is necessary for early diagnosis and better prognosis. The potential role of glypican-3 (GPC3) in human HCC is receiving increasing attention.

Research frontiers

GPC3 is highly expressed in HCC cells and tissues. However, little is known about the association of GPC3 expression with Wnt and other growth signaling molecules. In this study, the authors demonstrate that GPC3, in conjunction with matrix metalloproteinases (MMPs) and growth signaling molecules, might play an important role in the progression of HCC.

Innovations and breakthroughs

GPC3 was overexpressed in most HCCs at mRNA and protein levels and its serum levels were significantly higher in patients with HCC than in non-HCC subjects. Altered expressions of various MMPs and growth signaling molecules, some of which were correlated with GPC3 expression, were observed in HCCs. This is the first study to report an association of GPC3 expression with MMPs and growth signaling molecules in HCC.

Applications

Considering the tumor specific expression pattern of GPC3 together with its oncogenic function, GPC3 could be an attractive target for molecular diagnosis and/or therapy in clinical settings. Understanding of the direct and/or indirect interactions between GPC3 and growth signaling molecules may represent a future strategy for therapeutic intervention in the treatment of patients with HCC.

Terminology

GPC3: GPC3 is a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans. Sulfatase (SULF): Two recently identified human heparin-degrading endosulfatases, named SULF1 and SULF2, are extracellular neutral-pH SULFs. SULFs modulate several signaling pathways, including the promotion of Wnt signaling.

Peer review

This paper reports an association of GPC3 expression with MMPs and growth signaling molecules in HCC. The authors showed that GPC3, in conjunction with MMPs and growth signaling molecules, might play a key role in the progression of HCC. The study sounds interesting and significant in a potentially important area involving HCC.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3529

World J Gastroenterol 2010 July 28; 16(28): 3529-3540
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Probiotic Bio-Three induces Th1 and anti-inflammatory effects in PBMC and dendritic cells

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Supported by Chang Gung Memorial Hospital research project grant CMRPG470051 and National Science Council of Taiwan grant NSC-96-2314-B-182A-015-MY3
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Received: March 12, 2010 Revised: May 4, 2010
Accepted: May 11, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate the immune response of peripheral blood mononuclear cells (PBMCs) and dendritic cells (DCs) that were stimulated by probiotic preparations.

METHODS: PBMCs were isolated, cultured, and stimulated with Bio-Three (a mixture of *Bacillus mesentericus*, *Clostridium butyricum* and *Enterococcus faecalis*; 10^5, 10^6 and 10^7 CFU/mL for 24 h). Cytokine production of (1) circulating PBMCs; (2) PBMCs stimulated by probiotic preparation; (3) monocyte-derived DCs; and (4) DC and T cell co-culture was determined by enzyme-linked immunosorbent assay. Phenotypic analysis of circulating PBMCs was also investigated by flow cytometry. Blood was obtained from individuals who consumed Bio-Three (10^9 CFU/d *B. mesentericus*, *C. butyricum* and *E. faecalis*) for 2 wk, or those who did not take probiotics orally.

RESULTS: In culture supernatants, interferon-γ (IFN-γ) and interleukin (IL)-10 production increased, but IL-4 and tumor necrosis factor-α (TNF-α) production by PBMCs decreased after 1 and 2 wk of probiotic treatment. Flow cytometry was also performed on day 14 and detected enhanced expression of CD11b, HLA-DR, CD4, CD45RA, CD25, CD44 and CD69 in response to Bio-Three. Furthermore, IL-10 and IL-12 were upregulated in supernatants of monocyte-derived DCs, and IFN-γ and IL-10 were enhanced in supernatants of $CD4^+$ T cells co-cultured with DCs.

CONCLUSION: Bio-Three appeared to stimulate the Th1 immune response, downregulate pro-inflammatory cytokines (TNF-α) and upregulate anti-inflammatory cytokine (IL-10). Probiotics could be effective in activation of PBMCs and DCs.

Key words: Probiotics; Bio-Three; Peripheral blood mononuclear cells; Dendritic cells

Peer reviewers: Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, #301, Nok-Ji Building, Seoul 136-701, South Korea; Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46., Budapest 1088, Hungary

Hua MC, Lin TY, Lai MW, Kong MS, Chang HJ, Chen CC. Probiotic Bio-Three induces Th1 and anti-inflammatory effects in PBMC and dendritic cells. *World J Gastroenterol* 2010; 16(28): 3529-3540 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3529.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3529

INTRODUCTION

The use of probiotics to promote human health has been proposed for many years. Mechanisms of probiotic actions include effects on luminal microbial ecology and immune modulation, particularly through balance control of pro-inflammatory and anti-inflammatory cytokines[1-4]. Currently, species of lactobacillus and bifidobacteria are most widely used to prevent and treat allergy and intestinal disorders; other strains such as *Bacillus*, *Clostridium*, *Streptococcus*, *Escherichia coli* and *Saccharomyces have received* increased attention.

To date, the most extensively studied and best documented probiotic application is for the treatment of acute infectious diarrhea, prevention of antibiotic-associated diarrhea, and allergic diseases[5-7]. Many other benefits are largely unproven, including therapeutic use in necrotizing enterocolitis, irritable bowel syndrome, constipation, inflammatory bowel diseases, pouchitis, and *Helicobacter pylori* infection[5-7].

It has been proposed that many effects of probiotics are mediated *via* immune modulation[3]. There are host-specific and strain-specific differences in the activities of probiotic bacteria. Some strains can enhance or eliminate the activity of other strains *in vivo*[1,2,8]. Previously, most studies that have reported the beneficial effect of probiotics have been on single strain preparations. Few have examined the effect of multiple strain preparations (e.g. VSL#3)[9,10]. Our study was undertaken to investigate whether Bio-Three (a mixture of *Bacillus mesentericus*, *Clostridium butyricum* and *Enterococcus faecalis*) affects immune regulation in human peripheral blood mononuclear cells (PBMCs).

The primary effectors of the human gut are antigen-presenting cells [APCs, including monocytes, macrophages, and dendritic cells (DC)], which provide nonspecific innate immune protection. APCs are responsible for detecting microbes through Toll-like receptors (TLRs) and presenting their antigenic structures to T cells. This triggering process of APCs initiates a signal transduction cascade that leads to the release of cytokines and initiation of the acquired immune response[2,11,12]. Among the APCs, DCs are the most potent. These differentiate from CD14+ monocytes *in vitro* in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4[10,13,14]. DCs activated by microbes further stimulate the development of T helper type 1 (Th1) and T helper type 2 (Th2) cells or regulatory T cells[1,15].

Several cytokines are involved in immune modulation. Tumor necrosis factor-α (TNF-α) and IL-6 are pro-inflammatory cytokines that are involved in systemic inflammation and the acute phase reaction[12]. IL-12 together with interferon-γ (IFN-γ) causes a shift towards a Th1 response, which favors the development of cell-mediated and cytotoxic immunity[2]. Prostaglandin E_2 (PGE_2), together with IL-4, causes a shift towards a Th2 immune response, which favors the production of antibodies and the induction of IgE and allergic responses[2]. IL-10 (an anti-inflammatory cytokine) enhances the generation of regulatory T (Treg) cells[1,2]. Treg cells seem to suppress or regulate effector T cell function through production of cytokines such as IL-10 and transforming growth factor-β (TGF-β).

The purpose of this study was to determine whether a probiotic combination (Bio-Three) had immunomodulatory effects (altered the phenotype of circulating lymphocytes or monocytes) in human PBMCs. We observed the expression of specific cytokines and changes in PBMC phenotypes.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan, China.

PBMC preparation

Blood cells were separated from platelet-rich plasma and suspended in RPMI 1640 medium. Human PBMCs were isolated by centrifugation of buffy coats on Lymphoprep (Nycomed, Oslo, Norway) gradients. After washing, cells were resuspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium that contained 10% heat-inactivated fetal bovine serum.

Source of blood donors

All experiments were performed with cells obtained from 14 blood donor volunteers. Subjects were eligible if they were in good general health and were not currently taking medications, probiotics, and other supplements. These blood donors were divided randomly into two groups: group A ($n = 7$) consumed the probiotic preparation Bio-Three 5×10^8 CFU/dose twice daily (total 10^9 CFU/d) for 2 wk, and group B ($n = 7$), the negative control, did not consume probiotics. Peripheral blood samples were obtained from each subject by venipuncture on day 7 (week 1) and day 14 (week 2).

Probiotic preparation for stimulation experiments

The probiotic preparation Bio-Three was a lyophilized mixture that consisted of three different bacteria (*B. mesentericus*, *C. butyricum* and *E. faecalis)*, at a concentration of 3×10^8 live bacteria/packet. 3×10^8 live bacteria were reconstituted in 3 mL sterile PBS without additives, and serial dilutions (1:10) were made in sterile PBS for addition to cell cultures.

PBMC cultures and stimulation experiments

The concentration of PBMCs (obtained from groups A and B) was adjusted to 10^5 cells/mL in complete medium, and the cells were transferred to 24-well plates. Some wells were collected for cytokine detection [by enzyme-linked immunosorbent assay (ELISA)] after 12 h culture at 37℃. The remaining wells were then stimulated with Bio-Three for 24 h at 37℃ in an atmosphere that contained 5% CO_2. Initial dose-response experiments were performed by co-culturing 10^5 (1:1), 10^6 (1:10), and 10^7 CFU (1:100) of bacteria per mL (host cells: bacteria ratio), respectively. Culture supernatants were collected, and triplicates were pooled and kept at -20℃ until analyzed by ELISA. The remaining cells in the culture plates were mixed with

TRIzol Reagent (Gibco Life Technologies, Carlsbad, CA, USA) and stored at -20℃ for gene expression analysis. Repeated thawing and freezing were avoided.

Cytokine determination

Concentrations of IFN-γ, IL-4, TNF-α, IL-10, and IL12 p70 in the supernatants of cell cultures were determined by ELISA. All antibodies and standards were purchased from Pharmingen (San Diego, CA, USA). Costar plates (Invitrogen, San Diego, CA, USA) were coated with the following capture monoclonal antibodies (mAbs): anti-IFN-γ (MQ2-13A5), anti-IL-4 (8D4-8), anti-IL-12 p70 (20C2), anti-TNF-α (MAb1), and anti-IL-10 (JES3-9D7). Standard curves were generated using recombinant human IFN-γ, IL-4, IL-12 p70, TNF-α, and IL-10, respectively. The following biotinylated antibodies were used for detection: anti-IFN-γ (MQ2-39C3), anti-IL-4 (MP4-25D2), anti-IL-12 p40/p70 (C8.6), anti-TNF-α (MAb11), and anti-IL-10 (JES3-12G8). Samples, standards, biotinylated antibodies, and streptavidin-horseradish peroxidase were diluted in high-performance ELISA dilution buffer (Sanquin, Amsterdam, Netherlands).

Detection of PBMC cytokine mRNA expression

Cytokine mRNA expression in PBMCs was evaluated by real-time polymerase chain reaction (PCR). Total cellular RNA was isolated from frozen cultured PBMCs using TRIzol (Gibco Life Technologies) according to the manufacturer's instructions. cDNA was synthesized and used as templates for PCR using specific primers for human IFN-γ (forward: 5'-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC; reverse: 5'-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG), IL-4 (forward: 5'-TCTCACCTCCCAACTGCTTCC; reverse: 5'-CGTTTCAGGAATCGGATCAGC), TNF-α (forward: 5'-AGCCAGTAGCTCATGTTGTAGCAA; reverse: 5'-GGCACTATCAGCTGGTTGTCTGT), IL-10 (forward: 5'-GCTGGAGGACTTTAAGGGTTACCT; reverse: 5'-CTTGATGTCTGGGTCTTGGTTCT), IL-12 (forward: 5'-TGGATGCTATTCACAAGCTCAAGT; reverse: 5'-TGGTTTGATGATGTCTCTGATGAAG), and β-actin (forward: 5'-GCATGGAGTCCTGTGGCAT; reverse: 5'-CTAGAAGCATTTGCGGTGG). All experimental samples were amplified in duplicate. The results were normalized to β-actin expression.

Phenotypic analysis by flow cytometry

Flow cytometry was performed on day 14 after enrollment and detected the expression of immune phenotype distributions. White blood cells were stained using a panel of mAbs directed against surface antigens expressed by PBMCs, lymphocytes, monocytes and the appropriate species-specific IgG isotype controls. After blocking with FCγⅢ/ⅡR antibody (CD16/CD32; Pharmingen), the cells were stained with mAbs directed against CD4, CD45RA, CD45RO, CD14 fluorescein isothiocyanate, together with one of the activation markers: CD3, CD8, CD19, CD25, CD44, CD69, or CD11b, HLA-DR (all phycoerythrin-conjugated; Pharmingen). We analyzed 10000-20000 cells using a FACSCalibur system (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with CellQuest software (San Jose, CA, USA).

Generation of monocyte-derived DCs

CD14-positive monocytes were then purified from the mononuclear cells by magnetic cell sorting by using positive selection according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (10^6 cells/mL) were cultured in six-well plates in endotoxin-free RPMI 1640 medium supplemented with 250 U/mL recombinant IL-4 (R&D Systems, Abingdon, UK) and 250 U/mL recombinant GM-CSF (R&D Systems). The cells were cultured in the presence of 5% CO_2 at 37℃ for 7 d. Fresh medium that contained IL-4 and GM-CSF was added every second day. This procedure resulted in generation of immature DCs that were positive for CD11b but negative for CD14.

Isolation of human $CD4^+$ T cells and co-culture with DCs

PBMCs were isolated by centrifugation of buffy coats on Lymphoprep gradients. $CD4^+$ T cells were separated using negative selection affinity columns (R&D Systems), according to the manufacturer's instructions. After separation, the T cells were washed and resuspended in RPMI 1640 culture medium supplemented with 5% heat-inactivated human AB serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Purified $CD4^+$ T cells (1×10^6/mL) were stimulated by the combination of immobilized anti-CD3 (1 μg/mL) and soluble anti-CD28 (5 μg/mL) mAbs (Pharmingen). Subsequently, purified $CD4^+$ T cells were incubated with the above DCs at a ratio of 2.5×10^5/mL DCs to 10^6/mL T cells. After 48 h of co-culture, concentrations of IFN-γ, IL-4, IL-10, and IL12 p70 in the culture supernatants were determined by ELISA.

Statistical analysis

Statistical analysis was performed using a paired samples *t* test to reveal significant between-group differences in cytokine production. In all cases, $P < 0.05$ was considered as significant. Statistical calculations were performed using the GraphPad Software Prism 3.03 (San Diego, CA, USA) and SPSS for Windows 12.0 (Chicago, IL, USA).

RESULTS

Effects of Bio-Three on PBMCs isolated from blood donors

To determine the cytokine production of PBMCs, we examined the supernatants of cells isolated from blood donors. Group A consumed Bio-Three and group B was a negative control. In Figure 1, IFN-γ, IL-10 and IL-12 levels were upregulated in group A, but IL-4 and TNF-α levels were downregulated at 1 and 2 wk after Bio-Three consumption. This indicates that this probiotic preparation enhances cytokines associated with Th1 (IFN-γ, IL-12) and anti-inflammatory (IL-10) responses. In contrast, it might reduce cytokine production associated with Th2 (IL-4) and pro-inflammatory (TNF-α) responses.

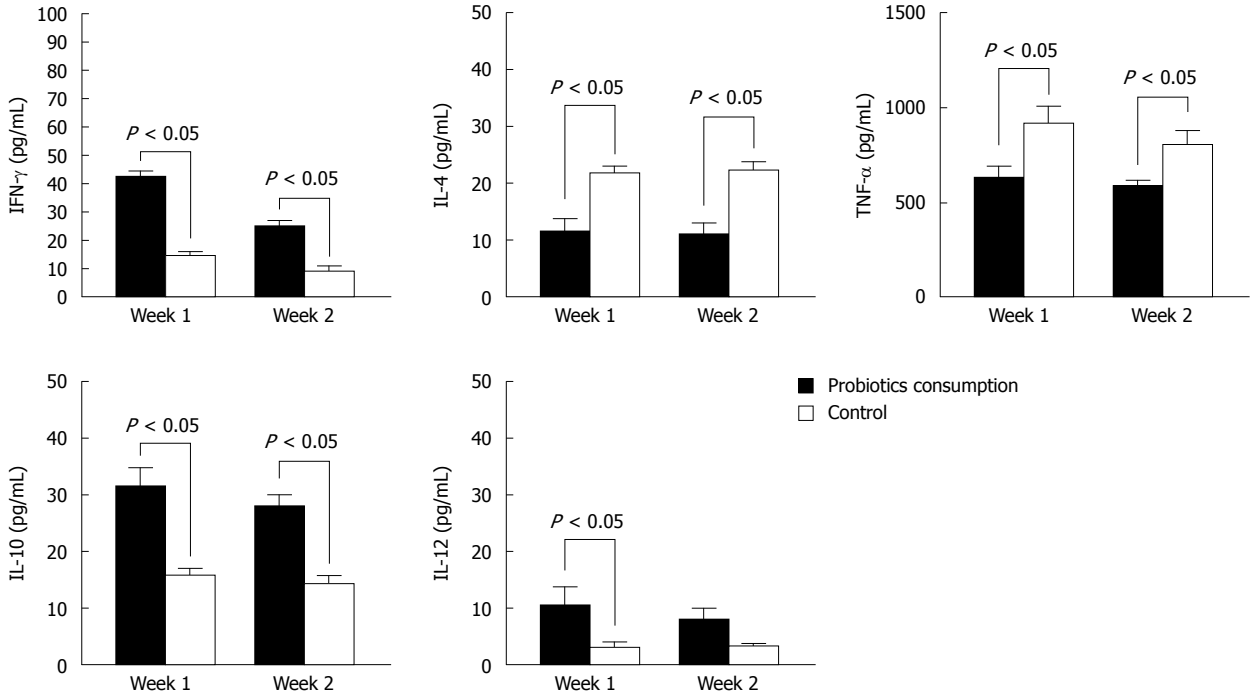

Figure 1 Effects of Bio-Three on peripheral blood mononuclear cells isolated from blood donors. Concentrations of interferon-γ (IFN-γ), interleukin (IL)-4, tumor necrosis factor-α (TNF-α), IL-10, and IL-12 p70 in the supernatants of peripheral blood mononuclear cells (PBMCs) (10^5 cells/mL) incubated for 12 h were determined by enzyme-linked immunosorbent assay. The data shown are the values of different cytokines in the supernatants of PBMCs, which were collected at 1 and 2 wk after Bio-Three consumption, compared with controls. The results are presented as the mean ± SD.

Enhancement of IFN-γ and IL-10 levels, but inhibition of TNF-α production in bacterial stimulation experiments

To explore the effects of probiotic bacteria on PBMCs, we performed stimulation experiments with different bacteria at concentrations of 10^5, 10^6 and 10^7 CFU/mL.

The level of IFN-γ was significantly higher in group A (probiotics) than group B (negative control) (Figures 2 and 3). Moreover, the level of IFN-γ revealed a dose-dependent effect of Bio-Three. There was no significant effect on IL-4 level, although it seemed slightly lower in the probiotic group. In contrast, TNF-α level was decreased at weeks 1 and 2 in the probiotic group (Figures 2 and 3). TNF-α level was significantly lower in response to restimulation with 10^7 CFU/mL probiotic bacteria after 2 wk of Bio-Three consumption. The probiotic group showed increased IL-10 production, which was maximal following restimulation with 10^6-10^7 CFU/mL probiotic bacteria at week 2. The level of IL-12 was low in both groups, which suggested that Bio-Three had no significant effect on IL-12 production (Figures 2 and 3). After stimulation with Bio-Three and co-culture *in vitro*, the IL-12 level in supernatants of PBMCs was upregulated (Figures 2 and 3). PBMCs can be activated by *in vivo* stimulation (probiotic consumption) and probiotic bacteria re-stimulation *in vitro*, which enhanced IFN-γ and IL-10 production, as well as downregulated TNF-α production.

Initial cytokine levels and immune phenotype distribution in PBMCs from blood donors

Initial concentrations of IFN-γ, IL-4, TNF-α, IL-10, and IL12 p70 in the supernatants of isolated human PBMCs were determined (Figure 4A) by ELISA. To determine the effect of probiotics (Bio-Three) on PBMCs, phenotypic analysis of immune responses was also studied. Figure 4B shows that probiotics might alter the expression of some T cell and DC surface phenotypes compared with controls, such as $CD4^+$ (54.2% ± 3.6% *vs* 43.4% ± 3.0%), $CD45RA^+$ (69.1% ± 4.2% *vs* 43.3% ± 3.6%), $CD25^+$ (15.1% ± 2.6% *vs* 9.8% ± 2.3%), $CD44^+$ (48.3% ± 3.8% *vs* 40.1% ± 3.2%), $CD69^+$ (45.6% ± 2.4% *vs* 34.3% ± 2.7%), $CD11b^+$ (63.6% ± 4.5% *vs* 51.8% ± 2.8%) and HLA-DR (29.9% ± 2.7% *vs* 22.3% ± 2.3%). Probiotics can enhance expression of CD4, CD45RB, CD44, CD69, CD25, CD11b and HLA-DR, which indicates alternation of co-stimulation with markers of Th cells and DCs.

Effect of probiotics on expression of CD14, CD 11b and HLA-DR

To determine the effect of Bio-Three on circulating monocytes and APCs (such as DCs), phenotypic analysis of CD14, CD 11b and HLA-DR was also studied. Probiotics can also enhance the expression of CD11b, and HLA-DR in gating monocytes and granulocytes (Figure 5), which indicates alternation of co-stimulation with marker of DCs. However, the expression of CD14 was similar in the probiotics and control groups.

Enhancement of CD4, CD45RA, CD44, CD69 and CD25 expression

To determine the effect of Bio-Three on circulating lymphocytes, phenotypic analysis of T cells and B cells was also studied. Figure 6 shows that probiotics can alter the

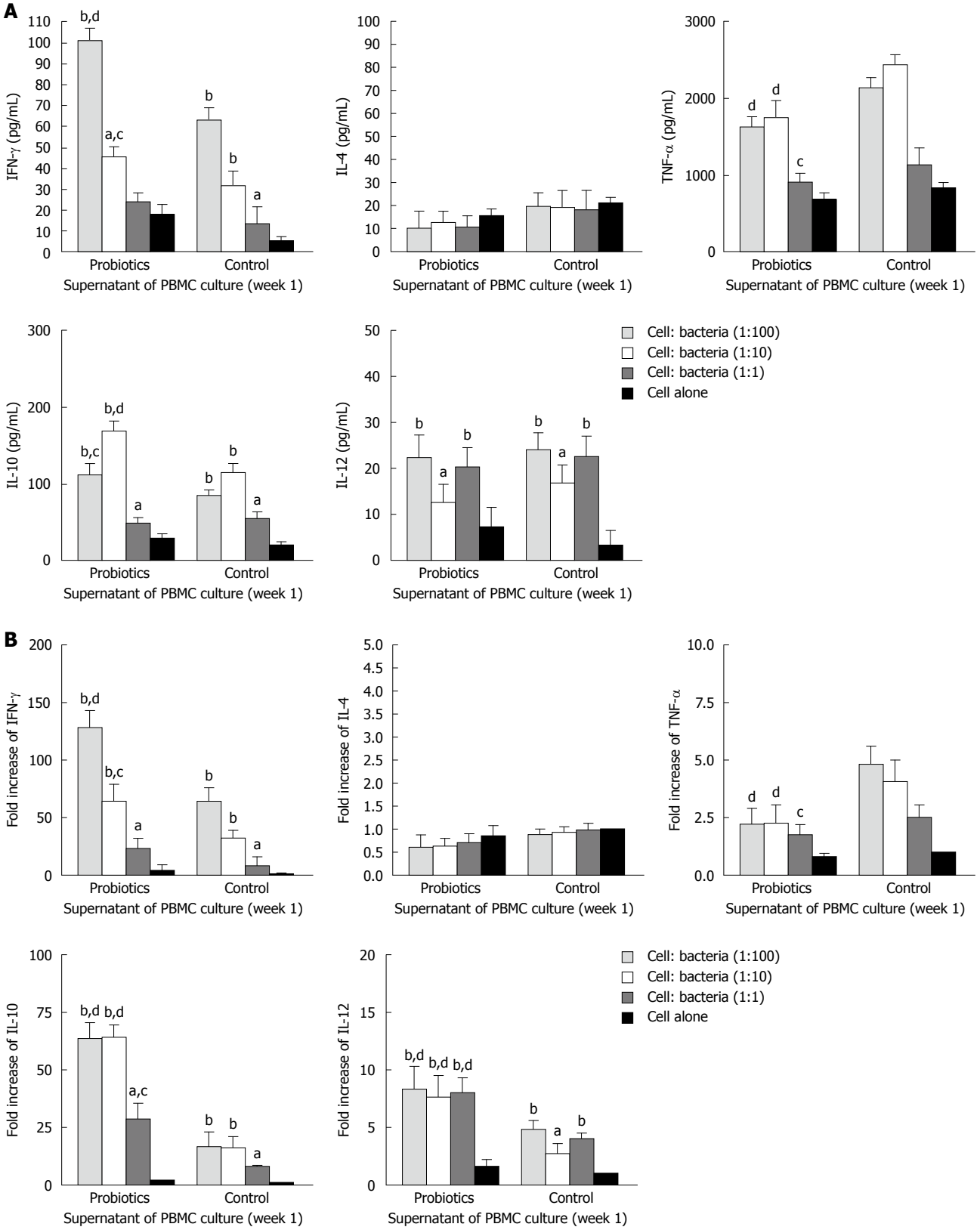

Figure 2 Effects of Bio-Three re-stimulation on cytokine production of peripheral blood mononuclear cells isolated at 1 wk after consumption, compared with controls. Peripheral blood mononuclear cells (PBMCs) (10^5 cells/mL) were stimulated at a host cell: bacteria ratio of 1:1, 1:10 and 1:100. A: At 24 h after bacterial stimulation, cell culture supernatants were collected and cytokine levels were determined by enzyme-linked immunosorbent assay; B: The remaining cells in the culture plates were mixed with TRIzol Reagent, and cytokine mRNA expression was analyzed by real-time polymerase chain reaction. The fold increases were compared to that of control cells, which was set at 1. The columns represent the means and the error bars indicate the SD. $^aP < 0.05$, $^bP < 0.01$ *vs* control cells; $^cP < 0.05$, $^dP < 0.01$ *vs* controls at the same host cell:bacteria ratio. IFN-γ: Interferon-γ; IL-4: Interleukin-4; TNF-α: Tumor necrosis factor-α.

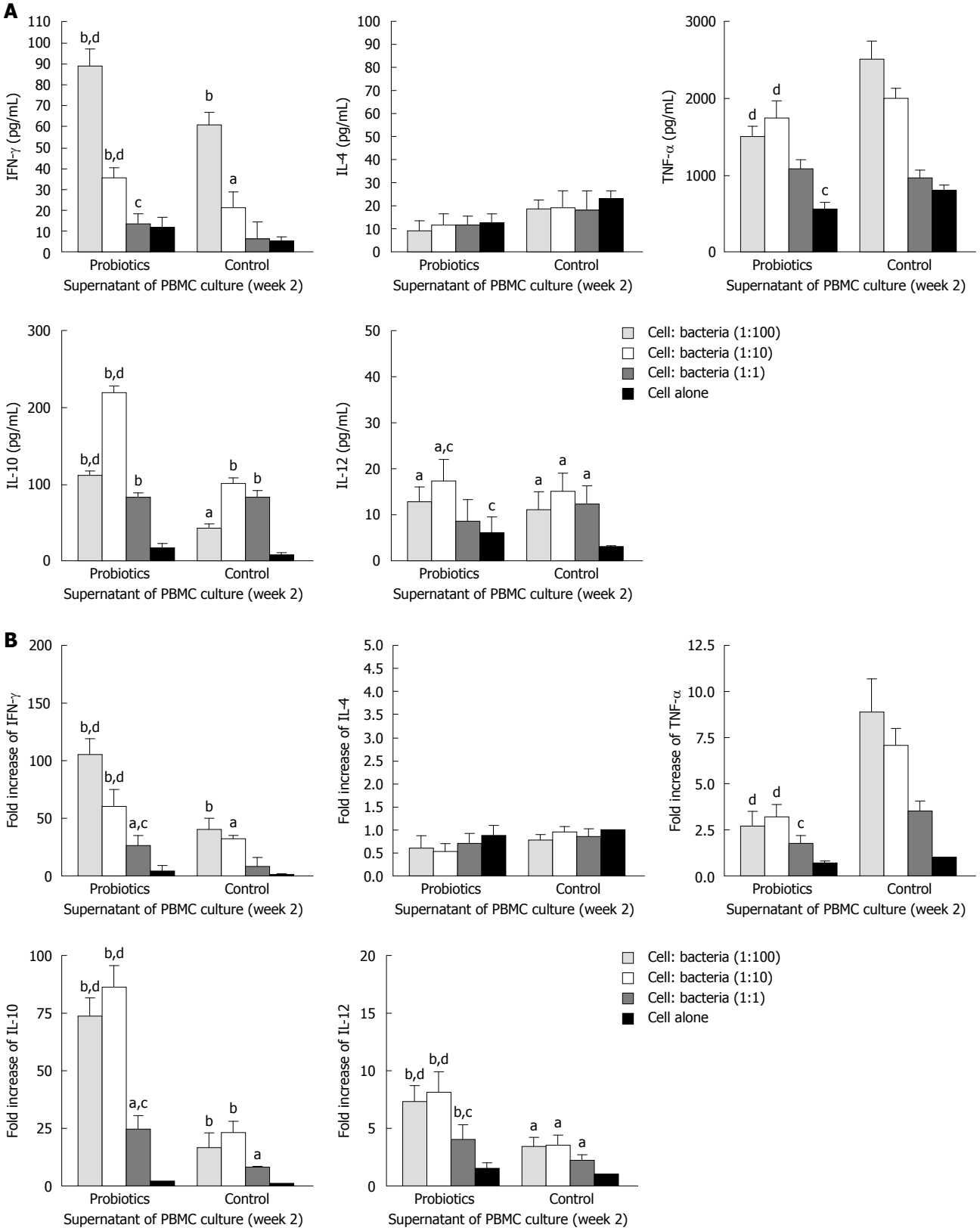

Figure 3 Effects of Bio-Three re-stimulation on cytokine production in peripheral blood mononuclear cells isolated at 2 wk after consumption, compared with controls. Peripheral blood mononuclear cells (PBMCs) (10^5 cells/mL) were stimulated at a host cell: bacteria ratio of 1:1, 1:10 and 1:100. A: At 24 h after bacterial stimulation, cell culture supernatants were collected and cytokine levels were determined by enzyme-linked immunosorbent assay; B: The remaining cells in the culture plates were mixed with TRIzol Reagent, and cytokine mRNA expression was analyzed by real-time polymerase chain reaction. The fold increases were compared to that of control cells, which was set at 1. The columns represent the means and error bars indicate the SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* controls at the same host cell:bacteria ratio. IFN-γ: Interferon-γ; IL-4: Interleukin-4; TNF-α: Tumor necrosis factor-α.

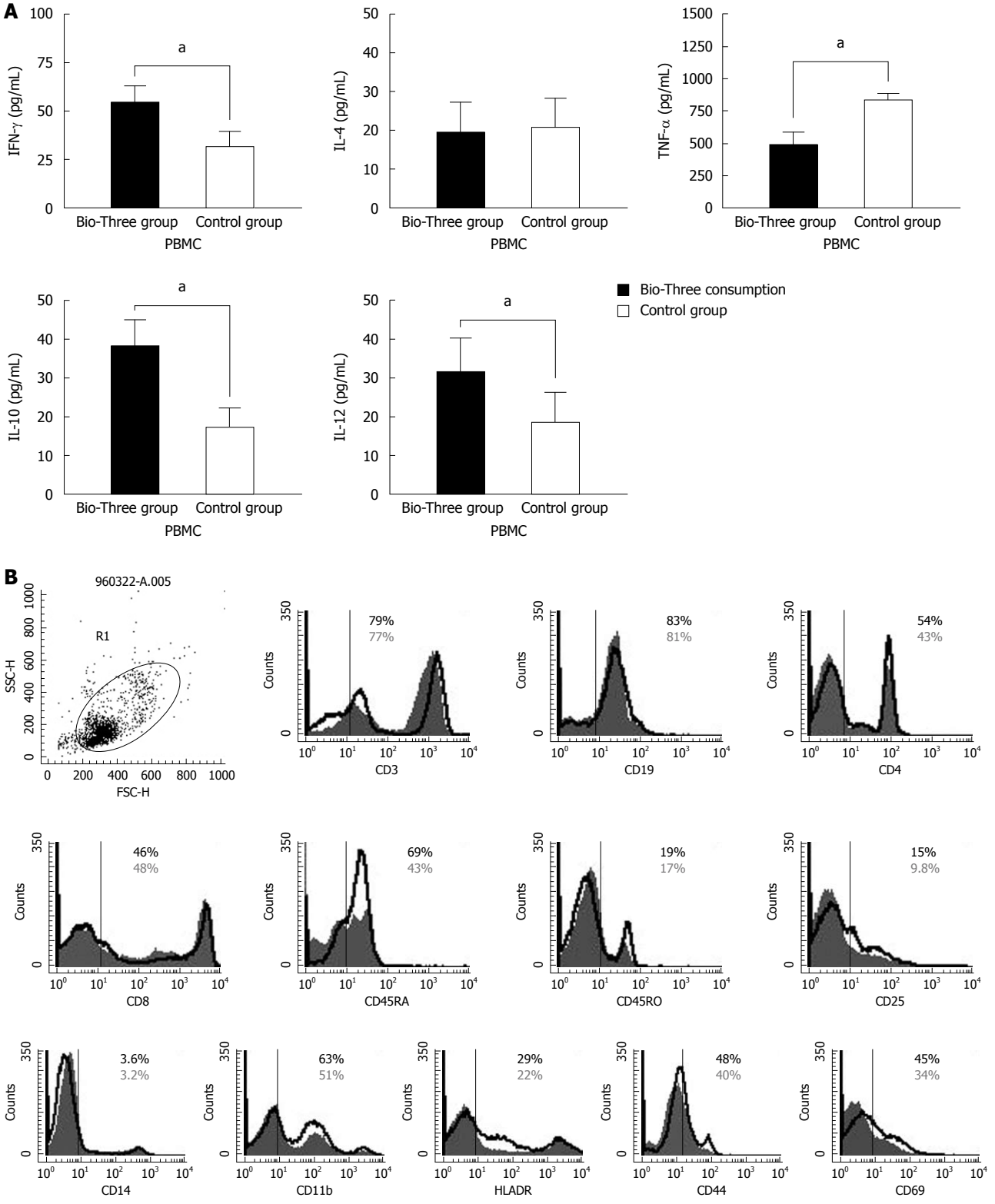

Figure 4 Measuring initial cytokine levels and determining immune phenotype distributions in peripheral blood mononuclear cells isolated from blood donors. A: Initial concentrations of interferon-γ (IFN-γ), interleukin (IL)-4, tumor necrosis factor-α (TNF-α), IL-10, and IL-12 p70 in the supernatants of human peripheral blood mononuclear cells (PBMCs) were determined by enzyme-linked immunosorbent assay. The results are presented as the mean ± SD. Statistically significant differences compared with the controls ($^aP < 0.05$); B: To determine the effect of Bio-Three on PBMCs, phenotypic analysis of the immune response was studied. The solid histogram shows the control results, and the unshaded area shows the level of expression of co-stimulatory molecules after Bio-Three consumption. The data shown are representative of three experiments performed. Probiotics might enhance expression of CD4, CD45RB, CD44, CD69, CD25, CD11b and HLA-DR, which indicates alternation of co-stimulatory markers of T helper cells and dendritic cells.

expression of some T-cell surface phenotypes compared with controls, such as $CD4^+$ (63.2% ± 4.6% *vs* 45.6% ± 3.1%), $CD45RA^+$ (69.2% ± 3.9% *vs* 42.3% ± 2.6%), $CD25^+$ (13.2% ± 2.5% *vs* 8.7% ± 2.1%), $CD44^+$ (47.3%

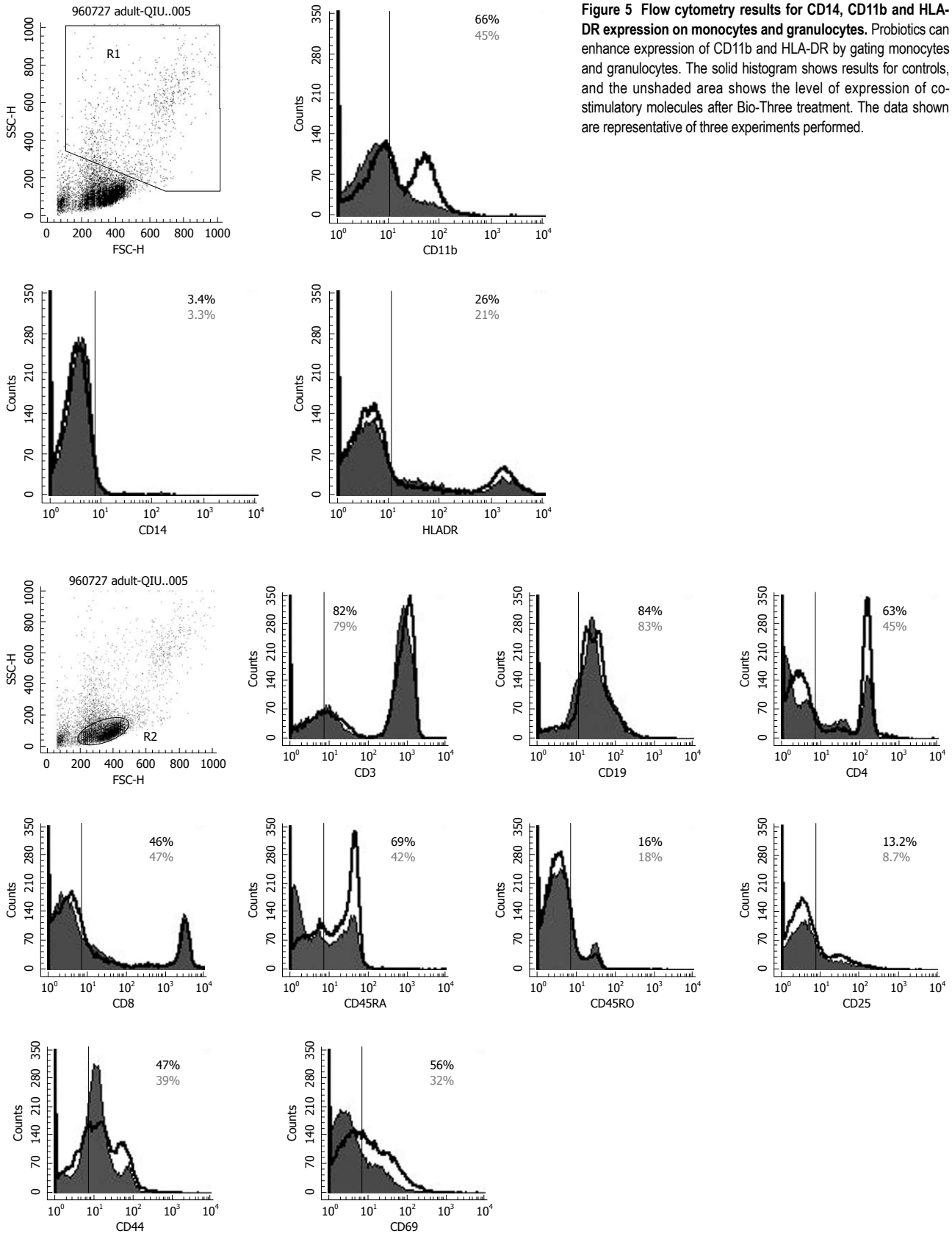

Figure 5 Flow cytometry results for CD14, CD11b and HLA-DR expression on monocytes and granulocytes. Probiotics can enhance expression of CD11b and HLA-DR by gating monocytes and granulocytes. The solid histogram shows results for controls, and the unshaded area shows the level of expression of co-stimulatory molecules after Bio-Three treatment. The data shown are representative of three experiments performed.

Figure 6 Flow cytometry results for CD3, CD4, CD45RA, CD45RO, CD8, CD19, CD25, CD44, CD69 expression on peripheral lymphocytes. The solid histogram shows the results for controls, and the unshaded area shows the level of expression of co-stimulatory molecules after Bio-Three treatment. The data shown are representative of three experiments performed. Bio-Three might alter the expression of T-cell surface phenotype, such as CD4, C45RA, CD44, CD69, and even CD25.

± 3.6% *vs* 39.8% ± 3.8%) and $CD69^+$ (56.6% ± 2.3% *vs* 32.1% ± 2.6%). Flow cytometry showed that probiotic treatment was associated with altered expression of CD4 (Th cells), CD45RA (naïve T cells), CD25 (Treg cells),

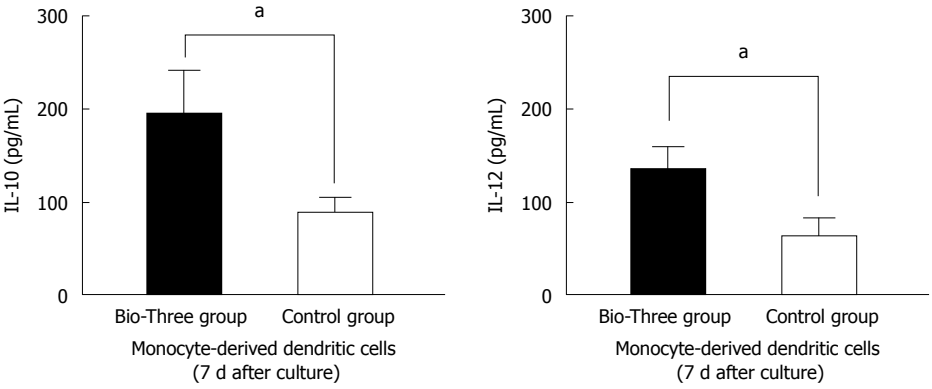

Figure 7 Interleukin-10 and interleukin-12 p70 levels in the supernatants of monocyte-derived dendritic cells. The data shown are the levels of cytokines in the probiotic group (black bar), and control group (white bar) collected on day 7 after Bio-Three consumption. Bio-Three upregulated interleukin (IL)-10 and IL-12 p70 levels in the supernatants of monocyte-derived dendritic cells. The results are presented as the mean ± SD. Statistically significant differences compared with the controls ($^aP < 0.05$).

CD44 and CD69 (T-cell activation markers) on lymphocytes, but there was no significant effect on the expression of CD3, CD8 or CD45RO.

Effect of probiotics on monocyte-derived DCs

To determine the effect of probiotics on DCs, we isolated monocytes and cultured them at 37℃ for 7 d. In the culture supernatants from monocyte-derived DCs, IL-10 and IL-12 levels were upregulated in the probiotic group compared to the control group (Figure 7). This indicates that Bio-Three can stimulate monocyte-derived DCs to produce more IL-10 and IL-12.

Probiotics enhance cytokine levels associated with Th1 and Treg cells in CD4$^+$ T cells co-cultured with DCs

To explore the effect of probiotic consumption on DC and CD4$^+$ T cell differentiation, we use a cell culture model to study cytokine levels in the supernatants of CD4$^+$ T cells co-cultured with monocyte-derived DCs. Bio-Three upregulated IFN-γ (associated with Th1) and IL-10 (associated with Treg cells) levels in the supernatants of DCs and CD4$^+$ T cells co-cultured for 48 h (Figure 8).

DISCUSSION

The role of intestinal microflora as a modulator of the immune response has been studied intensively in recent years. *Lactobacillus* species are the most well-studied both *in vitro* and *in vivo*, and could have clinical importance in inducing phagocytosis and IgA secretion, modifying T-cell responses, enhancing Th1 responses, and attenuating Th2 responses[2,15-17]. However, how intestinal microbes interact with the mucosal immune system remains unclear[15]. The ability of different strains of *Lactobacillus* to induce production of key cytokines such as IL-12 and IL-10 varies markedly[2,8].

The functionally different CD4$^+$ Th cell subsets known as Th1 and Th2 have different cytokine profiles. The Th1/Th2 paradigm is relevant to the pathogenesis of several pathological conditions and provides the rationale for the development of new strategies for treating and preventing diseases. The development of polarized Th1 or Th2 responses depends on environmental factors [e.g. dose of antigen, nature of the immunogen, and cytokines (IL-4, IL-12 or interferons) at the time of antigen presentation], or on other undefined factors that mainly affect so-called natural immunity[18]. Th1-dominated responses are potentially effective in eradicating infectious agents, including those hidden within the host cells.

In our study, probiotics upregulated IFN-γ levels and moderately downregulated IL-4 in the supernatant of cultured PBMCs, which suggests that probiotics enhance the Th1 immune response and suppress the Th2 immune response.

The effects of consuming probiotics other than lactic acid bacteria are relatively unexplored. *Bacillus* strains are used in the treatment of diarrhea[19]. Additionally, they have antimicrobial activities, induce secretory IgA, IFN-γ, IL-12, IL-10 and TGF-β production, stimulate CD4$^+$ T cell proliferation, and suppress IL-4 levels[20,21]. The clinical and immunomodulatory effects of *Clostridium* and *Enterococcus* species are well documented in animal models. Heat-inactivated *C. butyricum* enhanced IFN-γ production, polyclonal antibody formation, and phagocytosis in a mouse model[22,23]. Moreover, culture supernatants of *C. butyricum* TO-A downregulate TLR4 expression in human colonic epithelial cells[24]. Feeding *E. faecium* SF68 to mice has been documented to antagonize *Giardia intestinalis* infection and increase the percentage of CD4$^+$ T cells in the Peyer's patches and spleen[25]. It has also been suggested that adequate *E. faecium* after antibiotic treatment improves the intestinal ecosystem, and thereby prevents the shift to Th2 immunity in neonatal mice[26].

The present study focused on the probiotic mixture Bio-Three of *B. mesentericus*, *C. butyricum* and *E. faecalis*. We speculated that each species of Bio-Three would modify the immune function differently, thus leading to more complex effects. To date, few studies have examined the clinical effects of Bio-Three. One study has found that Bio-Three prevents enterohemorrhagic *Escherichia coli* O157: H7 infection in rabbits[27]. Another has found that Bio-Three is effective in patients with ulcerative colitis that is refractory to conventional therapy[28]. Administration of Bio-Three to infants changes the composition of

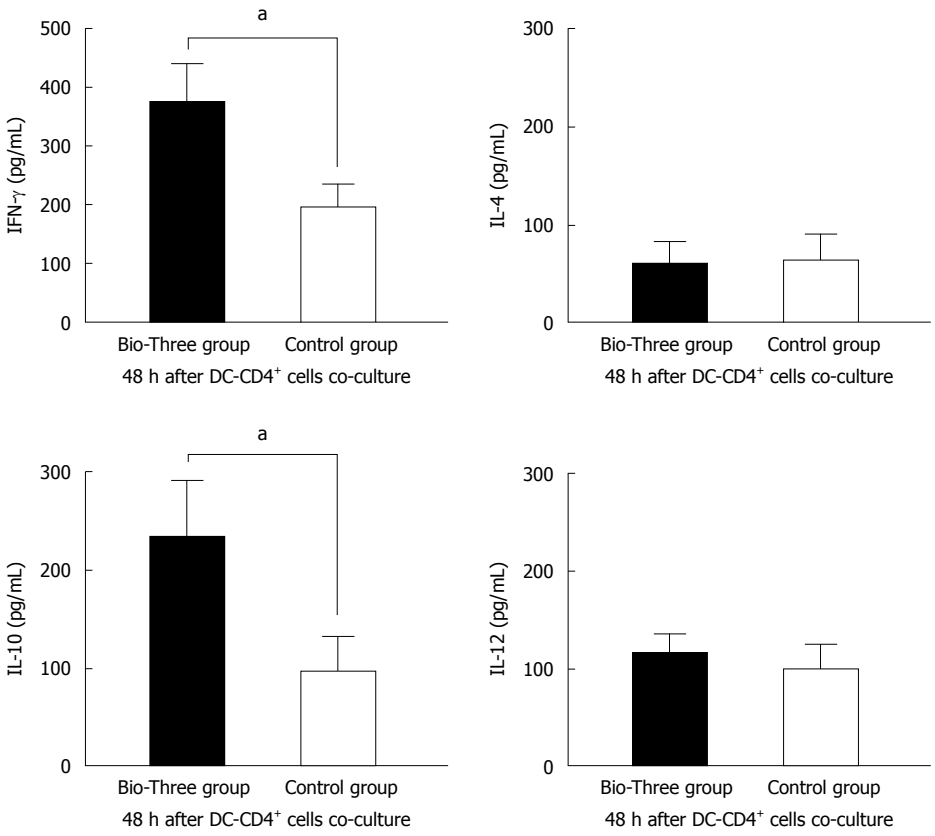

Figure 8 Interferon-γ, interleukin-4, interleukin-10, and interleukin-12 p70 cytokine profile of supernatants of human CD4$^+$ T cells co-cultured with dendritic cells. The data shown are the levels of cytokines in the probiotic group (black bar), and control group (white bar). Bio-Three upregulated interferon-γ (IFN-γ) and interleukin (IL)-10 levels in the supernatants of CD4$^+$ T cells co-cultured with dendritic cells at a ratio of 1:4 for 48 h. The results are presented as the mean ± SD. Statistically significant differences compared with the controls ($^aP < 0.05$).

intestinal flora and decreases serum endotoxin produced by potentially pathogenic microorganisms[29], and probably reduces infectious complications after pancreaticoduodenectomy[30].

In the present study, Bio-Three induced IL-10 production and increased the number of Treg cells (CD4$^+$ and CD25$^+$ T lymphocytes). IL-10 modulates immune responses by probiotic bacteria[2,15,31] by inhibiting synthesis of IL-2, IL-12 and TNF-α produced by cells such as APCs and Th1 cells[10,12,32]. Increased IL-10 production might explain why Bio-Three inhibits TNF-α production, which can be harmful at high levels[3,33].

IL-12 plays a central role in promoting the Th1 response[2,10]. Specific strains of *Lactobacillus* enhance IL-12 production by human mononuclear cells[34,35]. However, in our study, this effect was limited probably because of strains in Bio-Three were not stimulatory. Other explanations include inhibition by IL-10, shorter time of stimulation *in vitro* (24 h), and inadequate dose (around 10^9 organisms/d). In previous studies, daily doses of 10^{10}-10^{11} (but not < 10^9) organisms of probiotic lactic acid bacteria conferred physiological benefits[36,37]. In the present study, IL-12 level in both groups was low, therefore, the influence of Bio-Three dose on IL-12 production should be further investigated. Notably, not all probiotics species can induce IL-12 production.

We used a previously published method (10^5, 10^6 and 10^7 CFU/mL bacteria co-cultured with PBMCs (10^5/mL) for 1 d (the ratio of bacteria:PBMC was 1:1, 10:1 and 100:1) to investigate how the concentration of bacteria affects cytokine production[35,38,39]. A prior study has demonstrated that *Lactobacillus* dose-dependently stimulates PBMC expression of cytokines[39]. In our system, the optimum dose was around 10^6-10^7 CFU/mL, which was almost statistically significant ($P < 0.05$). However, cytokine production was less at the highest concentration (10^8 CFU/mL bacteria, data not shown), which suggested that stimulation of PBMCs was not fully dose-dependent. This inconsistency between the responses to Bio-Three and *Lactobacillus* may have been due to species differences. Moreover, the possibility that highly concentrated cell debris induces apoptosis, deletion, or cell death of PBMCs should be considered.

In our experiment, CD4$^+$, CD45RA$^+$ and CD25$^+$ T lymphocytes were upregulated, whereas CD14$^+$ cells showed no significant change. DCs are CD11b$^+$ cells that can differentiate from CD14$^+$ monocytes[10,40]. The expression of CD11b$^+$ cells in our study suggested that they were increased by exposure to Bio-Three. DCs regulate the development of T cell responses, especially the polarization of such responses[1,2]. It has been demonstrated that exposure to probiotic bacteria upregulates markers of DC maturation, such as HLA-DR and members of the B7 family (CD80, CD86)[10,31,41-44]. High but not low doses of probiotic organisms induce DC maturation, which suggests that different intracellular signal-

ing pathways are activated by high doses[10,45].

As described above, the level of HLA-DR (a marker of immune stimulation), which participates in DC signaling, is usually enhanced. In the present study, Bio-Three had an enhancing effect on expression of HLA-DR. Furthermore, co-culture with DCs and $CD4^+$ T cells, showed upregulation of IFN-γ (associated with Th1 cells) and IL-10 (regulatory cytokine) in the supernatants.

To date, the real pathways of probiotic immunomodulatory effects are not fully understood, and some types of immune cells that are primed by probiotics might be the connection between *in vivo* and *in vitro* stimulation. We proposed that PBMCs are involved in the *in vivo* sensitizing effect of Bio-Three and further *in vitro* stimulatory effects. The monocyte-derived DC transformation might play a key role in the mechanism of probiotic immunomodulation. DCs are the most potent APCs that are primed by probiotic bacteria, and they are the principal stimulators of naïve T cells to drive further immune responses.

In conclusion, we found that probiotics (Bio-Three) increased expression of IFN-γ (associated with Th1 cells), increased IL-10 production (anti-inflammatory cytokine), and decreased TNF-α level. The optimum concentration of Bio-Three for cytokine production was around 10^6-10^7 CFU/mL. It is reasonable to speculate that Bio-Three redirected the immune system toward an anti-inflammatory phenotype, rather than an aggressive immune response, even at a relatively low dose (10^9 organisms/d for 2 wk). Furthermore, the short term *in vivo* (2 wk) and *in vitro* (24 h) exposure was probably sufficient to promote a Th1 cell response and HLA-DR expression on DCs.

COMMENTS

Background

There is increasing evidence that probiotic bacteria influence host immune function, but the immune response in human peripheral blood after probiotic consumption is little known. Probiotic products, however, are usually consumed by the general population, but not much is known about the effects that they have on the immune system in healthy adults.

Research frontiers

It is not fully clarified how probiotics exert their beneficial effects, but one of the most probable mechanisms is the modulation of host immune responses. The possible action mechanism could be the ability to induce cytokines that further regulate innate and adaptive immune responses.

Innovations and breakthroughs

The present study was designed to explore the immune response of peripheral blood mononuclear cells (PBMCs) *in vivo* after stimulation by a probiotic preparation, such as cytokine production and phenotypic analysis of circulating PBMCs. The authors also investigated cytokine production of (1) PBMCs stimulated by probiotic preparation; (2) monocyte-derived dendritic cells (DCs); and (3) co-cultured DCs and T cells.

Applications

The authors found that the probiotic preparation Bio-Three (*Bacillus mesentericus*, *Clostridium butyricum* and *Enterococcus faecalis*) could direct immune responses to either a Th1 or anti-inflammatory response. More detailed information on the cytokine patterns elicited by probiotic bacteria could help in designing probiotic preparations for specific preventative or therapeutic purposes.

Peer review

This is a well-written paper with promising results that could be the basis of forthcoming new research on the therapeutic and immunomodulatory effects of probiotics.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3541

World J Gastroenterol 2010 July 28; 16(28): 3541-3545
ISSN 1007-9327 (print)

BRIEF ARTICLE

Hepcidin levels in hereditary hyperferritinemia: Insights into the iron-sensing mechanism in hepatocytes

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Received: March 29, 2010 Revised: May 19, 2010
Accepted: May 26, 2010
Published online: July 28, 2010

Abstract

AIM: To study the role of hepcidin in hereditary hyperferritinemia cataract syndrome (HHCS).

METHODS: Six patients from two families with HHCS, confirmed by genetic analysis showing A to G mutation at position +40 in the L-ferritin gene, were recruited to undergo serum hepcidin and prohepcidin measurements using radioimmunoassay and enzyme linked immunoassay, respectively, and measurements were compared with levels in serum from 25 healthy volunteers (14 females), mean age 36 ± 11.9 years.

RESULTS: The serum hepcidin and prohepcidin levels in patients with HHCS were 19.1 ± 18.6 and 187 ± 120.9 ng/mL, respectively. Serum ferritin was 1716.3 ± 376 μg/L. Liver biopsy in one patient did not show any evidence of iron overload. Serum hepcidin and prohepcidin values in healthy controls (HCs) were 15.30 ± 15.71 and 236.88 ± 83.68 ng/mL, respectively, while serum ferritin was 110 ± 128.08 μg/L. There was no statistical difference in serum hepcidin level between the two cohorts (19.1 ± 18.6 ng/mL *vs* 15.30 ± 15.71 ng/mL, P = 0.612) using two-tailed t-test.

CONCLUSION: Serum hepcidin levels in HHCS patients is similar to that in HCs. Our study suggests that circulating ferritin is not a factor influencing hepcidin synthesis and does not have a role in the iron-sensing mechanism in hepatocytes.

Key words: Hereditary hyperferritinemia; Hereditary hyperferritinemia cataract syndrome; Hepcidin; Hepcidin assay; Iron-sensing mechanism; Iron responsive element; Ferritin

Peer reviewers: Kostas Pantopoulos, Associate Professor, Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada; Sebastian Mueller, MD, PhD, Professor of Medicine, Department of Internal Medicine, Salem Medical Center, and Center for Alcohol Research, University of Heidelberg, Zeppelinstraße 11 - 33, Heidelberg 69121, Germany

Arnold J, Sangwaiya A, Manglam V, Thursz M, Beaumont C, Kannengiesser C, Busbridge M. Hepcidin levels in hereditary hyperferritinemia: Insights into the iron-sensing mechanism in hepatocytes. *World J Gastroenterol* 2010; 16(28): 3541-3545 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3541.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3541

INTRODUCTION

The iron-sensing mechanism has been widely studied but is not yet fully defined. In iron-replete states, circulating transferrin carrying iron to hepatocytes competes with hemochromatosis-Fe protein (HFE-protein) to bind to transferrin receptor-1 (TfR-1)[1]. Transferrin loaded with iron has greater affinity with TfR-1 than does HFE-protein. When transferrin binds to TfR-1, HFE-protein is freed up to bind to transferrin receptor-2 (TfR-2). This complex is thought to operate as an iron sensor mechanism and functions as an inducer of hepcidin production *via* SMAD (small mothers against decepentaplegic homologue) pathway[2]. Under normal circumstances, hepcidin expression and subsequent release into plasma prevents further absorption of iron from the duodenal enterocytes by preventing the efflux of iron by ferroportin channels and hence reduced amounts of iron delivery *via* transferrin to hepatocytes[3].

Ferritin is an iron storage protein and its synthesis is controlled at the level of transcription[4] and mRNA translation by an iron response mechanism. The control process depends on a highly conserved motif at the 5' non-coding region of ferritin mRNA. Studies have shown that ferritin is synthesized in the liver and has two subunits, L (19 kDa, Light) and H (21 kDa, Heavy)[5]. Different proportions of component L and H subunits give rise to isoferritins with tissue specific distributions, with H-ferritin as the major iron storage protein. The L-ferritin and H-ferritin genes are based in chromosomes 19 and 11, respectively.

Regulation of ferritin synthesis involves an interaction between an iron binding protein, termed as the iron regulatory protein (IRP), and ferritin mRNA[6,7]. The translational regulation of ferritin mRNA involves two IRPs, IRP-1 and IRP-2. Only IRP-1, but not IRP-2, contains an iron-sulphur complex and is bifunctional, registering intracellular iron status mainly through an iron-sulphur switch mechanism and alternating between an active cytosolic aconitase form and an apoprotein that binds iron responsive elements (IREs). Although IRP-2 is homologous to IRP-1, IRP-2 activity is regulated primarily by iron-dependent degradation through the ubiquitin-proteasomal system in iron-replete cells. Targeted deletions of IRP-1 and IRP-2 in animals suggest that IRP-2 is the chief physiologic iron sensor[8]. A constant region of the ferritin mRNA molecule, termed the IRE, binds with IRP. The resultant IRE-IRP complex inhibits ribosomal binding to mRNA and prevents translation of the ferritin coding sequence[6]. A critical CAGUGU sequence within IRE is important for binding with IRP.

In iron overload, as in hereditary hemochromatosis, the IRE-IRP inhibitory system is suppressed and ferritin synthesis is increased. When this form of iron storage is saturated, iron is deposited as hemosiderin in tissue[9]. Thus ferritin is a sequel of intracellular iron metabolism and not a part of the iron sensor mechanism itself.

Hereditary hyperferritinemia cataract syndrome (HHCS) is an autosomal dominant disorder characterized by premature cataract formation and raised serum L-ferritin in the absence of iron overload[10,11]. We have earlier described this syndrome in a family with 11 members, six members from three generations having genetic mutation at +40 (A to G mutation) corresponding to the critically conserved nucleotide motif in L-ferritin mRNA IRE on chromosome 19[12]. We have recently discovered another family with five members from two generations; two members confirmed as having the genetic mutation as described above. In this manuscript, we describe serum levels of hepcidin and prohepcidin in HHCS and discuss the iron-sensing mechanism in hepatocytes.

MATERIALS AND METHODS

A prospective study was performed with approval of our Regional Ethics Committee and written consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki. Patients and healthy volunteers were recruited from a single hospital with mixed ethnicity mainly comprising Caucasians and South Asians living in West London.

Healthy controls

The serum study comprised of 25 healthy controls (HCs); 14 were females, mean age was 36 ± 11.9 years (age range 21-62 years), who were hospital colleagues recruited to measure serum hepcidin-25, prohepcidin and ferritin as well as routine biochemical profile. HCs on supplemental vitamins and oral iron were excluded. None of the participants had received any blood products in the past. A morning fasting venous blood sample was collected and serum stored at -20℃ for analysis.

Patients

Six patients from two families with HHCS were recruited for serum hepcidin, prohepcidin and ferritin analysis. All six patients had cataracts with limited reduction in visual acuity and all had hyperferritinemia but normal transferrin saturation. Venous blood samples were obtained and serum separated and stored at -20℃ until analysis. None of the recruited patients had chronic inflammatory disease and none were on oral iron supplementation.

Liver biopsy was performed on one of the affected individuals (A Ⅱ-1) to determine tissue iron status using Perl's Prussian blue staining. Blood samples from family members were analyzed for genetic mutation within the L-ferritin IRE.

Genetic analysis

Polymerase chain reaction amplification and DNA sequencing were carried out as described previously[12,13]. Direct cycle sequencing of the 5' untranslated region of the L-ferritin gene from three affected members from Family A (A Ⅰ-2, A Ⅱ-1 and A Ⅱ-5) revealed that all three patients were heterozygous for A to G point mutation at position +40. Family B (B Ⅱ-1 and B Ⅱ-3) underwent genetic analysis and revealed the same mutation as mentioned above. This corresponds to position 2 of the CAGUGU motif within the L-ferritin IRE mRNA.

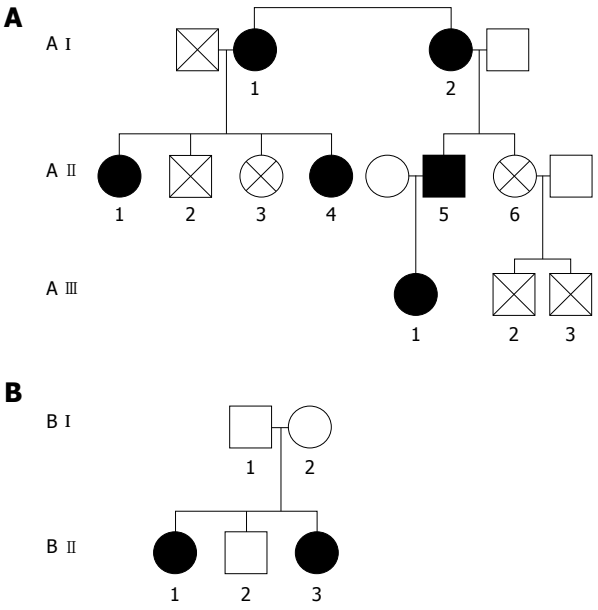

Figure 1 Family tree of two families Family A (A) and Family B (B) with hyperferritinemia. Solid symbols indicate those with ferritin in excess of 800 μg/mL; symbols with crosses indicate ferritin concentrations within the normal range; clear symbols indicate spouses in whom the ferritin concentrations have not been measured.

Hepcidin and prohepcidin analysis

We have earlier described development of a radioimmunoassay for measurement of serum hepcidin-25[14]. Serum prohepcidin was measured by enzyme linked immunoassay (DRG Diagnostics, UK). Serum ferritin was measured using a standard solid phase, two site chemiluminescent immunometric assay (Immulite).

Statistical analysis

Quantitative variables were compared using unpaired *t*-test. A value of $P < 0.05$ was considered significant. All statistical analyses were carried out using the statistical package GraphPad Prism, version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

The serum hepcidin and transferrin saturation values for all six patients with HHCS were similar to HCs. Figure 1 describes the relationship between the various family members and Table 1 describes the relation of serum ferritin to cataract. The mean ± SD serum hepcidin in patients with HHCS was 19.1 ± 18.6 ng/mL as compared to an HC mean value of 15.68 ± 15.7 ng/mL, and there was no statistically significant difference between cohorts ($P = 0.612$). However, there was a highly significant difference ($P < 0.0001$) in serum ferritin level between the two cohorts; mean serum ferritin for HHCS patients was 1716 ± 376 μg/L, as compared to mean value of 110 ± 128 μg/L for HCs (Table 2). Liver biopsy performed on one patient did not reveal iron overload.

Table 1 Relation between serum ferritin concentrations and cataracts

Family member	Serum ferritin (μg/L)	Transferrin saturation (%)	Cataract
A I 1	1542	18	Yes
A I 2	1581	17	Yes
A II 1	2049	22	Yes
A II 4	988	17	Yes
A II 5	1855	26	Yes
A III 1	902	37	Yes
A II 2	37	21	No
A II 3	49	NA	No
A III 2	15	16	No
A III 3	19	NA	No
B I 1	NA	NA	Yes
B I 2	NA	NA	No
B II 1	2143	9	Yes
B II 2	NA	NA	Yes
B II 3	1128	37	Yes

NA: Not available.

Table 2 Demographic and biochemical results for healthy controls and hereditary hyperferritinemia cataract syndrome patients (mean ± SD)

	Healthy controls	HHCS cohort	Reference values
No. (M:F)	25 (11:14)	6 (1:5)	
Age (range), yr	36 (21-62)	44.65 (21-75)	
Hb (g/dL)	13.8 ± 1.3	12.8 ± 2.3	13-17
MCV (fL)	87.8 ± 5.8	90.4 ± 3.6	80-100
Iron (μmol/L)	16.4 ± 4.0	15.6 ± 6.7	10.6-28.3
Ferritin (μg/L)	110 ± 128	1716.3 ± 376	30-400
Prohepcidin (ng/mL)	236.88 ± 83.68	187 ± 120.9	NA
Hepcidin (ng/mL)	15.68 ± 15.7	19.1 ± 18.6	NA
TIBC (μmol/L)	59.2 ± 9.6	70.67 ± 19.9	41-77
Tsat %	28.8 ± 9.6	21.5 ± 9.5	NA
Vitamin B12 (pg/mL)	280.4 ± 153	324.2 ± 127.2	180-914
Serum folate (ng/mL)	8.2 ± 4.0	5.5 ± 1.9	3.1-17.5
Bilirubin (μmol/L)	10.44 ± 5.5	5.3 ± 3	0-20
ALP (IU/L)	73.3 ± 26.6	77.5 ± 11	40-129
ALT (IU/L)	30.3 ± 22.4	17.7 ± 6.9	10-50
Albumin (g/L)	46.2 ± 2.4	42.7 ± 4.2	34-50

Reference range as followed by Department of Biochemistry, Ealing Hospital NHS Trust, University of London, United Kingdom. HHCS: Hereditary hyperferritinemia cataract syndrome; Hb: Hemoglobin; MCV: Mean corpuscular volume; TIBC: Total iron binding capacity; Tsat %: Transferrin saturation; ALP: Alkaline phosphatase; ALT: Alanine transaminase; NA: Not available.

DISCUSSION

HHCS is now a well recognized entity[15,16]. The only clinical manifestation in these patients appears to be early onset cataracts. Further studies on other family members who have undergone cataract extraction have confirmed that the cataracts represent deposits of L-ferritin subunits[17]. To our knowledge, this is the first ever study of hepcidin and prohepcidin levels in patients with HHCS. Our patients had normal transferrin saturation with liver biopsy on one patient ruling out tissue iron overload. Serum hepcidin levels in HHCS patients were similar to those in HCs.

The iron-sensing mechanism in hepatocytes is poorly understood. Unlike the ferritin mRNA, there is no iron regulatory element sequence in the hepcidin gene that is directly influenced by cellular iron[18]. Kemna *et al*[19] have put forward the hypothesis that several pathways are involved in hepcidin regulation. Three of these are active regulation pathways (erythropoietic activity derived regulation, iron store based regulation, and inflammation induced regulation) and one is an independent mandatory signaling pathway.

Hemojuvelin (HJV)-controlled transcription factors, such as the bone morphogenic protein (BMP)/SMAD signaling pathway, appears to be a mandatory signaling process for the influence of iron stores and erythropoiesis derived hepcidin regulation[19,20]. Any direct influence of intracellular iron on hepcidin regulation is not well elucidated. It is known that iron activates BMP-6, an upstream regulator of hepcidin. Moreover, it has also been suggested that hepcidin responds to increases in transferrin saturation. Recent reports suggest that a transmembrane protease (matriptase-2) inhibits hepcidin activation by cleaving membrane HJV[21,22].

The inflammation regulatory pathway for hepcidin regulation is mainly induced by interleukin-6 (IL-6) with a cascade involving IL-6 receptor Janus kinase, signal transducer and activator of transcription (STAT 3)[23].

A syndrome of non-hereditary liver iron overload in patients with modestly raised serum ferritin but normal transferrin saturation has also been reported. This disorder is distinct from both hereditary hemochromatosis and HHCS and seems to be associated with hyperlipidemia and impaired glucose tolerance[24]. The metabolic syndrome candidate genes, upstream stimulating factor (*USF*) *1* and *2* in chromosome 19, are in direct control of the hepcidin anti-microbial peptide gene (*HAMP* gene, chromosome 19 q13), suggesting a link between lipid, glucose, and iron metabolism.

Our study confirms that L-ferritin in patients with HHCS is not a factor in the regulation of hepcidin synthesis. It is therefore most likely that iron saturation of transferrin plays a key role in the iron-sensing mechanism in hepatocytes. The iron overload reported in patients with congenital atransferrinemia also supports this hypothesis[25].

ACKNOWLEDGMENTS

We would like to thank Dr. Bharati Bhatkal for helping us with the recruitment of patients and Dr. Frank Geoghegan, Department of Biochemistry, Ealing Hospital NHS Trust, for his helpful advice and discussion.

COMMENTS

Background

Hereditary hyperferritinemia cataract syndrome (HHCS) is an autosomal dominant disorder characterized by L-ferritin hyperferritinemia and premature cataract formation. The defect is due to a point mutation or deletion in the L-ferritin iron responsive element (IRE) and the resultant changes in the structure of the L-ferritin IRE preventing its interaction with the iron regulatory protein. Hepcidin is an iron regulatory peptide predominantly formed in the liver. Hepcidin has been shown to be low in the various types of hereditary hemochromatosis such as hemochromatosis-Fe-related C282Y/C282Y, hemojuvelin- and transferrin receptor-2-related hemochromatosis. No data has been available on the levels of hepcidin in HHCS to date. This research study examined serum levels of hepcidin in two families with HHCS and set out to explain the iron-sensing mechanism in the liver.

Research frontiers

Hepcidin and its role in iron metabolism has been a topic of intense research recently. The initial hurdle to measure hepcidin in serum has partly been resolved with the development of assays to measure the peptide in serum, e.g. radioimmunoassay and enzyme-linked immunosorbent assay. HHCS is a benign disorder of iron metabolism and we report for the first time serum levels of hepcidin in this disorder. This should help in further understanding the regulation of hepcidin in various disorders.

Innovations and breakthroughs

The authors report for the first time the level of serum hepcidin in patients with HHCS. Results prove that circulating ferritin has no role in the iron-sensing mechanism.

Applications

The authors used radioimmunoassay to measure serum levels of hepcidin in patients and healthy controls. Use of this assay would help explore the role of hepcidin in various iron disorders.

Peer review

In this work Arnold *et al* measure the iron regulatory hormone hepcidin (and its precursor pro-hepcidin) in sera from patients with HHCS. They report that levels of hepcidin are within the normal range in these patients and conclude that serum ferritin is not involved in body iron sensing. These findings are not surprising, but are nevertheless interesting and worthy of publication.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3546

World J Gastroenterol 2010 July 28; 16(28): 3546-3552
ISSN 1007-9327 (print)

BRIEF ARTICLE

Bacteriolytic therapy of experimental pancreatic carcinoma

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Supported by Grant Number 2006/A29 from the Else-Kröner Fresenius Stiftung to Linnebacher M and Klar E
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Received: January 15, 2010 Revised: February 4, 2010
Accepted: February 11, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate the effectiveness of *Clostridium novyi* (*C. novyi*)-*NT* spores for the treatment of established subcutaneous pancreatic tumor in the syngeneic, immunocompetent Panc02/C57Bl/6 model.

METHODS: *C. novyi-NT* spores were applied intravenously to animals carrying established pancreatic tumors of three different sizes. Systemic immune responses in peripheral blood and spleen were examined by flow cytometry. Supplementary, cytotoxic activity of lymphocytes against syngeneic tumor targets was analyzed.

RESULTS: Application of spores identified, that (1) small tumors (< 150 mm^3) were completely unaffected (n = 10); (2) very large tumors (> 450 mm^3) responded with substantial necrosis followed by shrinkage and significant lethality most likely due to tumor lysis syndrome (n = 6); and (3) an optimal treatment window exists for tumors of approximately 250 mm^3 (n = 21). In this latter group, all tumor-bearing animals had complete tumor regression and remained free of tumor recurrence. In subsequent tumor rechallenge experiments a significant delay in tumor growth compared to the initial tumor cell inoculation was observed (tumor volume at day 28: 197.8 ± 87.3 mm^3 *vs* 500.1 ± 50.9 mm^3, $P < 0.05$). These effects were accompanied by systemic activation of immune response mechanisms predominantly mediated by the innate arm of the immune system.

CONCLUSION: The observed complete tumor regression is encouraging and shows that immunotherapy with *C. novyi-NT* is an interesting strategy for the treatment of pancreatic carcinomas of defined sizes.

Key words: Bacteriolytic immunotherapy; *Clostridium novyi-NT*; Immune response; Pancreatic carcinoma

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Maletzki C, Gock M, Klier U, Klar E, Linnebacher M. Bacteriolytic therapy of experimental pancreatic carcinoma. *World J Gastroenterol* 2010; 16(28): 3546-3552 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3546.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3546

INTRODUCTION

Spontaneous regression of tumors in patients with concurrent bacterial infections was described for the first time over 300 years ago[1]. Two centuries later, it was William Coley who developed pioneering work on a treatment strategy using bacteria. He applied-with remarkable results-a combination of *Streptococcus pyogenes* and *Serratia marcescens* to patients suffering from inoperable tumors[2,3]. With the emergence of modern chemotherapy, bacteriological therapy of cancer fell more and more into oblivion. However, in 1976 Morales *et al*[4] reintroduced the principle into the clinic with the development of a treat-

ment regimen for superficial bladder cancer using bacillus Calmette-Guerin. Remarkably, this therapy is still in use today.

It is well known that hypoxic zones in poorly vascularised or necrotic tumors are a major handicap in cancer therapy preventing effective delivery of therapeutic medications to tumor cells or limiting radiation efficacy. On the other hand, these zones of severe hypoxia offer the opportunity for tumor-specific colonization with anaerobic bacteria along with subsequent tumor destruction[5]. Anaerobic bacteria are strictly limited to growth in oxygen-deprived areas and have several advantages compared to viruses such as (1) ease of production and storage stability; (2) no interference with the genome of the recipient; and (3) the possibility of complete eradication of bacteria with antibiotics[5]. In the 1960's several reports described the lytical effects of specific clostridial strains targeting tumors by colonization of hypoxic tumor areas[6,7]. In the past few years, several *Clostridium* species have been studied for their antitumor potential[8]. In 2001, the Vogelstein group investigated a variety of anaerobic bacteria, including several *Clostridium* strains[9]. They found *Clostridium novyi* to be the best at colonizing tumors. A detoxified strain of *C. novyi* [*C. novyi non-toxic* (*NT*)] was shown to germinate and grow within the avascular regions of xenografted human cancer and to destroy surrounding tumor cells in immunodeficient mice. Similar results were obtained for the treatment of experimental murine cancer in syngeneic, immunocompetent animals. They showed complete tumor regression in up to 30% of treated animals with renal and colorectal tumors[10]. Successfully treated animals even rejected rechallenged tumors and remained disease free. The authors presumed a substantial participation of immune mechanisms in order to explain their results[10].

So far, there are no experimental data regarding the antitumoral effects of *C. novyi-NT* against pancreatic carcinomas. Our study therefore examines the efficacy of *C. novyi-NT* treatment in a syngeneic pancreatic carcinoma mouse model. These experiments demonstrated that bacteriolytic therapy is effective in eradication of pancreatic tumors, although strictly dependent on the initial tumor size. We found that (1) small tumors (< 150 mm^3) were completely unaffected; (2) very large tumors (> 450 mm^3) responded with substantial necrosis followed by shrinkage accompanied by significant lethality; and (3) an optimal treatment window exists for tumors of approximately 250 mm^3 finally resulting in long-term tumor-free survival.

MATERIALS AND METHODS

Animals and tumor cell line

Experiments were performed on female 8-10-wk-old C57Bl/6N mice (Charles River, Fa. Wiga, Sulzfeld, Germany) weighing 18-20 g. All animals were fed standard laboratory chow and given free access to water. Experiments were performed in accordance with the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, vol.25, no.28, 1996).

The pancreatic tumor model using Panc02 cells was originally described in 1984 by Corbett *et al*[11]. Panc02 cells were cultivated in Dulbecco's modified Eagle's medium/Ham's F12 medium, supplemented with 10% FCS and 200 mmol/L Glutamine and were incubated at 37℃ in an atmosphere of 5% CO_2 under humidity of 95%. All media and supplements were from PAA unless stated otherwise (Cölbe, Germany).

Bacteria

Clostridium novyi-NT spores were kindly provided by Bert Vogelstein (Oncology Center, Johns Hopkins University, Baltimore, USA). Obtained spores were first cultivated on reinforced clostridial medium (Difco, Le Pont de Claix, France). About three to five days after cultivation in an anaerobic atmosphere, colonies of vegetative bacteria were transferred into cooked meat medium (Difco) for sporulation. Spores settled in the cooked meat particle layer and were purified from contaminating vegetative forms on a discontinuous Percoll gradient (70%, 55%). Aliquots of 7.5 × 10^7 spores were preserved in phosphate buffered saline (PBS) until injection.

Tumor model and treatment regimen

Under brief ether anaesthesia 1 × 10^6 Panc02 cells were injected subcutaneously (s.c.) into the right hind leg. Tumor growth was routinely controlled at least twice a week and tumor volume was estimated according to the formula: V = $width^2$ × length × 0.52.

Three groups with different tumor volumes were defined (150 mm^3, n = 10, 250 mm^3, n = 21 and 450 mm^3; n = 6). After reaching the predetermined tumor volumes, 7.5 × 10^7 spores were injected intravenously (i.v.) *via* tail vein injection in 50 μL PBS. As controls, one tumor-carrying group and one group without tumor received PBS (vehicle) alone.

Animals were euthanized at day 28 post-treatment or when they became moribund due to extensive tumor growth. Subsequently, complete tumor, spleen, mesenteric lymph nodes, femur bone marrow and blood samples were collected for further analysis.

Rechallenge experiment

After successful treatment (defined as macroscopic tumor disappearance), respective animals (n = 5) received a second dose of 1 × 10^6 Panc02 tumor cells into the left hind leg contralateral to the initial tumor cell injection site. Tumor volumes were evaluated twice a week as described above.

Flow cytometry

Flow cytometry was performed with leukocytes from peripheral blood, spleen and bone marrow. Leukocytes from spleens were isolated after homogenization and subsequent lysis of erythrocytes in lysis buffer (0.17 mol/L Tris, 0.16 mol/L NH_4Cl). Purified bone marrow cells were obtained after washing in PBS.

Whole blood cells and isolated leukocytes from spleens

and bone marrow were labelled using the following fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated rat anti-mouse monoclonal antibodies (mAbs): CD3ε FITC (1 μg, ImmunoTools, Friesoythe, Germany), CD11b FITC, CD11c FITC, CD19 FITC, CD4 PE, CD8 PE, CD62L PE, Gr1 PE (1 μg, Miltenyi Biotec, Bergisch-Gladbach, Germany) and NK1.1 FITC (0.5 μg BD Pharmingen) followed by lysis of erythrocytes in the case of whole blood cells (FACS Lysing Solution, BD Pharmingen, Heidelberg, Germany). Negative controls consisted of lymphocytes stained with the appropriate isotypes (BD Pharmingen). Samples were analyzed on a FACSCalibur Cytometer (BD Pharmingen). Data analysis was performed using CellQuest software (BD Pharmingen).

Flow cytometric cytotoxicity assay

Lytic activity of lymphocytes (spleen) from treated animals against target cells (Panc02, CMT-93, and MC3T3-E1) was determined by flow cytometry. Prior to co-culture, target cells were labelled with CFDA-SE (carboxyfluorescein diacetate succinimidyl ester, final concentration: 2 μmol/L). Target cells without effector cells were used as negative controls. Following co-incubation for five hours at an effector to target cell ratio of 30:1 and 10:1, propidium iodide (PI) was added to measure death of target cells based on carboxylfluorescein diacetate/propidium iodide (CFDA/PI) double positive cells. Cytotoxicity was calculated according to the following formula: % cytotoxicity = experimental release-spontaneous release of target cells.

Statistical analysis

All values are expressed as mean ± SE. After proving the assumption of normality, differences between tumor control and treated animals were determined by using the unpaired Student's *t*-test. If normality failed, the nonparametric Mann-Whitney *U*-Test was applied. The tests were performed by using Sigma-Stat 3.0 (Jandel Corporation, San Rafael, CA, USA). The criterion for significance was set to $P < 0.05$.

RESULTS

Therapeutic effectiveness of C. novyi-NT is related to tumor size

Earlier experiments described complete eradication of tumors subsequent to intravenous injection of *C. novyi* spores. However, there is a lack of experimental data for the treatment of pancreatic carcinomas. This has prompted the investigation of whether bacteriolytic therapy is also applicable for this tumor entity. Different sizes of the tumor were chosen to establish an effective treatment regimen (Figure 1A).

In the first series of experiments, animals with small tumors below 150 mm^3 were treated. Macroscopically, tumor growth was completely unaffected, with tumor sizes remaining comparable to controls until the endpoint (day 21: 1036.6 ± 232.9 mm^3 *vs* 1079.1 ± 164.3 mm^3). This was most likely due to the absence of intratumoral hypoxia and necrotic areas, thereby prohibiting germination of bacteria within the tumor. To overcome this, animals with large tumors (> 450 mm^3) were employed. All tumors responded with substantial necrosis and subsequent shrinkage within a few hours ($P < 0.05$ *vs* tumor control).

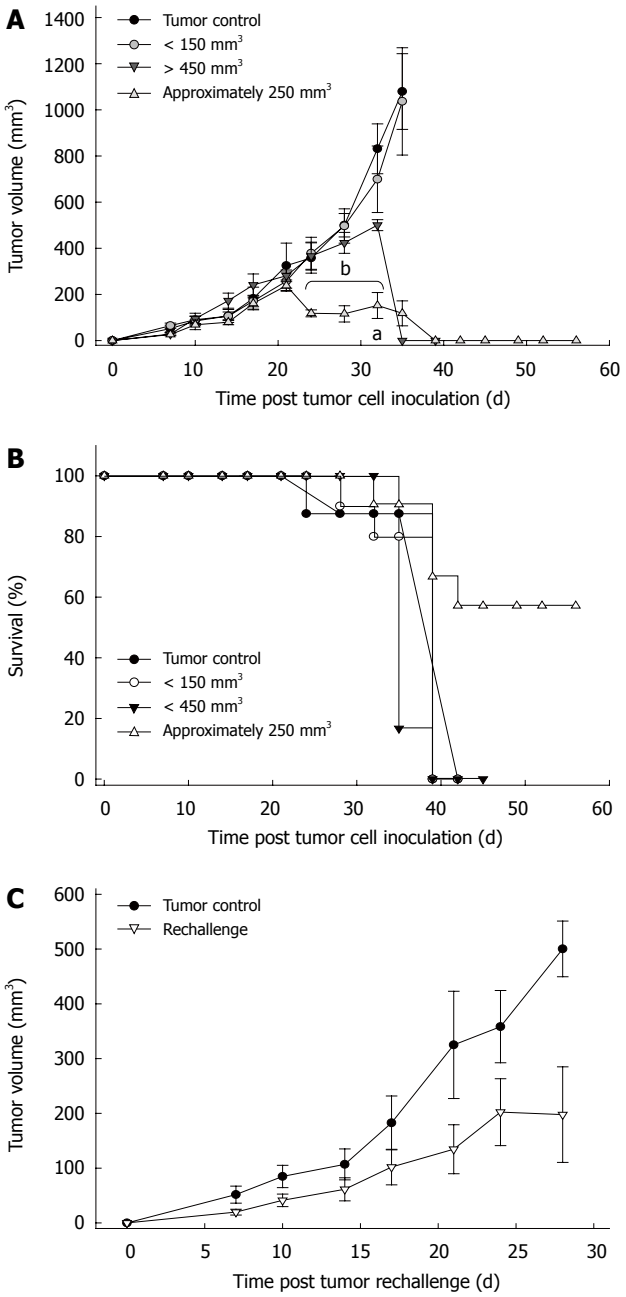

Figure 1 Data of *in vivo*-analyses of s.c. Panc02 tumors treated with *Clostridium novyi-NT* spores. A: Growth kinetics of s.c. Panc02 tumors; B: Survival curve of tumor-carrying mice after i.v. injection of *Clostridium novyi-NT* spores. For evaluation of the optimal therapeutic dose, different groups of tumor carrying mice were employed. These included tumor sizes below 150 mm^3 (n = 10), of about 250 mm^3 (n = 21) and larger than 450 mm^3 (n = 6). Experiments identified a tumor-size dependent toxicity and response rate. Small tumors were completely unaffected and remained comparable to controls. In contrast, larger tumors responded with substantial necrosis followed by shrinkage and subsequent complete regression; C: Growth kinetics of tumors after rechallenge. Successfully treated animals (n = 5) received a second tumorigenic dose of Panc02 cells at day 28 post therapy. Naïve animals were used as controls. As can be depicted from the graph, bacteriolytic therapy mediated partial protection from re-exposure to tumor cells. Values are given as the mean tumor volume (mm^3) ± SE; $^aP < 0.05$ vs tumor control; $^bP < 0.001$ *vs* tumor control; Mann-Whitney *U*-test.

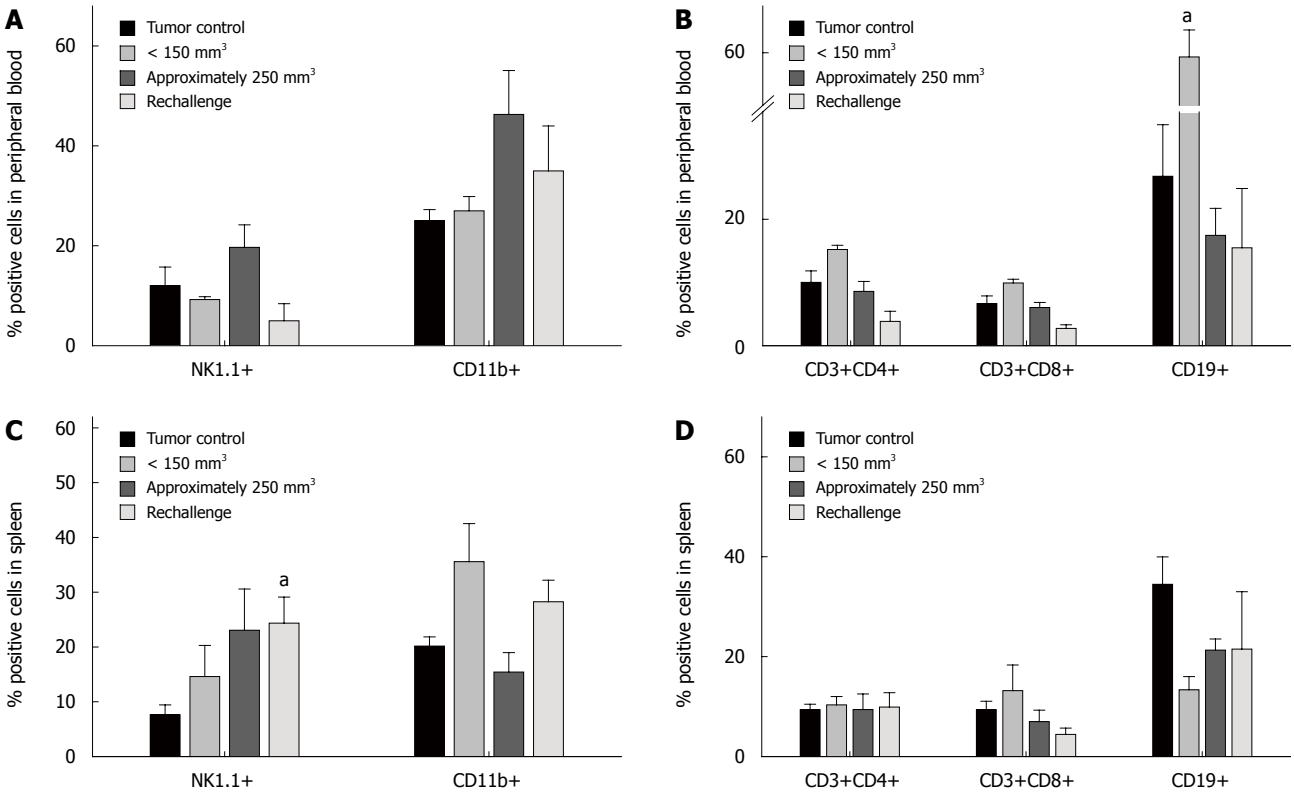

Figure 2 Flow cytometric analyses of leukocytes from peripheral blood (A, B) and spleens (C, D) in tumor control animals, of *Clostridium novyi-NT* treated animals (< 150 mm^3, approximately 250 mm^3) and after rechallenge. Bacteriolytic therapy predominantly activated the innate arm of the immune system. Values are given as mean ± SE. $^aP < 0.05$ *vs* tumor control; Mann-Whitney *U*-test.

Another group consisted of animals carrying tumors of about 250 mm^3. This tumor size was found to be optimal for *C. novyi* treatment. Tumors displayed rapid necrosis within 48 h and macroscopically disappeared 10-14 d later ($P < 0.05$ *vs* tumor control). There was no tumor regrowth until the experimental endpoint (day 31), indicative of complete tumor regression.

Toxicity of spores correlates with tumor size

Toxicity of *C. novyi-NT* spores is thought to be dependent on germination of bacteria within hypoxic regions of tumors. Accordingly, tumor-free control animals only receiving a single intravenous injection of spores displayed no clinical symptoms such as weight loss, lethargy or anorexia. Macroscopic examination of the inner organs revealed no abnormalities. Toxicity was also low in tumor-carrying animals where germination did not occur (Figure 1B). However, toxicity appeared to be greater when larger tumors were employed. We observed up to 40% lethality in mice carrying tumors of about 250 mm^3. Death was observed predominantly in the post-acute phase of infection between days 10-18. In this time, mice were cured of their tumors. Toxicity is thus related to germination of the bacteria within tumors. This finding is reinforced by the massive lethality post-treatment of larger tumors. All animals ($n = 6$) died within 24 h after spore administration.

Tumor rechallenge demonstrates partial protection

Next, we analyzed to what extent the bacteriolytic therapy not only led to tumor regression but also induced protective immunologic memory towards a re-exposure to Panc02 tumor cells. To perform these rechallenge experiments, successfully cured animals (approximately 250 mm^3) received a tumorigenic dose of Panc02 cells into the contralateral flank. Slowly growing tumors (197.8 ± 87.3 mm^3, $P < 0.05$ *vs* tumor control) developed in 4/5 mice four weeks after tumor inoculation, with one animal remaining tumor-free until the end of the experiment (Figure 1C). However, when compared with untreated, naïve mice (500.1 ± 50.3 mm^3), tumor development was significantly sustained (Figure 1C). Taken together, this indicates, that *C. novyi* therapy mediates potent antitumor reactivity *in vivo*. However, complete protective immunity was obtained in only 20% of the animals.

Immunologic effects by C. novyi NT treatment

Regression of tumors subsequent to *C. novyi-NT* treatment is thought to be, at least in part, due to the stimulation of immune response mechanisms. We therefore examined the influence of spore application on immune cells. Flow cytometric analysis of circulating leukocytes identified increased numbers of monocytes, granulocytes (CD11b$^+$) and NK cells (NK1.1$^+$) after treatment (250 mm^3 tumors), while levels of T (CD3$^+$/CD4$^+$, CD3$^+$/CD8$^+$) and B cells (CD19$^+$) were similar to controls (Figure 2A and B). Therapy of small tumors (< 150 mm^3), however, resulted in considerably elevated levels of CD19$^+$ B cells ($P < 0.05$ *vs* tumor control), but again, numbers of T cells remained

unchanged. Likewise, monocytes and NK cells showed no alteration (Figure 2B).

In spleens, *C. noyvi* mediated increases in NK cells (Figure 2C). Elevation of this cell population was highest after treatment of 250 mm^3 tumors. As well as in peripheral blood, levels of T and B cells were not affected by the application of bacteria (Figure 2D). This was also seen post-bacterial exposure in small tumors. Here, numbers of NK1.1 and CD11b positive cells were only slightly induced, while CD19 positive B cells were found to be down-regulated. Other analyzed cell populations, such as $CD11c^+$ dendritic cells and $Gr1^+$ granulocytes were also not induced (data not shown).

To sum up these findings, bacteriolytic treatment predominantly induced the innate arm of the immune system.

Functional immune responses evoked by C. novyi-NT treatment

We next examined whether immune cells isolated from treated animals were able to react against tumor cells. In a functional cytotoxicity assay, splenocytes were used as effectors. Panc02 tumor cells were lysed by immune cells from successfully treated mice. Similar results were obtained with the syngeneic colorectal cell line CMT-93 (Figure 3). However, this reaction was not restricted to tumor cells. Reactivity of splenocytes to the non-cancerous MC3T3-E1 fibroblasts was also observed, indicative of non-specific stimulation of immune cells. Thus, these effects were more likely mediated by activated NK cells than by tumor-antigen specific T cells.

DISCUSSION

The cure of cancer by severe bacterial infections was reported by W.B. Coley over a century ago. These historical observations have provided a basis for active microbial immunotherapy, which has been extensively studied in recent years. In 2001, the Vogelstein group demonstrated in pioneering work that spores of *C. noyvi-NT* are very efficient in eradicating established solid tumors[9]. These effects could be boosted when combined with standard chemotherapy[9], resulting in long-term protective immunologic memory. Similarly, other bacteria such as *Salmonella* and *Mycobacteria* have been shown to stimulate an inflammatory response with neutrophil-directed cytokines leading to a potent cellular antitumoral immune response[12-14].

Among the more frequent tumors, pancreatic cancer has an outstandingly poor prognosis. Recent treatment regimens have so far failed to substantially improve the clinical outcome of this tumor entity. Moreover, immunotherapeutic approaches aiming to specifically stimulate the host's immune system against tumor cells are rare in pancreatic malignancies. Here, we investigated the effectiveness of *Clostridium* spores for the treatment of established Panc02 tumors in the syngeneic, immunocompetent C57Bl/6 model. We found that (1) small tumors (< 150 mm^3) were completely unaffected; (2) very large tumors (> 450 mm^3) responded with substantial necrosis

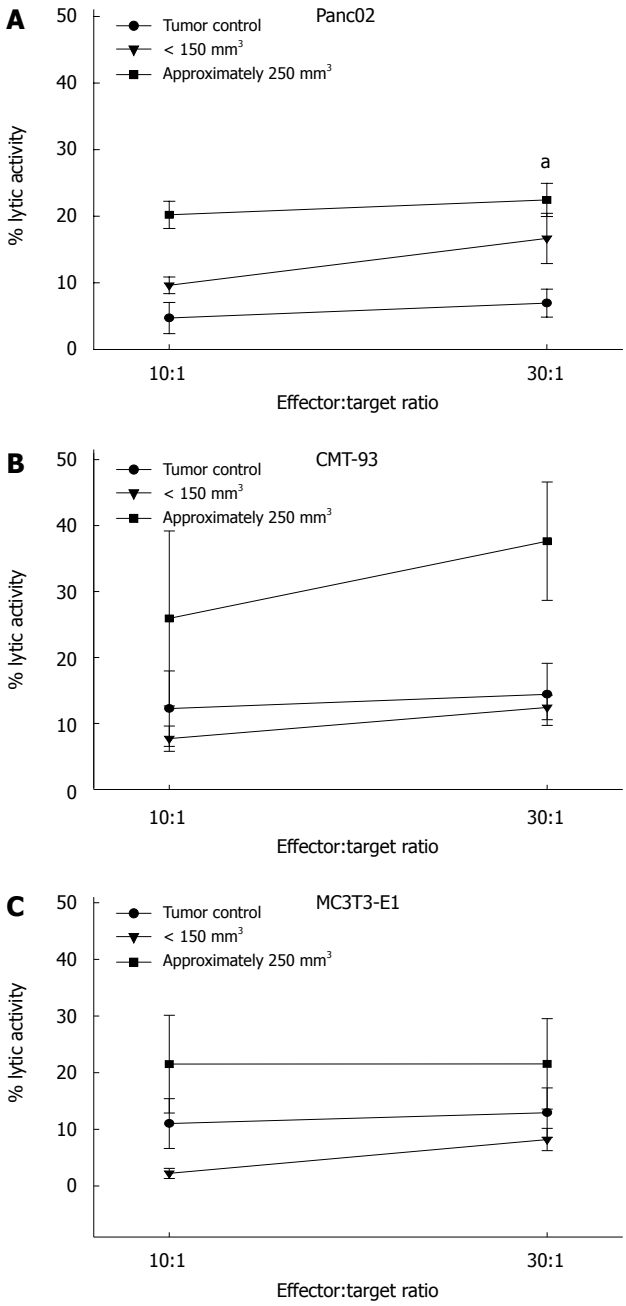

Figure 3 Quantitative analysis of cytotoxicity using flow cytometric carboxylfluorescein diacetate/propidium iodide staining. Lymphocytes were isolated from spleens and co-cultured with targets for five hours at E:T cell ratios of 10:1 and 30:1. Lymphocytes from successfully treated animals showed lytic activity against (A, B) syngeneic tumor cells with highest reactivity against CMT-93 cells at an E:T ratio of 30:1. However, cytotoxicity was found to be non-tumor specific, as reactivity was also observed against (C) non-malignant fibroblasts MC3T3-E1. Values are given as mean ± SE; $^aP < 0.05$ *vs* tumor control; *t*-test.

followed by shrinkage but accompanied by significant lethality (80%); and (3) an optimal treatment window exists for tumors of approximately 250 mm^3. These findings are, in part, consistent with previous studies in other tumor entities, where therapeutic effectiveness and toxicity were also found to be related to the size of the tumor. However, lethality was predominantly observed after combined bacteriolytic therapy, but not after administration of spores or chemotherapeutics alone even when large tu-

mors were employed[9]. In 2005, Diaz *et al*[15] described the relationship between toxicity, spore dose and tumor size. They found an increased mortality at higher spore doses injected into Balb/c mice carrying large tumors and concluded a germinating-dependent toxic effect of *C. novyi*. In our study, we also observed massive toxicity only after treatment of mice with large tumor burdens. On the other hand, mortality was absent in animals carrying no tumors or only small tumors, where anoxic areas allowing for bacterial germination were missing. It is therefore conceivable that toxicity was related to germination of the bacteria within tumors. This germination occurred rapidly in larger Panc02 tumors which contained extended areas of necrosis resulting from inadequate oxygenation and nutrition. As a consequence, massive destruction of neoplastic cells occurred, accompanied by the release of intracellular ions and metabolic by-products into the systemic circulation that may lead to metabolic complications and death. This phenomenon is recognized in the clinic as "tumor lysis syndrome" usually occurring in patients with bulky, rapidly proliferating tumors responding to treatment. Clinically, the syndrome is characterized by the development of hyperuricaemia, hyperkalaemia, hyperphosphataemia, hypocalcaemia, and acute renal failure[16]. Nevertheless, the tumor lysis syndrome appeared not to be the lethal cause in our experimental setting since clinical parameters, such as levels of serum uric acid and calcium were not altered (data not shown).

Another important fact of bacterial therapy is the activation of immune response mechanisms. It has been reported that *C. novyi* infection is associated with inflammation and subsequent development of a potent immune response. Consistent with these data, we also observed raised numbers of circulating NK cells, granulocytes and monocytes even four weeks post-infection. Of note, this immune stimulation was paralleled by additional signs of systemic inflammation like reactive splenomegaly. In subsequent rechallenge experiments, a growth retardation of tumors was observed. However, in a functional *in vitro* cytotoxicity assay, these effects were found not to be tumor specific as reactivity was also observed towards non-malignant syngeneic cells like fibroblasts. Thus, in our study, the bacterial treatment predominantly activated the innate arm of the immune system. Most likely, NK cells are the main cytotoxic cell type involved in recognition of Panc02 tumor cells. These observations are to some extent contrary to those described by Agrawal and coworkers, who achieved prevention of tumor growth subsequent to adoptive transfer of $CD8^+$ lymphocytes from tumor-carrying mice cured by *C. novyi-NT* application.

Recently, we provided convincing data that experimental pancreatic tumors can be successfully treated with the facultative anaerobic bacterium *S. pyogenes*. Living bacteria as well as bacterial lysates mediated regression of established tumors in the very same syngeneic mouse model[17,18]. Compared to these results, we found here that despite their ability to induce substantial tumor necrosis even after application of only small numbers into large tumors, *C. novyi* spores are substantially more toxic, and most importantly from the immunological point of view, are a less potent inducer of tumor-reactive T cells.

ACKNOWLEDGMENTS

We thank Professor B. Vogelstein for kindly supplying us with *C. novyi-NT* spores.

COMMENTS

Background

Pancreatic carcinoma has an outstandingly poor prognosis. This has prompted research on alternative treatment approaches that may complement conventional anticancer strategies. In this context, the therapeutic application of bacteria is equally simple and promising.

Research frontiers

Immunotherapy has repeatedly been shown to have a very strong antitumoral potential. Most groups focus on tumor antigens together with dendritic cell vaccination. Recently, several groups have developed novel antitumoral approaches using tumor-therapeutic bacterial strains.

Innovations and breakthroughs

Vogelstein and coworkers proved that anaerobic bacteria such as *Clostridium noyvi* (*C. noyvi*)-*NT* selectively germinate and grow within hypoxic regions of tumors. However, they always combined the i.v. administration of lethal toxin-free spores of *Clostridium novyi* with standard chemotherapy. This is the first report on the therapeutic effectiveness of *Clostridium novyi*-spores as a single agent for the treatment of pancreatic carcinoma. Additionally, the study showed that this effect is due to an immunological response dominated by the innate immune system.

Applications

The observed complete tumor regression is encouraging and shows that immunotherapy with *Clostridium novyi*-spores is an interesting strategy for the treatment of pancreatic carcinomas of defined sizes.

Peer review

The study investigates a novel approach for pancreatic cancer treatment through applying anaerobic bacteria. The idea behind this approach is that anaerobic bacteria which are strictly limited to growth in oxygen-deprived areas would colonize in tumors which have hypoxic areas but not in normal organs. The study investigates the effectiveness of *C. novyi-NT* spores on the established tumor in a subcutaneous model of pancreatic cancer. The authors show that the effects of *C. novyi-NT* on the pancreatic tumor depend on the size of the tumor.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3553

World J Gastroenterol 2010 July 28; 16(28): 3553-3560
ISSN 1007-9327 (print)

BRIEF ARTICLE

Promoter methylation status of *hMLH1*, *MGMT*, and *CDKN2A/p16* in colorectal adenomas

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Supported by A 2-year grant of the Greek Ministry of Health and Welfare, No. 111K/56
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Received: December 19, 2009 Revised: February 3, 2010
Accepted: February 10, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate aberrant DNA methylation of CpG islands and subsequent low- or high-level DNA microsatellite instability (MSI) which is assumed to drive colon carcinogenesis.

METHODS: DNA of healthy individuals, adenoma (tubular or villous/tubulovillous) patients, and colorectal carcinoma patients who underwent colonoscopy was used for assessing the prevalence of aberrant DNA methylation of human DNA mismatch repair gene mutator L homologue 1 (*hMLH1*), Cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*), and O-6-methylguanine DNA methyltransferase (*MGMT*), as well as their relation to MSI.

RESULTS: The frequency of promoter methylation for each locus increased in the sequence healthy tissue/adenoma/carcinoma. *MGMT* showed the highest frequency in each group. *MGMT* and *CDKN2A/p16* presented a statistically significant increase in promoter methylation between the less and more tumorigenic forms of colorectal adenomas (tubular *vs* tubullovillous and villous adenomas). All patients with tubulovillous/villous adenomas, as well as all colorectal cancer patients, showed promoter methylation in at least one of the examined loci. These findings suggest a potentially crucial role for methylation in the polyp/adenoma to cancer progression in colorectal carcinogenesis. MSI and methylation seem to be interdependent, as simultaneous *hMLH1*, *CDKN2A/p16*, and *MGMT* promoter methylation was present in 8/9 colorectal cancer patients showing the MSI phenotype.

CONCLUSION: Methylation analysis of *hMLH1*, *CDKN2A/p16*, and *MGMT* revealed specific methylation profiles for tubular adenomas, tubulovillous/villous adenomas, and colorectal cancers, supporting the use of these alterations in assessment of colorectal tumorigenesis.

Key words: Promoter methylation; Microsatellite instability; Human DNA mismatch repair gene mutator L homologue 1; O-6-methylguanine DNA methyltransferase; Cyclin-dependent kinase inhibitor 2A

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Psofaki V, Kalogera C, Tzambouras N, Stephanou D, Tsianos E, Seferiadis K, Kolios G. Promoter methylation status of *hMLH1*, *MGMT*, and *CDKN2A/p16* in colorectal adenomas. *World J Gastroenterol* 2010; 16(28): 3553-3560 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3553.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3553

INTRODUCTION

Chromosomal instability and microsatellite instability (MSI) are two well-known pathways of colorectal carcinogenesis[1,2]. Recently, the CpG island methylation phenotype has been added to these two pathways. This novel pathway is characterized by the concordant methylation of the promoter regions of multiple genes that play a role in carcinogenesis[3], an alteration that has also been associated with the process of aging. CpG island DNA methylation, the most extensively studied epigenetic alteration in neoplasms, represses gene transcription by excessive and aberrant methylation of CpG-rich regions, called "CpG islands", in the 5' region of genes, leading to transcriptional silencing of the promoter and therefore inactivation of the gene[4,5].

Aberrant DNA methylation of CpG islands (CpG island methylator phenotype, CIMP) is also linked with subsequent low- or high-level DNA MSI[6-8]. Methylation of the human DNA mismatch repair gene mutator L homologue 1 (*hMLH1*) is the principal mechanism underlying the pathogenesis of sporadic high-level MSI (MSI-H) colorectal cancer (CRC)[6] and methylation of another DNA repair gene, O-6-methylguanine DNA methyltransferase (*MGMT*), is linked with low-level MSI (MSI-L)[7,8]. Moreover, epigenetic inactivation of the cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*) by methylation has been observed in both adenomas and CRC[9,10]. These hypermethylated genes are not only probable pathogenic events in the polyp to cancer progression sequence, but are also neoplasm-specific molecular events that have the potential to be used as molecular markers for pre-malignant tumors in the colon.

In this study, we used high-sensitivity methylation-specific polymerase chain reaction (PCR) (MSP) assays for *hMLH1*, *CDKN2A/p16*, and *MGMT*, three genes previously shown to be aberrantly methylated in pre-malignant neoplasms in the colon[6-10]. We have applied these assays to DNA extracted from blood, normal tissue, adenomas (tubular, villous, or tubulovillous), and colon cancer of patients who underwent first time colonoscopy at the University Hospital of Ioannina, Greece. We assessed the prevalence of aberrant DNA methylation and its correlation to MSI status, as well as the temporal order and the time of their appearance during the different steps of adenoma to carcinoma progression, in an attempt to identify their possible use as molecular markers of colon carcinogenesis and furthermore their pathogenic role in the transformation of colon neoplastic adenomas to carcinoma.

MATERIALS AND METHODS

Study design

The study was designed to include patients undergoing first time colonoscopy for routine clinical indications, organized in four different groups, on account of their colonoscopic and histopathological data. Patients without colonoscopic findings and histologically normal mucosa consisted group I (normal individuals - G I). Patients with tubular adenomatous polyps comprised group II (G II). Patients with polyps of increased tumorigenic potential (tubulovillous or villous adenomatous polyps) formed group III (G III), and, finally, patients with colonoscopic findings and histological evidence of CRC comprised group IV (G IV).

Patients

Seventy nine patients (44 males and 35 females, mean age: 62.5 ± 13.9 years) who underwent colonoscopy for routine clinical indications or for colon cancer screening at the Hepatogastroenterology Unit of the University Hospital of Ioannina, were included in this study, after approval from the Review Board of the University Hospital of Ioannina. Each group consisted of approximately 20 consecutive patients defined by histopathological analysis. Thus, we obtained 18 patients with tubular adenomas (10 M/8 F), 21 patients with tubulovillous or villous adenomas (12 M/9 F), and 20 colon cancer cases (11 M/9 F). Finally, 20 adenoma-free patients matched by sex and age (11 M/9 F) formed the control group.

The study was performed on freshly obtained lesions from adenomas or cancerous tissue that were resected at the time of colonoscopy. Matched normal colorectal mucosa was obtained from the resection margin that was furthest from any malignant lesion. Blood samples withdrawn at the time of colonoscopy were also included in this study. Patients with a prior history of inflammatory bowel disease, genetic CRC syndromes, or any other cancers, were excluded from our study.

Collection of tissue and blood DNA samples

DNA from the blood, fresh normal, and abnormal tissue of patients who underwent colonoscopy was extracted using the QiaAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Methylation-specific PCR

Methylation of the CpG promoter region of the three genes *hMLH1*, *CDKN2A/p16*, and *MGMT* was determined by MSP after bisulfite-modification of DNA samples (blood, cancerous, and non-cancerous tissue) as previously described[11,12]. These three genes have been

Table 1 Primer sequences, annealing temperature, and product sizes for methylation-specific polymerase chain reaction assays

CpG status	Genes	Forward primer (5′→3′)	Reverse primer (5′→3′)	Genomic position[2]	Annealing temperature (℃)	Product size (bp)
M	*hMLH1*	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	-716 to -602	55	115
U		TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	-721 to -598	55	124
M	*MGMT*[1]	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	+142 to +223	62	121
U		TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	+137 to +230	62	133
M	*CDKN2A/p16*	TTATTAGAGGGTGGGGCGGATCGC	CCACCTAAATCGACCTCCGACCG	+167 to +401	65	234
U		TTATTAGAGGGTGGGGTGGATTGT	CCACCTAAATCAACCTCCAACCA	+167 to +401	55	234

[1]All of the primers for the *MGMT* gene were modified with a 20 bp GC-rich tail (5′-GCGGTCCCAAAAGGGTCAGT-3′) at their 5′ end; [2]GenBank (PubMed). M: Methylated; U: Unmethylated; *hMLH1*: AB017806; *MGMT*: DD183709; *CDKN2A/p16*: DQ406745.

employed in several earlier methylation studies of CIMP and CRC. In brief, the procedure was as follows: The extracted DNA underwent bisulfite modification (2 μg DNA is necessary for each experiment) using the EZ DNA methylation Gold kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The modified DNA was used immediately for MSP or stored at -20℃ for further analysis. The bisulfite-treated DNA was subject to MSP in a blinded manner using primer pairs designed to specifically amplify the methylated or unmethylated alleles for the respective genes (Table 1). Each PCR reaction mix consisted of a total volume of 50 μL containing: 3.5 mmol/L of $MgCl_2$ (2 mmol/L for the methylated reaction); 1 × PCR Gold Buffer (Applied Biosystems, Weiterstadt, Germany); 250 μmol/L deoxynucleotide triphosphates mixture (Promega, Madison, USA); 0.1 μmol/L of forward and reverse primers (Invitrogen GmbH, Karlsruhe, Germany); 2.5 Units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Weiterstadt, Germany), and the appropriate amount of bisulfite-treated DNA. The thermocycler conditions were in general as follows: 95℃ for 10 min; 55 cycles of 30 s each at 95℃, specific annealing temperature for 30 s, and 1 min at 72℃; and a final extension of 10 min at 72℃. The PCR products were then subjected to horizontal gel electrophoresis on a 25 g/L agarose gel, stained with ethidium bromide, and visualized under UV transillumination. All MSP assays were repeated at least twice to validate the results. A set of known methylated and unmethylated control DNA samples was included in each round of bisulfite treatment.

The specificities of the MSP assays were confirmed by sequence analysis of aberrant methylation bands using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, USA), in a LI-COR 4200 DNA sequencer (LI-COR Inc., USA), in parallel with the corresponding normal DNA samples, as described previously[12].

MSI testing

The MSI status of the 79 patients was assessed using the reference panel of five pairs of microsatellite primers: BAT25, BAT26, D2S123, D5S346, and D17S250, known as Bethesda markers[13]. PCR reactions and primer sequences have been described previously[14]. The 5'-labelled PCR products are loaded onto a 66 cm denaturing 6 mol/L urea-acrylamide gel and analyzed in a LI-COR 4200 DNA sequencer. Each PCR was run twice to ensure reproducibility of results in case the band shifts were not clearly informative in the first attempt. The MSI phenotype was identified by the presence of abnormal bands in the polyp/adenoma or carcinoma tissue DNA that were not present in blood or normal tissue DNA. MSI high (MSI-H) polyps/adenomas or carcinomas were defined as those where two of the five Bethesda markers were unstable. MSI low (MSI-L) samples were defined as a shift in only one of the five markers. Samples showing no allelic shifts were termed as MSI stable (MSS).

RESULTS

MSP for hMLH1, CDKN2A/p16 and MGMT

Table 2 shows the methylation patterns of the four distinct patient groups for the promoters of the genes examined. Promoter methylation was detected even in the G Ⅰ group (healthy individuals). In this case, only patients aged over sixty showed methylation of some promoters (with one exception for MGMT concerning a 28-year-old woman). This indicates that methylation in normal patients is correlated to the age. By contrast,, in the patients of the G Ⅱ, G Ⅲ, and G Ⅳ groups, the frequency of methylation is similar in older and younger patients (> 60 years and $\leqslant 60$ years respectively, statistical data not shown), indicating that factors other than age are probably responsible for promoter methylation in these groups (Figure 1).

Among all samples investigated, promoter methylation of *hMLH1* or *CDKN2A/p16* was detected only in tissue samples and not in blood (indicating that methylation of these promoters is somewhat tissue/site specific). *MGMT* methylation was detected in tissue as well as in blood samples. The frequency of *MGMT* methylation was similar in blood and tissue samples only for the G Ⅰ patient group (46% in tissues *vs* 40% in blood, $P = 0.704$), as has been reported before and is also age related. However, in patient groups G Ⅱ, G Ⅲ, and G Ⅳ a significantly increased ratio in tissue samples *vs* blood samples (39% in tissues *vs* 6% in blood, $P = 0.022$; 76% *vs* 41%, $P = 0.035$ and 90% *vs* 64%, $P = 0.043$ for G Ⅱ, G Ⅲ and G Ⅳ, respectively) was observed. This implies that methylation of *MGMT* is

Table 2 Promoter methylation profiles for *hMLH1*, *CDKN2A/p16*, and *MGMT* in the four patient groups *n* (%)

Patient group	*hMLH1* (tissue)		*CDKN2A/p16* (tissue)		*MGMT* (tissue)		*MGMT* (blood)	
	M	U	M	U	M	U	M	U
G I (n = 20)	6/20 (30)	14/20 (70)	3/20 (15)	17/20 (85)	9/20 (46)	11/20 (54)	8/20 (40)	12/20 (60)
G II (n = 18)	7/18 (39)	11/18 (61)	4/18 (22)	14/18 (78)	7/18 (39)	11/18 (61)	1/18 (6)	17/18 (94)
	P = 0.563		P = 0.581		P = 0.666		P = 0.019[1]	
G III (n = 21)	10/21 (48)	11/21 (52)	14/21 (67)	7/21 (33)	16/21 (76)	5/21 (24)	7/17 (41)	10/17 (59)
	P = 0.576		P = 0.008[1]		P = 0.025[1]		P = 0.019[1]	
G IV (n = 20)	13/20 (65)	7/20 (35)	14/20 (70)	6/20 (30)	18/20 (90)	2/20 (10)	11/18 (64)	7/18 (36)
	P = 0.279		P = 0.837		P = 0.242		P = 0.245	

[1]Statistically significant in comparison to the previous group. M: Methylated; U: Unmethylated.

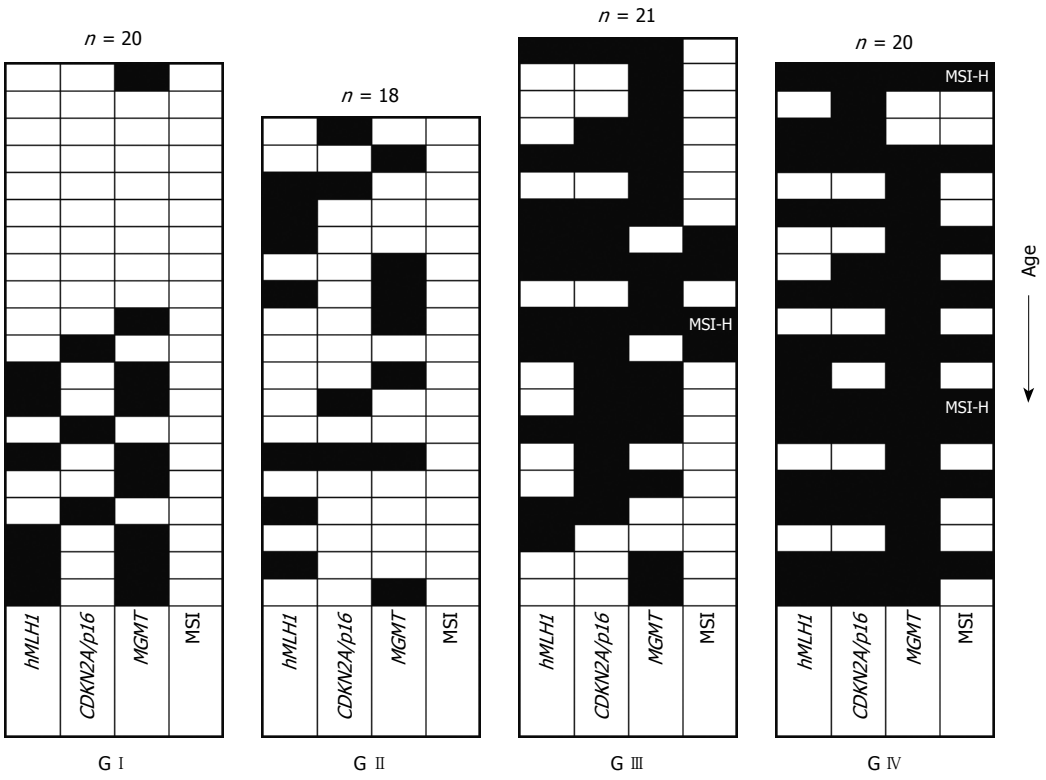

Figure 1 CpG island methylation at three loci and the microsatellite instability phenotype in G I (n = 20), G II (n = 18), G III (n = 21) and G IV (n = 20). The rows represent individual cases and the dark boxes indicate methylation or microsatellite instability (MSI) phenotype; the three high-level MSI (MSI-H) cases are also indicated.

also in part tissue/site specific. Another explanation for this could also be a low incidence of circulating cells having methylated promoters. In any case, this will require further investigation. The frequency of promoter methylation for each locus, as well as the number of methylated loci, increased from group G I to G IV (Table 2 and Figure 1), with *MGMT* showing a higher ratio. All of the methylated tissue samples also displayed evidence of unmethylated *hMLH1*, *CDKN2A/p16*, and *MGMT*, indicating that only one of the alleles is methylated or that only a part of the tissue contained cells that carried the methylated allele of the examined promoters (Figure 2).

It must be noted that *hMLH1* showed similar levels of promoter methylation between groups G II and G III (39% *vs* 48%, P = 0.576) in contrast to *CDKN2A/p16* and *MGMT*, which showed clearly increased levels of methylation in group G III (22% *vs* 67%, P = 0.008, and 39% *vs* 76%, P = 0.025, respectively), suggesting that *hMLH1* promoter methylation is an early phenomenon in comparison to polyp formation, while methylation of *CDKN2A/p16* and *MGMT* is correlated to the progression of polyps to more tumorigenic cases.

CDKN2A/p16 methylation was usually accompanied by methylation of another locus in tumorous and highly tumorigenic tissues (13/14 for G III and G IV), in contrast to the normal and low tumorigenic tissues (0/3 for GI and 2/4 for G II respectively). *MGMT* follows a rather different pattern, being in many cases the only methylated locus (5/7, 6/16, and 5/18 for G II, G III and G IV, respectively). The pattern for *hMLH1* is even more complicated (Figure 1).

Of the 18 tubular adenoma patients examined 14 (77%) showed methylation in at least one of the three tested loci, while four (22%) showed no evidence of pro-

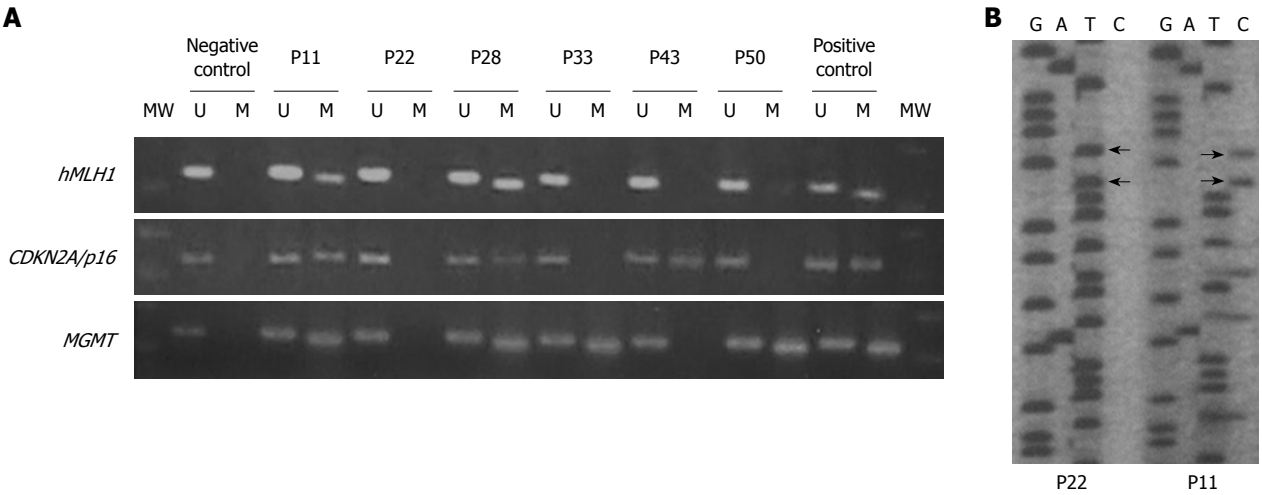

Figure 2 Methylation-specific polymerase chain reaction and sequence analysis. A: Methylation specific polymerase chain reaction (PCR) for *hMLH1*, *CDKN2A/p16* and *MGMT* promoter methylation. In this panel, the MSI-H patients P11 and P28 show promoter hypermethylation for *hMLH1*, *CDKN2A/p16*, and *MGMT*. The low-level MSI (MSI-L) patient P33 shows promoter hypermethylation for *MGMT*. The MSI stable (MSS) patient P22 does not show promoter hypermethylation for any gene, whereas the MSS patient P43 shows promoter hypermethylation only for *CDKN2A/p16*.The healthy MSS patient P50 shows promoter hypermethylation only for *MGMT*; B: Sequence analysis of methylation-specific PCR (MSP) PCR products of *hMLH1* promoter. The sequence analysis of MSP products for patients P22 (unmethylated) and P11 (methylated) reveals the complete transition of non-methylated cytosines to thymine (indicated by arrows) after bisulfite treatment. There also cytosines that are partially methylated, as indicated from the co-existence of T and C. The sequence from the bottom to top: 5'-TGG tGT TTG AtG TtG TGT TtG tGG GTA GT-3' P22 (unmethylated); 5'-TGG t(C)GT TTG At(C)G Tt(C)G TGT TCG CGG GTA GT-3' P11 (methylated); 5'-TGG CGT TTG ACG TCG TGT TCG CGG GTA GT-3' (wild type). Lower case letters represent thymines derived from unmethylated cytosines, the letters in parenthesis represent partially methylated cytosines and the bolds the CpG islands. MW: Molecular weight; M: Methylated promoter; U: Unmethylated promoter.

moter methylation, and only one (6%) showed methylation in all loci (Figure 1). All 21 G Ⅲ, as well as the 20 G Ⅳ patients (100%), showed promoter methylation in at least one locus. In particular, 6/21 (28%) and 11/20 (55%) for group G Ⅲ and G Ⅳ, respectively, were methylated in all loci, suggesting a potentially crucial role of methylation in the progression of CRC.

MSI status and hMLH1, CDKN2A/p16, and MGMT promoter methylation

According to our introductory remarks concerning the linkage between methylation status and MSI phenotype, we analysed the MSI status using the five Bethesda markers recommended by the NCI workshop[13]. Of the 79 samples examined, 66 (83%) were MSS, 10 (13%) were MSI-L and three (4%) were MSI-H (Figure 1). Only the G Ⅲ and G Ⅳ patients showed MSI, which reached 14% (3/21) for MSI-L and 5% (1/21) for MSI-H for the patient group G Ⅲ, whereas the corresponding percentages were 35% (7/20) and 10% (2/20) for MSI-L and MSI-H, respectively, in the G Ⅳ patient group.

Of the five Bethesda markers used for MSI analysis, BAT25 and BAT26 showed instability only in the MSI-H patients. On the other hand, the MSI-L phenotype was exclusively restricted to dinucleotide markers D2S123, D5S346, and D17S250 (3, 4 and 3 cases respectively). In the 13 cases with MSI-H or MSI-L, the following positive rates were obtained: 23% (3/13) in BAT26, 23% (3/13) in BAT25, 46% (6/13) in D2S123, 54% (7/13) in D5S346, and 46% (6/13) in D17S250, which is in accordance with previous references for cancer patients[15], as well as for patients with adenomatous polyps[16,17]. In a larger cohort of MSI-H cancer patients, Arnold *et al*[18] showed that the specificity of Bethesda markers was best for BAT26 and BAT25, with 99% and 95%, respectively. It is important to note that simultaneous *hMLH1*, *CDKN2A/p16*, and *MGMT* promoter methylation was present in 8/9 G Ⅳ patients, with the MSI phenotype as also being present in 2/4 patients of group G Ⅲ. All MSI-H patients showed methylation in all loci. From the remaining three patients, the two in group G Ⅲ showed methylation in two promoters (*hMLH1* and *CDKN2A/p16*) and the one G Ⅳ patient only in the *MGMT* promoter. The latter case also showed instability for the D2S123 marker. In the previous work of Arnold *et al*[18], all Bethesda markers, except the dinucleotide repeat D2S123, had a high detection rate (up to 90%) for the combination of MSI-H cancers and *hMLH1* methylation. This suggests that MSI-H cancers might originate from different pathways, e.g. one being caused by silencing the *hMLH1* gene and others by as yet unrecognized mechanisms.

DISCUSSION

The transformation of normal colon epithelial cells to adenomas, and then to cancer, is believed to be an evolutionary process in which neoplastic cells acquire heritable genetic and epigenetic alterations that drive the process of carcinogenesis[19]. Gene promoter hypermethylation is increasingly recognized to play an important role in cancer development through silencing gene transcription. It is also likely that the genetic and epigenetic alterations co-operate to promote tumor formation, and that the detection of colon polyps or adenomas that present aberrant promoter methylation might identify colonic epithelium that is at significant risk of acquiring genetic alterations

that will lead to colon tumor formation[20].

A number of studies have investigated the concordant methylation of multiple genes in colon adenoma[10,21,22]. Recently, aberrant promoter methylation found in different polyp forms[23,24] serves for a better understanding of aberrant CpG island methylation in the polyp/adenoma/carcinoma sequence of colorectal tumorigenesis. Thus, CIMP has been identified during several key stages in colon tumorigenesis, including aberrant crypt foci (ACF, the earliest identifiable neoplastic lesions in the colon), hyperplastic, tubular and tubulovillous/villous polyps, sporadic serrated adenomas, and tumors[3,10,22,25,26], suggesting that DNA methylation might be a pivotal event in the development of CRC. Furthermore, the aberrant methylation of *MGMT* and *hMLH1* has been shown to silence some genes and to result in cancer promoting events, such as an MSI phenotype or *k-RAS* mutation[27,28].

The molecular genetics of colorectal neoplasms have been studied extensively, but few studies have addressed the clinical and pathological associations in prospectively defined patient populations. Therefore, we studied specific promoter-methylation in a cohort that underwent colonoscopy including healthy individuals, polyp-bearing patients (more or less tumorigenic), as well as CRC patients.

Although we employed the MSP assay to detect methylated *hMLH1*, *CDKN2A/p16*, and *MGMT* DNA in the blood samples of patients of our cohort, we were able to detect methylated DNA in blood samples only for the *MGMT* promoter in all patient groups, including those with CRC. We note that Grady *et al*[11] used MSP in a study of 20 patients, detected methylated *hMLH1* DNA in the serum in 30% of patients with sporadic MSI colon cancer. This difference with our results might be attributed to the small number of MSI cancer patients of our cohort or to a different methylation site on the promoter.

We also observed increasing frequencies of promoter methylation as well as an increased number of methylated loci moving from group G Ⅰ to group G Ⅳ, for the three genes examined, which is in accordance with the data of Lee *et al*[29]. They also observed a stepwise increase in the number and frequency of methylated genes through the stages of multistep colorectal carcinogenesis in a study including twelve loci.

Adenomas with a villous component are generally larger than tubular adenomas and have been associated with a higher risk of CRC[30-32]. A number of studies have reported higher rates of methylation in adenomas with tubulovillous or villous histology (TVAs and VAs)[10,23,24,33,34] as compared to tubular adenomas (TA). Our study also demonstrated that methylation occurs more frequently in patients with TVAs/VAs (G Ⅲ) in comparison to patients with TAs for *MGMT* and *CDKN2A/p16* (statistically significant). By contrast, for *hMLH1,* the two groups showed a similar ratio. An increase in *MGMT* methylation has been demonstrated by other studies: 38% to 61% by Petko *et al*[23], 30% to 65% by Kim *et al*[33], and 37% to 87% by Kakar *et al*[24]. The higher rates of *MGMT* methylation in our study in comparison to previous ones could be attributed in part to the use of fresh tissue samples instead of paraffin-embedded tissue samples. Our results also showed an increased frequency of CDKN2A/p16 comparable with that of Petko *et al*[23], who found a ratio of 10% in the methylation of *CDKN2A/p16* for HPs and up to 30% for the adenomatous cases.

Furthermore, in our study, methylation, at least in one locus, reached 100% for TVAs/VAs and cancer patients, which is a higher value than that shown by Rashid *et al*[10].

It is likely that several different methylation pathways operate in CRC progression. Methylation of *MGMT* and *CDKN2A/p16* loci that are found to increase from TAs to TVA/VA could be a result of the adenoma to carcinoma progression, whereas methylation of *hMLH1* might be an initial step strongly associated with MSI-mediated carcinogenesis. The higher frequency of methylation in all loci for cancer patients compared to TVAs/VAs patients, as well as the increase in methylation from TAs to TVAs/VAs for *MGMT* and *CDKN2A/p16* supports this hypothesis.

The fact that MSI is evident in TVAs/VAs and that MSI and aberrant promoter methylation are observed simultaneously, suggests that MSI and hypermethylation are dependent on each other. As it has been shown before, that among 10% to 15% of the patients with colon cancer who have MSI, approximately 70% to 80% exhibit epigenetic gene silencing of the mismatch repair gene, *hMLH1*[6,27,35]. Moreover, a minor fraction of MSI-L and MSS cancers also appear to be methylated at the *hMLH1* promoter[36]. In our study, all MSI-H patients and 9/10 MSI-L patients showed simultaneous promoter methylation of *hMLH1,* while the remaining one MSI-L patient showed methylation of *MGMT*. However, not all MSI-L patients show inactivation of *MGMT* by promoter methylation, as we demonstrated for two of the G Ⅲ patients with the MSI-L phenotype (Figure 2).

The present study used MSP for detecting methylated alleles. MSP is a qualitative assay and does not provide quantitative information. Thus, the methylation detected by the MSP assay might not reflect gene expression, because the assay can detect only one methylated allele among 1000 unmethylated ones, and thus the vast majority of tumor cells may not harbor CpG island methylation of the given gene. The present study also shows that the methylated alleles of certain genes are present at an early stage of tumorigenesis, and that the number of genes with methylated alleles increases along the polyp/adenoma/carcinoma sequence, with *MGMT* being the most methylated gene.

In conclusion, our data indicates that aberrant CpG island hypermethylation occurs early and accumulates during multistep colorectal carcinogenesis, and that a temporal order exists in the methylation of tumor related genes. Furthermore, MSI is tightly connected to *hMLH1* as well as to *MGMT* promoter methylation, indicating that inactivation and/or overloading of the mismatch repair system might have a crucial role in driving CRC progression.

COMMENTS

Background

Colorectal carcinogenesis is a multistep process in which the progressive accumulation of genetic and epigenetic changes leads to a malignant transformation of normal epithelial cells to adenoma and, moreover, to cancer of the colon. CpG island DNA methylation, the most extensively studied epigenetic alteration in neoplasms, is characterized by the concordant methylation of the promoter region of many tumor suppressor and DNA repair genes, such as *hMLH1*, *CDKN2A/p16*, and *MGMT*, although their influence on disease progression remains inconclusive.

Research frontiers

Epigenetic changes usually begin very early in carcinogenesis, they are potentially reversible, and they can be thought of as one hit of the two-hits required for inactivation of carcinogenesis-related genes. For this reason, detection of aberrant methylation is important for early diagnosis, prognosis, and subsequent treatment of patients affected by this disease.

Innovations and breakthroughs

The frequency of promoter methylation for each promoter locus increases in the healthy tissue/adenoma/carcinoma sequence. *MGMT* shows the highest frequency in each group. *MGMT* and *CDKN2A*/*p16* present a statistically significant increase in promoter methylation between the less and more tumorigenic form of colorectal adenomas (tubular *vs* tubullovillous and villous adenomas). All tubulovillous/villous adenomas bearing patients, as well as all colorectal cancer patients, showed promoter methylation in at least one of the examined loci. These findings suggest a potentially crucial role for methylation in the polyp/adenoma to cancer progression in colorectal carcinogenesis. Microsatellite instability (MSI) and methylation seem to be dependent on each other, as simultaneous *hMLH1*, *CDKN2A*/*p16*, and *MGMT* promoter methylation was present in 8/9 colorectal cancer patients showing the MSI phenotype.

Applications

The results presented here show that a series of genetic and epigenetic molecular changes drives the next step during tumorigenesis in colorectal cancers, which results in a different molecular profile for each step. This underlines the need for a detailed record of the molecular situation, in order to establish the most efficient treatment for the patient. Prospective studies supplemented by the conventional study of prognostic factors, could improve the quality and accuracy of patients' prognosis and aid design of more efficient treatments.

Terminology

Epigenetic changes: Heritable changes in gene structure that do not include the changes in DNA sequence. CpG islands: CpG rich areas located in the promoter regions of many genes. CpG island methylation: The addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. Aberrant *de novo* methylation of CpG islands within the promoter region might lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation. MSI is a change in the length of DNA microsatellites due to the insertion or deletion of repeating units (usually 1-5 nucleotides long), caused by defects in mismatch repair genes (*MLH1*, *MSH2*, or *MSH6*, and others) or methylation of the *MLH1* promoter.

Peer review

In this study, Psofaki *et al* investigated promoter methylation of *hMLH1*, *p16* and *MGMT*, as well as MSI, in a cohort of samples from patients underwent colonoscopy. They found accumulation of promoter methylation events during colorectal tumor progression. Promoter methylation and MSI seem to be dependent on each other. These results confirm that epigenetic inactivation is an important mechanism of tumor suppressor gene silencing. The study was well designed and the results are interesting. The manuscript is well written and includes potentially interesting findings.

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S- Editor Wang JL **L- Editor** Stewart GJ **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3561

World J Gastroenterol 2010 July 28; 16(28): 3561-3566
ISSN 1007-9327 (print)

BRIEF ARTICLE

Primary site resection is superior for incurable metastatic colorectal cancer

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Received: February 21, 2010 Revised: April 6, 2010
Accepted: April 13, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate survival in patients treated with FOLFOX followed by primary site resection or palliative surgery for incurable metastatic colorectal cancer.

METHODS: Between 2001 and 2009, a total of 98 patients with colorectal adenocarcinoma and non-resectable metastases were diagnosed and treated with the new systemic agent chemotherapy regimen FOLFOX. Primary site resection was carried out in 38 patients, creation of a colostomy or bypass without resection was carried out in 36 patients, and 23 were not operated on because of advanced disease. The survival times of patients in different groups were analyzed.

RESULTS: There were no differences between the patients regarding their general condition, concurrent disease, or tumor stage according to AJCC classification. The median survivals of the three groups were 30.6, 20.8, and 12.7 mo (log-rank *P* value < 0.05), respectively. The postoperative complication rate was higher in the primary site resection group than in the palliative surgery group.

CONCLUSION: The results indicate that there are benefits from primary site resection for incurable metastatic colorectal cancer with systemic chemotherapy.

Key words: Colorectal cancer; Chemotherapy; Primary resection; Palliative surgery; FOLFOX; FOLFIRI

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Tanoue Y, Tanaka N, Nomura Y. Primary site resection is superior for incurable metastatic colorectal cancer. *World J Gastroenterol* 2010; 16(28): 3561-3566 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3561.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3561

INTRODUCTION

Colorectal cancer (CRC) is the most common cancer worldwide. The estimated 5-year survival rate ranges from 90% in patients with stage 1 disease to 10% in those with metastatic CRC (mCRC). Approximately 20% of patients with primary CRC present with stage 4 metastatic disease. For many years, the standard treatment for patients with mCRC has been systemic chemotherapy with fluorouracil (FU) and combination therapy. The standard treatment remains 5-FU based. In the past five years, chemotherapy has progressed dramatically and shifted from FU to the newer agents irinotecan, oxaliplatin, bevacizumab, and cetuximab. Patients treated with oxaliplatin, 5-fluorouracil, and leucovorin (FOLFOX) displayed a median survival of 19.5 mo which is the longest survival reported in a phase 3 trial[1]. Data from this and other trials suggest that patient survival might be prolonged with adjuvant chemotherapy; therefore the role of surgical treatment in these patients is controversial.

In localized (non-metastatic) disease, surgery is the primary treatment and can be curative. On the other hand, surgical resection of the primary lesion for patients with mCRC is indicated mainly to manage symptoms such as obstruction, perforation, or bleeding, but is of uncertain

benefit in the absence of these symptoms. Patient-dependent factors, such as age, medical comorbidity, extent of distant metastases, and localized invasion are among the factors that influence the decision to perform elective resection. The possible benefit of chemotherapy in conjunction with surgical management is unproven.

This study was designed to compare long-term survival and perioperative outcome in patients with incurable metastatic CRC treated with FOLFOX followed by either primary lesion resection, or palliative surgical management (colostomy or bypass).

MATERIALS AND METHODS

Patients

This retrospective, observational study was done using our hospital cancer database. From 2005 to 2009, all patients who underwent systemic treatment with FOLFOX for CRC at our hospital were identified and reviewed. Patients with non-resectable CRC at the time of diagnosis or operation were included in the study. Patients were excluded if they had metachronous metastasis from previous completely resected CRC, or they could not undergo any operation due to severe comorbidity, progression of cancer, or unwillingness to accept surgery. The remaining patients were stratified into two groups. In addition to chemotherapy, patients undergoing resection of their primary lesion were included in the primary site resection group, whereas those who underwent a bypass or stoma operation without resection for relief of obstruction or bleeding were included in the palliative surgery group. Demographics, comorbidity, and cancer-specific information were evaluated for each patient, including tumor location, grade, lymph node metastasis, liver, lung and peritoneal metastasis, survival, and mortality. Comorbidity was graded using the Charlson index. The location and volume of metastatic disease were evaluated by computer tomography and by direct visualization during surgery. Each tumor stage was coded according to the TNM classification as described in the AJCC. Patients with hepatic metastatic disease were further classified according to the volume of hepatic parenchymal replacement: < 25% (H1), 25 to 50% (H2), and > 50% replacement (H3). Patients with pulmonary and peritoneal involvement were stratified according to the appearance of their CT scan's (Table 1).

The FOLFOX4 protocol of systemic chemotherapy is as follows: Leucovorin (LV; 200 mg/m^2 per day in a 2-h infusion), followed by bolus 5-FU (400 mg/m^2 per day), and 600 mg/m^2 5-FU daily in a 22-h infusion, day 1 and 2 every 2 wk, plus oxaliplatin, 85 mg/m^2 (2-h infusion) on day 1, were administered through an implantable port and a disposable or electronic pump. Treatment was continued until progressive disease occurred (PD) or unacceptable toxicity occurred, or until the patient chose to discontinue treatment.

Statistical analysis

The principal outcome was survival. Survival was defined as the time from initiation of treatment, either chemotherapy or surgical operation, to the time of death. For patients who were alive at the time of analysis, data on survival were censored at the time of the last contact. The primary statistical analysis compared survival times between the two groups. Secondary analysis was conducted by comparing subgroups after excluding T4 patients. The Kaplan-Meier method was used to estimate actual control and survival curves. The log-rank test was used to evaluate the survival difference between two groups. All the *P* values were 2-sided and the level of significance was set at 0.05. The statistical analysis was conducted using JMP version 6.

Table 1 Patient characteristics *n* (%)

Parameter	Total	Primary resection	Palliative surgery
Total cohort	99	38 (100)	36 (100)
Age (median, range)	61.5	61 (50-79)	61 (31-83)
Sex			
M	39	20 (53)	19 (53)
F	36	19 (50)	17 (47)
Comorbidity[1]			
0	57	28 (74)	29 (81)
1	12	8 (21)	4 (11)
2	5	2 (5)	3 (8)
3	1	1 (3)	0 (0)
Primary site			
Cecum	3	2 (5)	1 (3)
Ascending colon	12	8 (21)	4 (11)
Transverse colon	6	2 (5)	4 (11)
Descending colon	6	5 (13)	1 (3)
Sigmoid colon	12	5 (13)	7 (19)
Rectum	35	16 (42)	19 (53)
Stage[2]			
T			
1	0	0 (0)	0 (0)
2	16	10 (26)	6 (17)
3	29	20 (53)	9 (25)
4	29	8 (21)	21 (58)
N			
0	18	8 (21)	10 (28)
1	31	19 (50)	12 (33)
2	35	11 (29)	14 (39)
H[3]			
0	31	11 (29)	20 (56)
1	10	5 (13)	5 (14)
2	7	5 (13)	2 (6)
3	26	17 (45)	9 (25)
Lung metastasis			
Yes	23	12 (32)	11 (31)
No	51	26 (68)	25 (69)
Peritoneal metastasis			
Yes	22	10 (26)	12 (33)
No	52	28 (74)	24 (67)

[1]Comorbidity defined using the Charlson index; [2]Obtained from the UICC classification; [3]The extent of liver involvement is defined: H1: Liver involvement less than 25%; H2: From 25% to 50%; H3: More than 50%.

RESULTS

Patient characteristics

Between 2005 and 2009, 172 patients were treated with FOLFOX in our hospital. Of these, 98 patients had

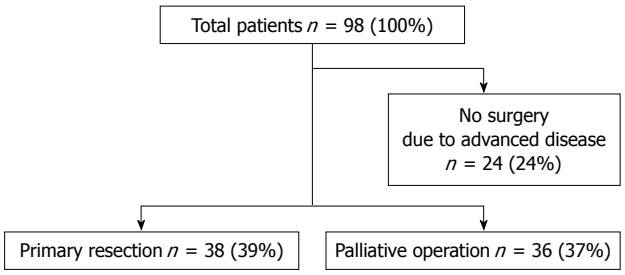

Figure 1 Breakdown of cohort by primary resection or palliative operation.

metastatic, locally advanced non-resectable CRC. Of these, 38 patients underwent initial resection of only their primary lesion without concomitant hepatic or pulmonary metastasectomy. Thirty-six patients did not undergo resection of their primary lesion, but had a palliative operation to alleviate symptoms of bleeding and obstruction, and 24 patients were excluded because they were not surgical candidates. This last group had a poor prognosis, with a median survival of 12.7 mo (Figure 1). Median age of the primary site resection group was 61 years (range 50-79 years) compared to 61 years (range 31-83 years) in the palliative surgery group. Primary tumor sites in both groups are shown in Table 1. There were no significant differences in the distribution of age, sex, primary site location, comorbidity, and metastatic disease, except for the T factor. Patients in the palliative surgery group were found to have more invasive and higher T factor lesions compared with the primary site resection group.

Chemotherapy

All patients were initially treated with FOLFOX4. The median duration of FOLFOX was 10 courses in primary site resection group and 11 courses in the palliative surgery group. The corresponding rates of response were 23% and 22%, respectively (P = 0.48). A total of 26 patients changed treatment to FOLFIRI (Irinotecan, Folinic acid, and 5-FU), 12 primary site resection patients and 14 palliative surgery patients. Bevacizumab and cetuximab were available from 2008. Four patients in each group were candidates for this new agent, and received the treatment without serious complications.

Survival time

Median survival time for the primary site resection group was 30.6 ± 7.8 mo, whereas in the palliative surgery group was 20.8 ± 10.2 mo. These median survival times were significantly different (P = 0.0094, log-rank test). The two-year actuarial survival in the primary site resection group was 67.2%, compared to 31.9% in the palliative surgery group (Figure 2A). We re-examined the mortality of the two groups to exclude the influence of localized invasion. After excluding all T4 patients, we found longer survival and higher rate of two-year survival in the primary site resection group. The median survival time was 30.6 ± 10.3 mo *vs* 17.3 ± 8.5 mo (P = 0.0149, log-rank test). Figure 2B shown the overall survival curves by treatment of primary resection or palliative surgery with FOLFOX (Modified group of T2-3 patients).

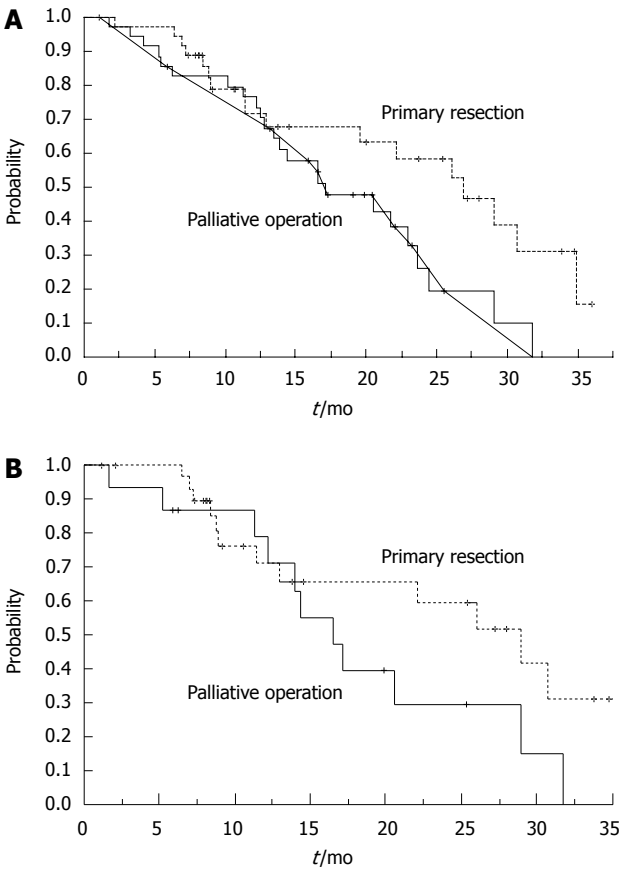

Figure 2 Overall survival curves by treatment of primary resection or palliative operation with FOLFOX (A), modified group of T2-3 patients (B).

Surgery

The operations performed are listed in Table 2. Of these, eight (21%) were emergent surgery in the primary site resection group and 10 (27%) in the palliative surgery group. In the primary site resection group, 16 of 38 patients experienced one or more postoperative complications *vs* 12 of 36 patients in the palliative surgery group. This relatively higher complication rate resulted in longer hospitalization (22.7 ± 12.9 d *vs* 18.1 ± 7.9 d) in the primary site resection group (Table 3). The two most common complications were wound infection and prolonged ileus, followed by urinary tract infection, pneumonia, catheter infection, gastric hemorrhage, and liver failure. In the primary site resection group, two patients (5.3%) developed anastomotic leaks and required reoperation for drainage and diversion. Among the patients who had undergone palliative surgery, two complications (5.5%) related to the primary tumor (one perforation and one bleeding) were observed. Both were managed conservatively.

DISCUSSION

The treatment of patients who present with mCRC is controversial. Although resection has often been advocat-

Table 2 Surgical procedure performed

Group	Procedure	*n*
Primary resection	Resection with anastomosis	27
	Resection with anastomosis + stoma	3
	Resection without anastomosis	8
Palliative operation	Creation of stoma	27
	Stoma + bypass	3
	Bypass	6

Table 3 Postoperative complications

Postoperative morbidity	Primary resection	Palliative surgery
Wound infection	6	5
Prolonged ileus	4	1
Intraabdominal/pelvic infection	1	2
Urinary tract infection	3	0
Anastomotic leak	2	0
Pneumonia	0	1
Catheter infection	1	0
Gastric hemorrhage	1	0
Liver failure	0	1
Perforation of cancer	0	1
Bleeding from cancer	0	1
Operational death	0	1
Total	18	13
Hospitalization (d) (P = 0.05)	22.7 ± 12.9	18.1 ± 7.9

ed to eliminate the source of symptoms such as bleeding, perforation and obstruction, management of asymptomatic patients has not been well defined. The purpose of our study was to define optimal primary tumor management in this subset of patients with metastatic disease. Our study demonstrated that the resection of the primary site significantly extends the median survival time in incurable CRC patients. Previous authors have addressed similar issues focusing in appropriate selection of surgical candidates, potential benefits of resection, and rates of operative morbidity and mortality. Some authors recommended resection to potentially improve survival[1-4].

Ruo *et al*[2] found a median survival of 16 mo in those undergoing resection and 9 mo in those never resected. Liu *et al*[3] also presented a retrospective series of 68 cases with longer mean survival of 10.6 mo in the resection group compared with 3.4 mo in patients who had a bypass. These findings are in agreement with our results. Others have reported similar results in terms of mortality between primary site resection and non-operative management, and recommend non-operative management of patients with metastatic CRC with synchronous metastases[5-7].

Charles *et al*[5] suggested that appropriately selected patients with incurable stage 4 CRC can be safely managed without primary tumor resection, with a median survival of 16.6 mo for the non-resection group. Johnson *et al*[7] found that in patients with rectal cancer and non-resectable liver metastases, there was no significant difference in cancer-specific survival after palliative resection or colostomy. Supporters of non-operative management argue that as metastatic tumor burden is the life-limiting factor, protracted postsurgical recovery might delay initiation of therapy targeting the disseminated disease, resulting in decreased survival. However, these studies have not addressed the effects of adjuvant chemotherapy including FOLFOX and FOLFIRI. In the study of George *et al*[2] of 233 patients with synchronous stage IV CRC treated by FOLFOX and FOLFIRI, 93% were managed non-operatively with 18 mo median overall survival. In comparison with our results, they might have obtained better outcomes using primary tumor resection, despite the patients' symptoms.

We believe that the role of primary resection in mCRC in the new era of chemotherapy and in the setting of incurable metastatic disease should be re-examined. We thought that new chemotherapies, including FOLFOX and FOLFIRI, would improve patients' prognosis and provide additional treatment options, including surgical resection. Indeed, the combination of oxaliplatin or irinotecan with 5-FU and leucovorin has proved to significantly increase response rates, prognosis, and survival compared with previous chemotherapies, such as 5-FU+LV[8-10], oxaliplatin alone[11] and IFL[1]. To compare the efficacy of FOLFOX and FOLFIRI, Tournigand *et al*[12] and Colucci *et al*[13] performed randomized trials in which patients received either FOLFOX followed by FOLFORI, or *vice versa*. Both investigators concluded that the regimens had similar efficacy when used as first-line therapy (median progression-free survival of 8 mo). Therefore FOLFOX followed by FOLFORI was chosen as the main chemotherapy in this study.

This study showed a significant difference in overall survival between the group of patients who underwent primary tumor resection without concomitant metastasectomy and the group of patients who underwent palliative surgery. However, patients with severe comorbidities or progressive metastasis who could not undergo an operation were excluded. We thought it was reasonable to separately compare patients who could undergo surgery. The two groups were well matched in terms of population and extent of tumor metastasis, but differed in their T factor. Thus, this observational survival benefit might be the result of patient selection. Therefore we additionally analyzed modified groups in which patients with T4 invasive CRC were excluded. Even in this analysis, the benefit of primary lesion resection was clearly shown. Postoperative complications in this study occurred in 28% of patients, which was similar to other reports[14]. Postoperative complications occurred more often after primary resection than after palliative surgery. This resulted in a significantly (P = 0.05) longer hospital stay in the primary resection group. A laparoscopic approach, when possible, might decrease morbidity[15]; however, we did not address this in our study. Laparoscopic surgery, as an alternative to open surgery, is safer with no added morbidity. In our study, two of 36 patients who were initially managed with palliative surgery developed perforation or significant gastrointestinal bleeding from their intact primary lesions and were successfully treated with no additional

surgical management. Such patients should be closely observed, because recent studies suggest that up to one-third of patients without resection will require subsequent intervention for symptoms related to an intact primary lesion[4,6,16]. Mild events can be managed medically, but life-threatening events require immediate surgical intervention. The issue of these tumor-dependent complications is still important. Until now it has been thought that most patients would succumb to systemic disease before developing a complication, based on high mortality and a poor 5-year survival of patients with stage 4 CRC. However, improved multidrug regimens such as FOLFOX and FOLFIRI would probably raise the incidence of severe complications indirectly by prolonging survival. Surgical treatment of primary disease to control severe complications including bleeding, perforation, and obstruction might also become increasingly important. Furthermore, whether perforation of the primary tumor is the result of tumor progression or a side effect of chemotherapy remains to be evaluated. Bevacizumab, a monoclonal antibody against vascular endothelial growth factor, is recognized to improve survival with chemotherapy but also has serious complications, such as gastrointestinal perforation. Hedrick *et al*[17] and Sugrue *et al*[18] found that 1.7% of patients who used bevacizumab experienced GI perforation. Intra-abdominal inflammation due to tumor necrosis was thought to be the common feature of these GI perforations. In addition, both the BRiTE[14] study and the First BEATrial[17] suggested that incidence of GI perforation was higher in patients with intact primary tumors. Therefore, perforation of the primary lesion is a severe problem and requires protective treatment for patients with invasive CRC. To gain a benefit from bevacizumab treatment without concern for perforation and bleeding from the primary tumor, resection of the primary tumor, if possible, is definitely favorable. In our study, eight patients have been treated with bevacizumab without severe toxicities since 2008. However, caution must be exercised in the management of the patients requiring surgery and receiving adjuvant treatment with bevacizumab.

In conclusion, this study supports the view that resection of the primary lesions in patients with non-resectable, metastatic colorectal cancer is advisable, as there is a significant improvement in survival with primary site resection over non-resected palliative surgery. We believe that all suitable patients should undergo this combined approach to avoid primary cancer complications and improve prognosis.

COMMENTS

Background

Colorectal cancer (CRC) is the most common cancer worldwide. Although chemotherapy has progressed dramatically, the role of surgical treatment in patients with incurable metastasis CRC is controversial. The resection of the primary lesions in these patients is recommended due to a significant improvement in survival.

Research frontiers

Previous studies have mentioned the comparison of primary site resection and palliative surgery. However, they have not addressed the effects of new adjuvant chemotherapy, including FOLFOX and FOLFIRI. In this study, the authors proved the superiority of primary site resection combined with FOLFOX.

Innovations and breakthroughs

This is the first study to report the combined effect of primary site resection and new chemotherapy. Furthermore, this study suggests that primary site resection might be associated with survival in patients with metastatic CRC.

Applications

This study might represent a future strategy for combined therapies for patients with CRC.

Peer review

This is an institutional experience that reviews the outcomes of patients with synchronous presentation of metastatic colorectal cancer who were treated with systemic chemotherapy for which subsequent management included a resection of the primary tumor or palliative surgical procedure to manage the primary tumor.

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S- Editor Wang JL **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3567

World J Gastroenterol 2010 July 28; 16(28): 3567-3572
ISSN 1007-9327 (print)

BRIEF ARTICLE

Rational therapeutic strategy for T2 gallbladder carcinoma based on tumor spread

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Received: January 13, 2010 Revised: February 22, 2010
Accepted: February 29, 2010
Published online: July 28, 2010

Abstract

AIM: To evaluate the adequacy of surgical treatment of T2 gallbladder carcinoma (GBCa) according to tumor spread in the subserosal layer.

METHODS: A series of 84 patients with GBCa were treated at Saga University Hospital, Japan between April 1989 and October 2008. The tumor stage was graded according to the TNM staging for GBCa from the American Joint Committee on Cancer Manual 6th edition. Tumor staging revealed 30 patients with T2 tumors. T2 GBCa was divided into three groups histologically by the extent of tumor spread in the subserosal layer, using a score of ss minimum (ss min), ss medium (ss med) or ss massive (ss mas).

RESULTS: For ss min GBCa, there was no positive pathological factor and patient survival was satisfactory with simple cholecystectomy, with or without extra-hepatic bile duct resection. For ss med GBCa, some pathological factors, h-inf (hepatic infiltration), ly (lymphatic invasion) and n (lymph node metastasis), were positive. For ss mas GBCa, there was a high incidence of positive pathological factors. The patient group with extra-hepatic bile duct resection with D2 lymph node dissection (BDR with D2) and those with S4a5 hepatectomy had significantly better survival rates.

CONCLUSION: We suggest that radical surgery is not necessary for ss min GBCa, and partial hepatectomy and BDR are necessary for both ss med and ss mas GBCa.

Key words: Hepatectomy; Bile duct resection; Gallbladder carcinoma; Tumor spread

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Kohya N, Kitahara K, Miyazaki K. Rational therapeutic strategy for T2 gallbladder carcinoma based on tumor spread. *World J Gastroenterol* 2010; 16(28): 3567-3572 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3567.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3567

INTRODUCTION

Gallbladder carcinoma (GBCa) is a relatively rare tumor[1-3], however, its mortality has increased worldwide over the past few decades[4], and the prognosis still remains poor[1,2]. There is no effective therapy for GBCa, except for surgical resection. However, the overall 5-year survival rate is 5%-42.3%, even after radical resection of the tumor[1,2,5,6]. The prognosis for patients with early GBCa, defined as pT1a/b lesions, shows a 5-year survival rate of 82%-100%[6-9]. Due to the anatomical proximity to important organs, surgery for advanced gallbladder cancer requires an aggressive approach. For pT2 or more advanced tumors, many authors advocate radical resection with lymph node dissection[10-14]. Previous reports have shown a second radical resection to be associated with significantly better survival than cholecystectomy alone in pT2 GBCa

patients whose cancers were incidentally found after cholecystectomy[15-18], whereas, Wakai *et al*[19] have reported that 40.5% of patients with unapparent pT2 tumors survived > 5 years after cholecystectomy alone. S4a5 hepatectomy combined with extra-hepatic bile duct resection (BDR) and D2 lymph node dissection is a highly recommended operation for the treatment of T2 and T3 GBCa[6], although, in T2 GBCa, the surgical procedure remains controversial, and there is no standard operation.

Recent reports have shown improved survival in patients with bile duct cancer who were treated with newly developed chemotherapy agents, gemcitabine and S-1. Several studies of single-agent gemcitabine have reported response rates of 8%-60%, and a median survival time ranging from 6.5 to 11.5 mo[20-25]. S-1 is an oral anticancer drug that contains two biochemical modulators, 5-chloro-2,4-dihydroxypridine and potassium oxonate, which improve the tumor-selective toxicity of 5-fluorouracil (5-FU)[26]. A phase Ⅱ study of S-1 has shown promising results with response rates ranging from 21% to 35% in biliary tract cancer[27,28].

The present retrospective study evaluated the limits of extended resection, such as hepatectomy, extra-hepatic BDR, and pancreatoduodenectomy (PD), especially for T2 GBCa, according to the extent of tumor spread in the subserosal layer, and to the characteristics of the clinicopathological or the prognostic factors. The good candidates were therefore recommended to receive adjuvant chemotherapy in T2 GBCa to obtain better survival.

MATERIALS AND METHODS

Between April 1989 and October 2008, 84 consecutive patients, 27 men and 57 women, with GBCa underwent surgical resection at Saga University Hospital, Japan. The mean age was 67.6 years, with a range of 45-87 years. The clinical and histopathological staging was based on the 6th edition of the American Joint Committee on Cancer Manual[29]. Nine (10.7%) patients were classified as T1a, eight (9.5%) as T1b, 30 (35.7%) as T2, 31 (36.9%) as T3, and six (7.1%) as T4. We evaluated the 30 patients with T2 GBCa who were treated with resection. These patients were divided into three groups histologically, according to the extent of tumor spread in the subserosal layer, using resected specimens. The pathological sections were examined and diagnosed using the most invaded slice.

In Table 1, the subserosal cancer invasion score (ss score) was histologically determined by dividing the vertical and horizontal tumor spread in the subserosal layer into three groups according to the ss score. Finally the extent of the subserosal invasion was divided subjectively into three categories: namely, ss minimum (ss min), ss medium (ss med), and ss massive (ss mas). As a result, the tumors were classified as ss min in four specimens, ss med in 10, and ss mas in 16.

Our fundamental strategy of S4a5 hepatectomy for T2 GBCa was indicated for highly suspected subserosal or serosal invasion preoperatively, and BDR for highly suspected lymph node metastases along the hepatoduodenal ligament. PD or pylorus-preserving PD (PPPD) was added for highly suspected retro-pancreatic lymph node metastases or direct invasion to the duodenum.

Table 1 Extent of tumor spread by ss score

Vertical invasion	< 1/3 in depth	α: score 1
	⩾ 1/3 and < 2/3	β: score 2
	⩾ 2/3	γ: score 3
Horizontal invasion	< 5 mm	A: score 1
	⩾ 5 mm and < 10 mm	B: score 2
	⩾ 10 mm	C: score 3

The sum total of ss score was calculated. ss minimum (ss min): 2; ss medium (ss med): 3-4; ss massive (ss mas): 5-6.

Table 2 ss score and clinicopathological factors *n* (%)

	ss min (*n* = 4)	ss med (*n* = 10)	ss mas (*n* = 16)
h-inf (+)	0	5 (50.0)	2 (12.5)
b-inf (+)	0	0	1 (6.3)
ly (+)	0	6 (60.0)	15 (93.8)
v (+)	0	0	5 (35.7)
pn (+)	0	1 (10.0)	6 (37.5)
n (+)	0	3 (30.0)	4 (25.0)

h-inf: Hepatic invasion; b-inf: Bile duct invasion; ly: Lymphatic invasion; v: Venous invasion; pn: Peri-neural invasion; n: Lymph node metastasis.

Statistical analysis

The clinicopathological factors and patient survival were statistically analyzed. The χ^2 and Fisher's exact tests were used to compare the two groups, and the Mann-Whitney *U* test was used for differences between the means. The survival was calculated according to the Kaplan-Meier method and compared between the groups by the log-rank test. Cox proportional hazards models were applied for the multivariate analysis. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Relationship between ss score and pathological factors

Table 2 describes the relationship between ss classification and clinicopathological factors. The ss min group had no positive pathological factors. In the ss med group, pathological factors of h-inf (hepatic infiltration), ly (lymphatic invasion), pn (perineural invasion) and n (lymph node metastasis) were positive in 50%, 60%, 10%, and 30% of the patients, respectively. All pathological factors were positive at a high rate in the ss mas group. The positive rate of pathological factors increased along with the degree of the ss score.

Survival according to ss score

Figure 1A shows the disease-specific survival curve of the patients with T2 GBCa by ss classification. All the patients with ss min and ss med survived until the end of the follow-up period and the 5-year survival rate was 100% in

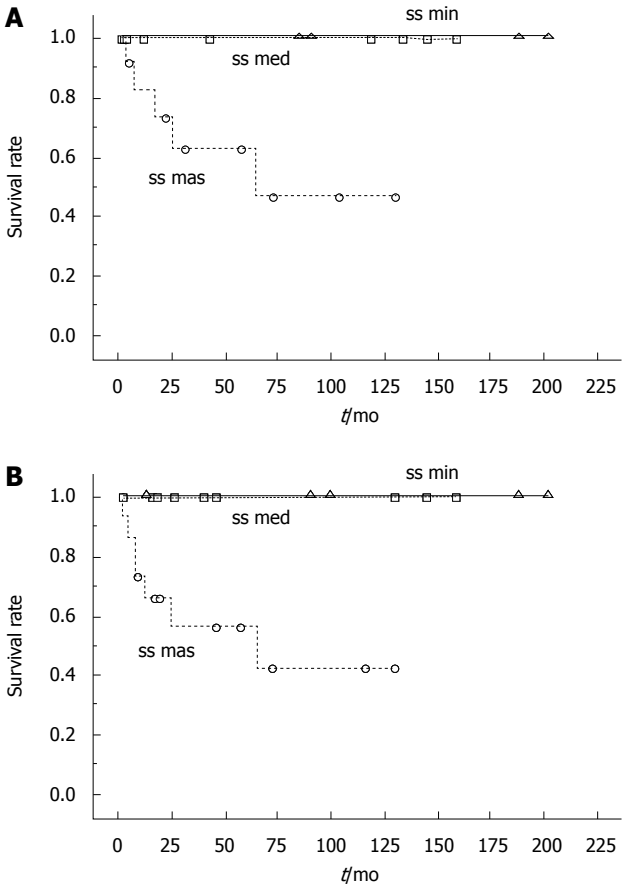

Figure 1 Kaplan-Meier survival analysis of patients with T2 gallbladder carcinoma by ss score. A: Disease-specific survival; the 5-year survival rate for ss min and ss med was 100%. In ss mas gallbladder carcinoma (GBCa), the 5-year survival rate was 59.7%; B: Disease-free survival; the 5-year survival rate for ss min and ss med was 100%. In ss mas GBCa, the 5-year survival rate was 56.6%.

the ss min and ss med groups. In ss mas GBCa, the survival was significantly worse than for ss min and ss med GBCa, and the 5-year survival rate was 59.7%. Figure 1B shows the disease-free survival curve. The 5-year survival rate in the ss min and ss med groups was 100%. In ss mas GBCa, the disease-free 5-year survival rate was 56.6%. There were seven patients with cancer recurrence in the ss mas group. The pattern of recurrence was three patients with lymph node recurrence, two with local recurrence, one with liver metastasis, and one with peritoneal dissemination.

Evaluation of surgical procedures in T2 GBCa

Table 3 summarizes the surgical procedures in each ss group. The procedure in ss min GBCa was based on simple cholecystectomy, and there was no hepatectomy, PD or PPPD. Six of 10 (60.0%) patients with ss med GBCa underwent S4a5 hepatectomy. In ss mas GBCa, the surgical procedures varied from simple cholecystectomy to extended right hepatectomy or PD, according to the mode of cancer spread.

Surgical procedures and survival in ss mas GBCa

To evaluate the appropriate surgical procedure for ss mas

Table 3 Surgical procedures in T2 gallbladder carcinoma

Surgical procedure	ss min (n = 4)	ss med (n = 10)	ss mas (n = 16)
Cx	3	2	3
Cx + Liver bed resection + D2ex			1
Cx + BDR + D2ex	1	2	1
S4a5 hepatectomy + D2ex		2	3
S4a5 hepatectomy + BDR+ D2ex		2	5
S4a5 hepatectomy + PD or PPPD		2	1
Extended right hepatectomy			1
PD alone			1

BDR: Bile duct resection; PD: Pancreatoduodenectomy; PPPD: Pylorus-preserving PD.

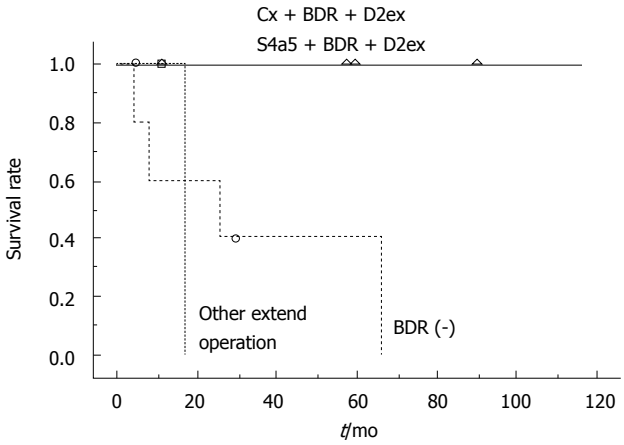

Figure 2 Kaplan-Meier survival analysis by surgical procedure for T2 gallbladder carcinoma. BDR: With extra-hepatic BDR; BDR (-): Without extra-hepatic BDR; S4a5: S4a5 hepatectomy; Cx: Simple cholecystectomy; D2ex: D2 lymph node dissection with para-aortic lymph node sampling. BDR: Bile duct resection.

GBCa, survival was analyzed according to the surgical procedures: simple cholecystectomy + extra-hepatic BDR + D2 lymph node dissection (Cx + BDR + D2ex); S4a5 hepatectomy + BDR + D2ex; S4a5 hepatectomy + PD (HPD); and cholecystectomy without BDR [Cx BDR (-)]. In ss mas GBCa, the Cx + BDR + D2ex and S4a5 + BDR + D2ex groups showed significantly better survival than the other groups (Figure 2). The 5-year survival of the Cx BDR(-) group was 33.3%, which was worse than for the Cx + BDR + D2ex group. Other extended operations, including HPD, PD, and extended hepatectomy, showed a dismal outcome. Surgery in these patients revealed massive lymph node metastasis during the operation.

DISCUSSION

In spite of the recent progress in diagnostic modalities, GBCa still tends to be found at an advanced stage[30], and only 15%-40% of patients who present with GBCa are candidates for surgical intervention[31]. Surgical management of T1 GBCa reveals almost no lymph node involvement and shows a relatively sufficient 5-year survival rate of 82%-100% with simple cholecystectomy[8,9,19].

The surgical management of T2 GBCa remains controversial. In these patients, the appropriateness of simple cholecystectomy versus radical resection remains the subject of debate. Some groups believe that most T2 lesions require only simple cholecystectomy, thus contending that radical resection is unnecessary and should be reserved for only a small subset of patients who meet certain pathological criteria[32-34]. On the other hand, proponents of radical resection believe that all T2 lesions should be treated with radical resection, because 40% of these patients will have residual lymphatic disease after resection. Radical cholecystectomy is associated with a significant survival benefit when liver surgery can be performed with minimal mortality and acceptable morbidity[35-37]. Radical surgery consisting of partial hepatectomy around the gallbladder fossa, and regional lymphadenectomy with or without resection of the extra-hepatic bile duct, yields 50%-86% 5-year survival rates[38-41]. Wakai *et al*[33] have reported the significance of the depth of subserosal invasion in patients with pT2 GBCa. The incidence of lymph node metastasis is significantly higher in patients with subserosal invasion > 2 mm (63%) than in patients with invasion < 2 mm (27%). Sasaki *et al*[42] have reported that lymph node involvement is seen in 33.3% of ss1, the upper third of subserosal invasion, 37.5% of ss2, middle third of subserosal invasion, and 85.7% of ss3, lower third of subserosal invasion. In the current study, the 5-year survival rate for T2 GBCa was 78.3%, including patients who underwent whole surgical procedures (data not shown). No positive pathological factors were observed in the ss min patient group, and simple cholecystectomy with or without extrahepatic BDR was associated with good survival (Table 3 and Figure 1). These data indicate that radical surgery, such as a hepatectomy or PD, is not necessary for ss min GBCa. For ss med GBCa, there were some positive pathological factors, h-inf, ly, pn, and n (Table 1). Partial hepatectomy, such as S4a5 hepatectomy or liver bed resection, and complete lymphadenectomy including BDR might therefore be necessary for ss med GBCa. For ss mas GBCa, a high incidence of multiple pathological factors was observed. Figure 2 shows that patients without BDR had a dismal outcome. To achieve better survival for ss mas GBCa, partial hepatectomy, such as S4a5 hepatectomy or liver bed resection, and complete lymphadenectomy including BDR, are the minimum requirement. However, radical surgery, such as major hepatectomy or hepatectomy with PD, had no survival benefit. In addition, the importance of S4a5 hepatectomy and BDR for T2 and T3 GBCa has also been previously reported[6].

To avoid unnecessary surgery, such as extended resection for ss min GBCa, the actual depth of GBCa in the subserosal layer should be determined pre- or intraoperatively. No report has previously described an effective pre- or intraoperative method to determine the actual extent of subserosal invasion. In addition, previous studies have employed intraoperative ultrasonography and frozen section histology to detect the actual depth of subserosal invasion, but it is still not sufficiently accurate, as well as being very difficult to determine the actual depth of invasion intraoperatively. A new method for the pre- or intraoperative determination of the actual extent of subserosal invasion is necessary to avoid unnecessary operation.

There is no current standard chemotherapy in advanced gallbladder cancer. In previous studies that have used chemotherapeutic agents, 5-FU, mitomycin C, methotrexate, etoposide, doxorubicin, or cisplatin, against biliary tract tumors, only 10%-20% revealed a partial response[43-46]. However, gemcitabine has shown remarkable biological activity against biliary tract cancers in some clinical studies. Several reports have described the efficacy of single-agent gemcitabine, with a response rate of approximately 30% and a median survival time of approximately 15 mo, and phase Ⅱ investigations into a gemcitabine-based combination have increased[47,48]. Gemcitabine is a novel nucleoside analog that demonstrates biological activities in a broad spectrum of solid tumors[49]. The ribonucleotide reductase subunit M1 (RRM1) plays an important role in gemcitabine resistance for biliary tract carcinomas. The expression of RRM1 has been investigated as a drug sensitivity marker for gemcitabine therapy of biliary tract carcinoma, through *in vitro* study and clinical analysis[50]. These results indicate that ss mas cancer with low RRM1 expression is therefore a good candidate for gemcitabine adjuvant chemotherapy to achieve better survival after surgical resection. S-1 has greater inhibition of thymidylate synthase (TS) and pemetrexed is classified as a multi-targeted antifolate. Orotate phosphoribosyl transferase (OPRT), dihydropyrimidine dehydrogenase (DPD) and TS play a critical role in the efficacy of antifolates. A low level of DPD and TS activity, and a high level of OPRT activity enhance the antitumor effect of S-1[51]. A phase Ⅱ study of S-1 in biliary tract cancer has shown promising results with a response rate of 21%-35%[27,28], and S-1 can be expected to have a good effect on gallbladder cancer. In T2 GBCa, ss mas cancers showed a high rate of recurrence, regardless of the radical surgical approach. Therefore, patients with ss mas cancer are thus considered to be good candidates for gemcitabine or S-1 adjuvant chemotherapy. Further studies of adjuvant chemotherapy against gallbladder cancer are necessary. The current algorithm of therapeutic strategy for T2 GBCa is shown in Figure 3.

In conclusion, the surgical management of T2 GBCa remains controversial. Radical surgery is not necessary for ss min GBCa. Furthermore, BDR may be necessary to complete lymphadenectomy for the hepato-duodenal ligament to achieve better survival for ss med and ss mas GBCa. S4a5 hepatectomy also contributed to better survival for ss med and ss mas GBCa. S4a5 hepatectomy with extra-hepatic BDR and lymphadenectomy should therefore be a standard operation for the treatment of ss med and ss mas GBCa. However, new methods for the pre- or intraoperative detection of the actual extent of subserosal invasion are still necessary to avoid unnecessary operations. In ss mas GBCa, survival remains unsatisfactory. Patients with ss mas GBCa are therefore

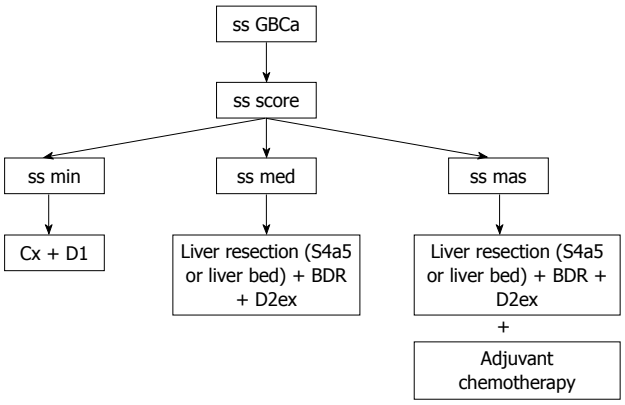

Figure 3 Algorithm of therapeutic strategy for T2 gallbladder carcinoma. Cx: Simple cholecystectomy; BDR: With extra-hepatic BDR; S4a5: S4a5 hepatectomy; D2ex: D2 lymph node dissection with para-aortic lymph node sampling.

considered to be good candidates for gemcitabine or S-1 adjuvant chemotherapy to achieve better survival.

COMMENTS

Background

Gallbladder carcinoma (GBCa) is a relatively rare tumor, however, the mortality of this tumor has increased worldwide over the past few decades, and the prognosis still remains poor. There is no effective therapy for GBCa, except for surgical resection. The prognosis for patients with early GBCa is good even with only cholecystectomy, but that for patients with advanced GBCa is poor even after radical surgery. The surgical management of T2 GBCa remains controversial.

Research frontiers

T2 GBCa was divided into three groups histologically by the extent of tumor spread in subserosal layer using the ss score. The ss score was histologically determined by dividing the vertical and horizontal tumor spread in the subserosal layer. Finally the extent of the subserosal invasion was divided subjectively into three categories: ss minimum (ss min), ss medium (ss med) and ss massive (ss mas).

Innovations and breakthroughs

For ss min GBCa, there was no positive pathological factor and survival was satisfactory after simple cholecystectomy. For ss med GBCa, some pathological factors were positive. For ss mas GBCa, there was a high incidence of positive pathological factors.

Applications

After surgical procedure analysis of T2 GBCa, the patient group with extra-hepatic bile duct resection with D2 lymph node dissection, and the group with S4a5 hepatectomy had significantly better survival rates. In ss mas GBCa, the survival of the patients remains unsatisfactory. Patients with ss mas GBCa are therefore considered to be good candidates for chemotherapy to achieve better survival.

Terminology

S4a5 hepatectomy is a type of hepatectomy for advanced GBCa. S4 is the lower half of the left medial segment of the liver, and S5 is the anterior medial segment of the liver, according to the Couinaud classification of liver segments. D2 lymph node dissection is based on the method described in the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract from the Japanese Society of Biliary Surgery, 5th edition, 2003.

Peer review

This study investigated an important subject, namely, the best therapeutic approach for GBCa.

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3573

World J Gastroenterol 2010 July 28; 16(28): 3573-3577
ISSN 1007-9327 (print)

BRIEF ARTICLE

Proton pump inhibitors as a risk factor for recurrence of *Clostridium-difficile*-associated diarrhea

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Supported by A Seoul National University Boramae Hospital Grant (03-2007-1)
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Received: February 25, 2010 Revised: April 13, 2010
Accepted: April 20, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate the risk factors for *Clostridium-difficile*-associated diarrhea (CDAD) recurrence, and its relationship with proton pump inhibitors (PPIs).

METHODS: Retrospective data of 125 consecutive hospitalized patients diagnosed with CDAD between January 2006 and December 2007 were collected by medical chart review. Collected data included patient characteristics at baseline, underlying medical disease, antibiotic history before receiving a diagnosis of CDAD, duration of hospital stay, severity of CDAD, concurrent treatment with PPIs, laboratory parameters, response to CDAD therapy, and recurrence of disease within 90 d of successful treatment. Various clinical and laboratory parameters were compared in patients in whom CDAD did or did not recur.

RESULTS: Of the 125 patients (mean age, 67.6 ± 13.9 years) that developed CDAD, 98 (78.4%) did not experience recurrence (non-recurrent group) and 27 (21.6%) experienced one or more recurrences (recurrent group). Prior to the development of CDAD, 96% of the 125 patients were prescribed antibiotics, and 56 (44.8%) of the patients received PPIs. Age older than 65 years (P = 0.021), feeding *via* nasogastric tube (NGT) (P = 0.045), low serum albumin level (P = 0.025), and concurrent use of PPIs (P = 0.014) were found to be risk factors for CDAD recurrence by univariate analysis. However, sex, length of hospital stay, duration and type of antibiotics used, severity of disease, leukocyte count and C-reactive protein (CRP) were not associated with risk of CDAD recurrence. On multivariate analysis, the important risk factors were advanced age (> 65 years, adjusted OR: 1.32, 95% CI: 1.12-3.87, P = 0.031), low serum albumin level (< 2.5 g/dL, adjusted OR: 1.85, 95% CI: 1.35-4.91, P = 0.028), and concurrent use of PPIs (adjusted OR: 3.48, 95% CI: 1.64-7.69, P = 0.016).

CONCLUSION: Advanced age, serum albumin level < 2.5 g/dL, and concomitant use of PPIs were found to be significant risk factors for CDAD recurrence.

Key words: *Clostridium difficile*; Diarrhea; Recurrence; Risk factors; Proton pump inhibitors

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HC, Song IS. Proton pump inhibitors as a risk factor for recurrence of *Clostridium-difficile*-associated diarrhea. *World J Gastroenterol* 2010; 16(28): 3573-3577 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3573.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3573

INTRODUCTION

Clostridium difficile is a spore-forming Gram-positive anaerobic bacillus and is the most common cause of hospital-acquired diarrhea[1]. *C. difficile* infection occurs when a susceptible host ingests spores, which then colonize the large bowel and release endotoxin. Specific antibiotic treatments with metronidazole or vancomycin show high levels of efficacy and reduce morbidity and mortality[2]. However, although initial response rates to antibiotic therapy exceed 90%, 10%-30% of patients experience *C. difficile*-associated diarrhea (CDAD) recurrence[3], which increases the cost of medical care, and causes re-hospitalization, complications, and mortality[4]. The complications of recurrent CDAD included toxic megacolon, septicemia, and C. *difficile*-associated arthritis. Furthermore, the average length of stay due to admission for recurrent CDAD among outpatients has been reported to be 8.8 d (range, 3-26)[5,6]. It has been reported that the elderly, and patients with chronic renal failure, multiple previous CDAD episodes, or a high level of white blood cell count ($\geqslant 15 \times 10^9$/L), and those that continue to use systemic antibiotics after a diagnosis of CDAD, are at high risk of recurrence[7,8].

Gastric acid inhibits the germination of ingested spores and the survival of *C. difficile*, and recent studies have found that gastric acid suppressive agents, such as PPIs, increase the risk of CDAD development in hospitalized patients[9,10]. However, relatively few studies have attempted to determine whether the use of gastric acid suppressive agents is associated with an elevated risk of CDAD recurrence[11,12].

The purpose of this study was to determine the risk factors of CDAD recurrence in hospitalized patients, and to investigate the relation between PPIs use and CDAD recurrence.

MATERIALS AND METHODS

Identification of subjects and collection of clinical data

This retrospective study was performed at Seoul National University Boramae Hospital, a 500-bed teaching hospital, between January 1, 2006 and December 31, 2007.

The medical records of all patients diagnosed with CDAD based on typical symptoms, that is, three or more bowel movements per day, abdominal pain, fever, leukocytosis at least 3 d after admission, and a positive ELISA result for *C. difficile* toxins A and B (Wampole TOX A/B Quic Check, Techlab, Blacksburg, VA, USA). The exclusion criteria applied were: age < 18 years, CDAD during the previous 3 mo, failure to complete at least 7 d of antibiotic therapy, a diagnosis of CDAD within 3 d of admission, or the presence of any other cause of diarrhea, such as, laxative use, the presence of another infectious pathogen, and inflammatory bowel disease.

Medical records included the following information: age; sex; type of underlying disease; duration, number and type of antibiotics prescribed before diagnosis of CDAD; hematological and biochemical parameters; CDAD severity; PPI use; specific therapy used to treat CDAD; time to resolution of CDAD symptoms; and disease recurrence within 90 d of cure. CDAD was considered severe if two or more of the following factors were present: (1) a frequency of stool of > 10/d; (2) fever (> 38.3℃); and (3) a leukocyte count of > 15000 cells/mm^3. PPI use was defined as at least 3 d treatment before the development of CDAD and continuous use thereafter.

In the majority of patients, oral metronidazole for 10-14 d was initially administered. Vancomycin was reserved for those that did not respond to metronidazole or had severe CDAD. The main causes of antibiotic prescription were pneumonia, urinary tract infection, postoperative wound infection, osteomyelitis, and cellulitis. Patients were classified into two groups based on recurrence within 90 d of cure (the recurrent and non-recurrent groups). Patients were regarded as cured when stool frequencies and consistencies were normal for at least three consecutive days. Recurrence was defined as diarrhea recurrence with a positive ELISA result for cytotoxin A, within 90 d after therapy completion, and the complete resolution of signs and symptoms. Patients were monitored for recurrence throughout this 90-d period.

Statistical analysis

SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Patients were divided into recurrent and non-recurrent groups. Data are presented as mean ± SD or percentage frequencies. Student's *t* test, χ^2 test and Fisher's exact test were used to analyze continuous and categorical variables. Logistic regression analysis was used to determine the effects of continuous variables on recurrence. For all analyses, $P < 0.05$ was considered significant. This study was approved by the institutional review board of Seoul National University Boramae Hospital.

RESULTS

A total of 125 patients who received full course therapy for CDAD were included in this study. There were 57 (45.6%) men and 68 (54.4%) women, and mean patient age was 67.6 years (range 35-92). One hundred and twenty (96%) were prescribed antibiotics before CDAD was diagnosed. The most common antibiotics administered were cephalosporins (80.8%), clindamycin (25.6%), penicillin analogues (20%), and quinolones (19.2%). Five patients (4%) did not receive antibiotics prior to diagnosis of CDAD, and 81 (64.8%) were treated with more than one antibiotic. Forty-eight (38.4%) patients had diabetes, 41 (32.8%) had malignant disease, and 17 patients (13.6%) had chronic renal failure. Thirty-five patients (28%) were fed *via* an NGT before diagnosis. Of the

Table 1 Univariate analysis of risk factors for recurrence of *Clostridium-difficile*-associated diarrhea (mean ± SD) *n* (%)

	Recurrence group (*n* = 27)	Non-recurrence group (*n* = 98)	*P* value
Age (≥ 65 yr)	16 (59.3)	31 (31.6)	0.021[a]
Female	15 (55.6)	53 (54.1)	0.905
Length of hospital stay (d)	36.5 ± 9.7	32.8 ± 8.5	0.152
Duration of antibiotics used (d)	13.1 ± 6.8	12.2 ± 5.8	0.105
Admission from institution	6 (22.2)	17 (17.4)	0.379
Severe CDAD	13 (48.2)	30 (30.6)	0.156
Comorbidities			
Diabetes mellitus	11 (40.7)	37 (37.8)	0.572
Malignancy	10 (37.1)	31 (31.6)	0.560
Renal failure	5 (18.5)	12 (12.3)	0.215
Active tuberculosis	2 (7.4)	6 (6.1)	0.719
NGT feeding	13 (48.1)	23 (23.5)	0.045[a]
WBC count, (mean) × 10^3/mm^3	15.1 ± 9.7	12.3 ± 8.1	0.413
WBC count > 15 × 10^3/mm^3	(44.4)	(40.8)	
Hb (g/dL)	10.9 ± 1.9	11.4 ± 1.6	0.279
Serum albumin (g/dL)	2.3 ± 0.5	3.1 ± 0.4	0.025[a]
Serum albumin < 2.5 g/dL	(59.3)	(36.7)	
Creatinine (mg/dL)	1.5 ± 1.4	1.2 ± 1.6	0.513
C-reactive protein (g/dL)	7.3 ± 4.8	5.6 ± 4.5	0.579
Use of PPIs	17 (63.0)	39 (39.8)	0.014[a]

[a]*P* < 0.05. CDAD: *Clostridium-difficile*-associated diarrhea; WBC: White blood cells; NGT: Nasogastric tube.

125 subjects, 56 (44.8%) received PPIs for ≥ 3 d. One hundred and thirteen (90.4%) were initially treated with metronidazole, and 12 (9.6%) with vancomycin.

Of the 125 study subjects, 27 (21.6%) experienced disease recurrence within 90 d after cure, and the remaining 98 (78.4%) did not experience recurrence. The recurrence rate among patients treated with metronidazole was no different from that among patients treated with vancomycin (21.2% *vs* 16.7%). Univariate analysis showed that five parameters were significantly associated with recurrence (Table 1). Patients aged ≥ 65 years were found to be more likely to develop recurrence than those < 65 years (59.3% *vs* 31.6%, *P* = 0.021). Patients fed *via* an NGT were also more likely to develop recurrence (48.1% *vs* 23.5%, *P* = 0.045). The mean serum albumin level at the time of CDAD diagnosis was lower in patients with recurrence than in patients without recurrence (2.3 ± 0.5 g/dL *vs* 3.1 ± 0.4 g/dL, *P* = 0.028). Furthermore, significantly more patients in the recurrent group received concurrent PPIs therapy (63.0% *vs* 39.8%, *P* = 0.014). However, sex, length of hospital stay, duration of antibiotics used before diagnosis of CDAD, the presence of diabetes mellitus or renal failure, severity of CDAD, leukocyte count, hemoglobin and CRP levels were not found to be associated with an increased risk of recurrence.

Multivariate analysis showed that age > 65 years (OR: 1.32, 95% CI: 1.12-3.87, *P* = 0.031), serum albumin level of < 2.5 g/dL (OR: 1.85, 95% CI: 1.35-4.91, *P* = 0.028), and concurrent use of PPIs (OR: 3.48, 95% CI: 1.64-7.69, *P* = 0.016) were associated with the risk of recurrence (Table 2).

Table 3 summarizes antibiotic use before CDAD was diagnosed. The mean number of antibiotic types prescribed to patients was not found to be a risk factor for recurrence (2.58 in the recurrent group *vs* 2.43 in the non-recurrent group, *P* = 0.56). Furthermore, the two study groups were similar in terms of exposure to high-risk antibiotics, such as, clindamycin, cephalosporins, or quinolones.

Table 2 Multivariate analysis of risk factors for recurrence of *Clostridium-difficile*-associated diarrhea

Risk factor	Adjusted OR	95% CI	*P* value
Age (≥ 65 yr)	1.32	1.12-3.87	0.031[a]
Low serum albumin of < 2.5 g/dL	1.85	1.35-4.91	0.028[a]
Concurrent use of PPI	3.48	1.64-7.69	0.016[a]
NGT feeding	1.25	0.91-2.65	0.068

[a]*P* < 0.05. NGT: Nasogastric tube; PPI: Proton pump inhibitor.

Table 3 Comparison of antibiotics used before diagnosis of *Clostridium-difficile*-associated diarrhea between non-recurrent and recurrent group (mean ± SD) *n* (%)

Type of antibiotics used	Non-recurrent CDAD (*n* = 98)	Recurrent CDAD (*n* = 27)	*P* value
No. of antibiotics used	2.58 ± 0.9	2.43 ± 0.8	0.560
No antibiotics	4 (4.1)	1 (3.7)	0.638
1 antibiotics	28 (28.6)	9 (33.3)	0.497
≥ 2 antibiotics	64 (65.3)	19 (70.4)	0.435
Penicillin analogue	19 (19.4)	6 (22.2)	0.561
Cephalosporin	79 (80.6)	22 (81.4)	0.613
Clindamycin	26 (26.5)	6 (22.2)	0.265
Quinolone	17 (17.3)	7 (25.9)	0.217
Macrolide	8 (8.2)	2 (7.4)	0.691
Carbapenem	11 (11.2)	5 (18.5)	0.215
Metronidazole	6 (6.1)	2 (7.4)	0.563
Vancomycin	3 (3.1)	1 (3.7)	0.729

CDAD: *Clostridium-difficile*-associated diarrhea.

DISCUSSION

Recurrent CDAD is one of the most difficult problems related to *C. difficile* infection. Despite an initial successful response in more than 90% of patients, CDAD recurs in 15%-30%[13,14]. In a previous study of 124 CDAD patients, 24% experienced recurrence[15], which concurs with the 20.8% observed in the present study. Patients treated with metronidazole (21.2%) had a greater tendency to recur than patients treated with vancomycin (16.7%), but this was not significant (*P* = 0.09). Studies have shown that the rates of treatment failure and recurrence are greater for patients treated with initial metronidazole than for patients treated with vancomycin, especially since 2000[16]. Because of its low cost and of concerns about vancomycin-resistant enterococci, metronidazole is recommended as first-line therapy. However, our finding that 21% of patients initially treated with metronidazole experienced recurrence suggests that vancomycin might be helpful as a first-line treatment in patients with multiple risk factors for recurrent CDAD. Although the pathogenesis of recur-

rence is poorly understood, several risk factors have been described. The important risk factors were advanced age, longer hospital stay, continued use of antibiotics for the treatment of non-*C. difficile* diarrhea after a first episode of CDAD, inadequate antitoxin antibody response, persistent disruption of colonic flora, and concomitant receipt of antacid medications[17-19].

Several studies have reported that agents that suppress gastric acid secretion, especially PPIs, increase the risk of CDAD development[20-22]. According to one study, the adjusted risk ratios for CDAD development for PPIs and H2-receptor antagonist (H_2RA) usage are 2.9 (95% CI: 2.4-3.4) and 2.0 (95% CI: 1.6-2.7), respectively. However, another study on the relationship between CDAD development and exposure to acid suppressive therapy in hospitalized patients has revealed an association with PPIs (OR: 3.6, 95% CI: 1.7-8.3) but not with H2RAs. In the present study, we found that PPI use was a significant risk factor of CDAD recurrence, which is in line with previous reports. In one study of 140 CDAD patients treated between 2004 and 2005, those receiving PPIs were found to be 4.17 times more likely to recur, which is greater than the OR found in the present study[11]. This result may be due to differences between study populations. In the study conducted by Cadle *et al*[11], most patients (98.6%) were men and the proportion of patients who received PPIs was greater than in the present study (69% *vs* 55%). In a recent meta-analysis, continued use of non-*C. difficile* antibiotics after a diagnosis of CDAD (OR: 4.23, 95% CI: 2.10-8.55), concomitant receipt of antacid medication (OR: 2.15, 95% CI: 1.13-4.08), and older age (OR: 1.62, 95% CI: 1.11-2.36) were found to be significantly associated with the risk of CDAD recurrence[12]. However, in this meta-analysis, PPIs and H2RAs were not differentiated, and thus, their specific effects on CDAD recurrence could not be evaluated.

The mechanisms by which acid suppressive agents increase the risk of CDAD development and recurrence are poorly understood[23]. However, although the spores of *C. difficile* are resistant to gastric acid, it has been suggested the survival and germination of *C. difficile* spores are greater at lower acidity, and that higher gastric pH increases vegetative bacteria counts in the small and large intestine[24]. Previous studies have found that gastric acid suppression increases stomach and small bowel colonization by bacteria and *C. difficile*[25,26]. Alternatively, it is also possible that PPIs directly affect host immune function, and thus, increase susceptibility to CDAD recurrence[27].

In the present study, age was found to be a risk factor for recurrence. A retrospective study of hospitalized patients in Quebec also has shown that patients aged > 65 years have a higher risk of CDAD recurrence after metronidazole and vancomycin therapy[28]. The reason why advanced age increases the risk of recurrence may be that these patients have comorbidities and are likely to be administered additional antibiotics for concomitant infections. A low serum albumin level was also found to be a significant risk factor for recurrence in our study. Hypoalbuminemia reflects a poor nutritional status, and may compromise immune response by diminishing antibody response to *C. difficile*. In one study, patients with recurrent disease were found to have significantly lower levels of IgG to toxin A than patients that did not recur[29]. Later increased serum levels of IgM and IgG to toxin A are known to be associated with a substantial reduction in the risk of CDAD recurrence[16].

In contrast to previous reports, we found no relationship between renal failure, leukocytosis, or CDAD severity and recurrence. However, we defined renal failure based on creatinine level alone, rather than on creatinine clearance, and this does not reflect the range of renal failure. With regard to leukocytosis and disease severity, a significant proportion of our study subjects were elderly, due to a failure to increase leukocytes in response to infection in some elderly patients, which may have influenced our results. Previous reports have shown that clindamycin, penicillin and cephalosporin use is an important risk factor for CDAD development, and recently, ciprofloxacin use also has been found to be a significant risk factor for CDAD in hospitalized patients[30-32]. In the present study, however, antibiotic use prior to CDAD was similar in our two patient groups, and this suggested that type and number of antibiotics used prior to CDAD might have influenced CDAD development, but not CDAD recurrence.

This study had several limitations that should be considered. First, the study was inherently limited by its retrospective nature, and in particular, stool cultures were not conducted, which prevented our determining whether recurrent CDAD was due to reinfection or relapse. Second, the confidence interval regarding the use of PPIs as a risk factor for recurrent CDAD was relatively large, which was probably due to the small number of patients enrolled in this study. Third, actual compliance to PPIs could not be assessed beyond the prescription data contained in medical charts.

In conclusion, older age (> 65 years) and a low serum albumin level (< 2.5 g/dL) were identified as risk factors for CDAD recurrence. The concomitant use of PPIs further enhanced the risk of recurrence. Of these risk factors, the use of PPIs is modifiable, and thus, it is appropriate to review constantly the necessity for concomitant use of PPIs in patients with CDAD. Prevention of unwarranted PPI therapy may be helpful in reducing the risk of recurrence, and additional larger studies are necessary in order to understand better the relationship between PPI use and CDAD recurrence.

COMMENTS

Background

The incidence and severity of *Clostridium-difficile*-associated diarrhea (CDAD) is increasing worldwide due to increased use of antibiotics and the introduction of hypervirulent strains. After successful initial therapy, approximately 10%-30% of patients experience symptomatic recurrence. Recurrence of CDAD causes increasing cost of medical care, re-hospitalization and complications. It is known that older patients, patients with chronic renal failure and multiple episode of previous CDAD are high risk groups for disease recurrence. Recent reports have suggested that the use of proton pump inhibitors (PPIs) is associated with CDAD recurrence in hospitalized patients, although the results appear to be conflicting. The objective of this study was to evaluate the risk factors for CDAD recurrence and to investigate the relationship of PPI use and recurrence of CDAD.

Research frontiers

Risk factors that are associated with CDAD recurrence and the association between use of PPIs and CDAD recurrence have been hot topics in recent studies. Of the 125 patients that developed CDAD, 98 did not experience recurrence and 27 experienced one or more recurrences. The important risk factors for CDAD recurrence were age > 65 years, low serum albumin level < 2.5 g/dL, and use of PPIs.

Innovations and breakthroughs

This study showed that advanced age, low serum albumin level and use of PPIs were associated with increased risk of CDAD recurrence.

Applications

This retrospective analysis of the risk factors for CDAD recurrence imply that avoidance of unwarranted PPI therapy may be helpful in reducing the risk of CDAD recurrence.

Peer review

This is a timely study extending present knowledge that PPIs are a risk factor for *C. difficile* reinfection.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3578

World J Gastroenterol 2010 July 28; 16(28): 3578-3583
ISSN 1007-9327 (print)

BRIEF ARTICLE

Correlation between *pre-miR-146a* C/G polymorphism and gastric cancer risk in Chinese population

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Supported by National Natural Science Foundation of China, No. 30873099; Natural Science Foundation of Jiangsu Province, No. BK2006525; No. 08KJB320004, "333 Project" and "Qinglan Project" Funds for the Young Academic Leader of Jiangsu Province to Wang B
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Received: March 17, 2010 Revised: May 20, 2010
Accepted: May 27, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate the association between *pre-miR-146a* C/G polymorphism and gastric cancer risk.

METHODS: We performed a hospital-based, case-control study using polymerase chain reaction-restriction fragment length polymorphism method in 608 individuals (304 gastric cancer patients and 304 age and sex matched cancer-free controls).

RESULTS: The frequencies of *pre-miR-146a* C/G genotypes in the case group were significantly different from those in the control groups (P = 0.037). Compared with CC genotype carriers, subjects with the variant genotypes (GC + GG) had a 58% increased risk of gastric cancer (adjusted OR = 1.58, 95% CI: 1.11-2.20, P = 0.009). Moreover, a higher gastric cancer risk was especially evident in younger individuals aged ⩽ 58 years, nonsmokers, and males (adjusted OR = 1.76, 95% CI: 1.08-2.87, P = 0.024; adjusted OR = 1.55, 95% CI: 1.06-2.28, P = 0.025; adjusted OR = 1.53, 95% CI: 1.04-2.27, P = 0.033; respectively).

CONCLUSION: *Pre-miR-146a* C/G polymorphism might be associated with an elevated risk of gastric cancer in Chinese population.

Key words: Pre-miR-146a; miR-146a; Gastric cancer; Polymorphism; Genotype

Peer reviewer: Carlos J Pirola, PhD, FAHA, Medical Research Institute A Lanari, Combatientes de Malvinas 3150, Buenos Aires-1427, Argentina

Zeng Y, Sun QM, Liu NN, Dong GH, Chen J, Yang L, Wang B. Correlation between *pre-miR-146a* C/G polymorphism and gastric cancer risk in Chinese population. *World J Gastroenterol* 2010; 16(28): 3578-3583 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3578.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3578

INTRODUCTION

MicroRNAs (miRNAs) are endogenous 19- to 25-nt noncoding RNAs, which could negatively regulate gene expression through suppressing translation or degrading mRNA[1]. Bioinformatic data identified that a single miRNA could bind to a large number of mRNA targets,

which could be implicated in disease development[2-8]. Interestingly, recent evidences indicated that miR-146a can play a role of mediator in a wide spectrum of biological processes, such as cell proliferation, differentiation, apoptosis, immune response and tumorigenesis[9-15]. Aberrant expression of miR-146a has been reported as a signature in breast cancer, pancreatic cancer, prostate cancer, and cervical cancer[16-18].

Single nucleotide polymorphism (SNP) is the most common type of genetic variation in the human genome. Polymorphisms in human pre-miRNA genome lesion modify the processing and/or target selection of human miRNAs, which are implicated in cell cycle regulation, and thereby play critical roles in carcinogenesis[19,20]. Calin *et al*[21] found that the *pre-miR-206* precursor mutation was associated with the risk of chronic lymphocytic leukemias (CLL). They also found a germ-line mutation in the miR-16-1-miR-15a primary precursor, which caused low levels of miRNA expression *in vivo* and *in vitro*[21].

Pre-miR-146a C/G polymorphism designated rs2910164 is located on chromosome 5, in the stem region opposite to the mature miR-146a sequence[22]. Several epidemiological studies have examined the role of the *pre-miR-146a* polymorphism in many human cancers, such as hepatocellular carcinoma (HCC), prostate cancer, breast cancer and papillary thyroid carcinoma (PTC)[22-26]. However, the results of these studies were inconsistent. Furthermore, no data are available concerning the association between *pre-miR-146a* C/G polymorphism and the risk of gastric cancer. Therefore, we have conducted a hospital-based case-control study to investigate the potential link between this polymorphism and gastric cancer in Chinese population.

MATERIALS AND METHODS

Subjects

This is a hospital-based case-control study, which comprised 304 gastric cancer patients and 304 cancer-free controls, consecutively recruited at the Affiliated Hospital of Nanjing Medical University. All the participants were genetically unrelated Han Chinese and were from Jiangsu Province or its surrounding regions. The diagnoses of gastric cancer were all confirmed by endoscopic biopsy or surgical specimens. Patients with secondary or recurrent tumors were excluded. Control subjects matched to gastric cancer cases by gender and age (within 5 years), were selected from patients hospitalized because of a variety of nonmalignant diseases during the time of case collection. Patients with previous histories of cancer or severe clinical symptoms and genetic disease were excluded. A structured questionnaire was administered by interviewers to collect information on demographic information and personal medical history. Those who formerly or currently smoked $\geqslant$ 10 cigarettes per day on average were defined as smokers. Pathologic variables were obtained after histopathological investigation. Depth of tumor invasion and local lymph node status were classified according to the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer (UICC)[27]. Differentiation grade was classified according to WHO classification. The study was approved by the Ethics Committee of the First Affiliated Hospital, Nanjing Medical University and informed consent was obtained from each participant.

Genotyping

The protocol for genomic DNA extraction was described in our previous study[28]. The polymorphism was genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. To control the quality of genotyping, the RFLP assay was performed without knowing the status of the cases or controls. The 372-bp DNA fragment containing the polymorphic site was amplified using two primers 5'-CATGGGTTGTGTCAGTGTTAGA-3' and 5'-CCAAGAGTCTCGTATAACAGCA-3'. PCR was done in 20 reaction mixtures containing 2 μL of 10 × PCR buffer (MBI Fermentas), 1.375 mmol/L $MaCl_2$, 0.1 mmol/L dNTPs, 1 unit Taq polymerase (MBI Fermentas), 200 ng genomic DNA, and 0.25 μmol/L of each primer. The PCR conditions were 95℃ for 8 min, followed by 35 cycles of 30 s at 95℃, 30 s at 54℃, and 30 s at 72℃, with a final elongation at 72℃ for 10 min. PCR product was digested with *HPY188*I (New England BioLabs), 5 units for 12 h at 37℃, followed by electrophoresis on a 3% agarose gel. The CC genotype yielded three bands at 211, 134 and 27 bp, and GG had two bands at 211 and 161 bp, while the GC produced four bands at 211, 161, 134, and 27 bp. Two research assistants independently examined the gel pictures and performed the repeated assays until they reached a consensus on the tested genotype.

In addition, 10% of the samples were randomly selected for retest, and the results were 100% concordant.

Statistical analysis

All the statistical analyses were performed using Stata Version 8.0 (STATA Corporation, College Station, TX). The Shapiro-Wilk statistics were used to estimate the normality of distribution. Quantitative variables departing from the normal distribution were summarized as median and estimated by the Mann-Whitney rank sum test. Pearson χ^2 test was used to investigate the difference in the distribution of categorical variables and genotype frequencies between the cases and controls. Hardy-Weinberg equilibrium of the *pre-miR-146a* genotypes was analyzed by the goodness-of-fit χ^2 test. Odds ratio (OR) and 95% confidence interval (CI) were calculated to evaluate the association between the polymorphism and the risk of gastric cancer. CC homozygotes were used as the reference. Crude OR was assessed by the Woolf approximation method, and the adjusted OR was computed using unconditional logistic regression with adjustment for age, gender, smoking status, residence, hypertension and diabetes. All statistical tests were two-tailed and considered statistically significant at a value of $P < 0.05$.

RESULTS

Demographic information

The demographic characteristics of the study partici-

Table 1 The characteristics of cases and controls *n* (%)

Characteristics	Cases	Controls	*P* value
Overall	304	304	
Gender (male)	228 (75)	228 (75)	1.000
Age[1] (yr)	59 (51-66)	58 (50-66)	0.865
Hypertension	54 (17.76)	59 (19.41)	0.602
Diabetes	17 (5.59)	27 (8.88)	0.118
Smoking	73 (24.01)	58 (19.08)	0.111
Residence			
Rural	158 (51.97)	165 (54.46)	0.540
Urban	146 (48.03)	138 (45.54)	

[1]Median (25th-75th percentiles).

pants are listed in Table 1. Cases and controls were well matched in terms of gender and age (within 5 years). Moreover, the two groups were similar with regard to smoking status, residence, history of hypertension, and diabetes. The number of patients with cancer of the gastric cardia and noncardia was 85 and 219, respectively. Most of the cases were adenocarcinoma (96.05%). Among those 304 gastric cancers with available clinicopathologic data, 52, 36, 142 and 67 were T1, T2, T3, and T4, respectively and 41, 165, and 91 were reported to be well, moderately, and poorly differentiated. Positive lymph nodes were identified in 190 cases.

Distributions of pre-miR-146a C/G genotype in cases and controls

Table 2 presents the genotype frequency and the association between *pre-miR-146a* C/G polymorphism and the risk of gastric cancer. The genotype distributions complied well with Hardy-Weinberg equilibrium in cases and controls (P = 0.7993 and P = 0.1223, respectively), indicating that there was no genetic drift or any selective advantage for particular *pre-miR-146a* alleles. A significantly different distribution of the *pre-miR-146a* genotypes was demonstrated between the cases and controls (χ^2 = 6.5768, P = 0.037). Furthermore, a higher frequency of G allele frequency was found in cases compared with the controls (χ^2 = 5.1232, P = 0.024).

Risk estimates for the variant pre-miR-146a genotypes

The association between gastric cancer risk and the polymorphism is presented in Table 2. With the CC genotype as reference, the OR for the variant genotypes (GG + GC) was 1.58 (95% CI: 1.12-2.22, P = 0.009) after adjusting for age, sex, smoking status, residence, hypertension, and diabetes. Moreover, the GG homozygotes had a 62% elevated risk (adjusted OR = 1.62, 95% CI: 1.01-2.58, P = 0.045), and the GC heterozygotes had a 55% increased risk (adjusted OR = 1.55, 95% CI: 1.07-2.22, P = 0.019).

Stratified analyses for the variant genotypes in cases and controls

Table 3 shows the results of stratified analyses by the median age of controls (58 years), sex, smoking status, and residence with the *pre-miR-146a* variant genotypes. A significantly increased risk associated with the variant genotypes was observed in only subjects aged ⩽ 58 years (adjusted OR = 1.76, 95% CI: 1.08-2.87, P = 0.024). In males, possession of the variant genotypes was associated with a 53% increased risk of gastric cancer (adjusted OR = 1.53, 95% CI: 1.04-2.27, P = 0.033), whereas the association was not statistically significant in females. In addition, stratification by smoking status revealed a significant cancer risk for nonsmokers (adjusted OR = 1.55, 95% CI: 1.06-2.28, P = 0.025) but not in smokers. However, no statistically significant difference was observed in rural and urban areas in the association between the polymorphism and susceptibility to gastric cancer.

We also evaluated the correlations of the variant genotypes with clinicopathologic features of gastric cancer, including tumor differentiation, depth of tumor infiltration, lymph node status and tumor location, however, no statistically significant association was observed (Table 4).

DISCUSSION

Recent genome-wide SNP polymorphisms are encouraging and widely performed to help in developing more accurate diagnostic and therapeutic strategies of various kinds of human disorders[29]. In the present study, we for the first time found that the C to G variant in *pre-miR-146a* conferred an increased risk of gastric cancer in Chinese population. We also found that the elevated gastric cancer risk was especially evident in the individuals aged ⩽ 58 years, nonsmokers and males.

The human miR-146a is located on chromosome 5[22]. In general, one miRNA targets thousands of mRNAs and one mRNA is regulated by many miRNAs. Therefore, it is very hard to understand the precise mechanisms of the miRNA in the pathogenesis of human disorders. Nevertheless, various studies have suggested that miR-146a is a NF-κB-dependent gene[30,31]. Upon processing, it could down-regulate levels of IRAK1 and TRAF6 proteins, reducing the activity of the NF-κB signaling pathway, which has been implicated as an important causal link between inflammation and carcinogenesis[9,10,32-34]. To date, aberrant miR-146a expressions have been described in several diseases[16-18,35-38]. Recent reports have indicated that miR-146a levels were increased in the tissues associated with chronic inflammatory diseases such as rheumatoid arthritis and psoriasis, whereas there was no increase of miR-146a levels in tissues obtained from patients with other chronic inflammatory diseases such as the skin from atopic eczema or lung biopsies from mild asthmatics[35-37]. Moreover, elevated miR-146a levels have been reported in PTC, cervical cancer, breast cancer and pancreatic cancer, whereas decreased miR-146a expression is associated with prostate cancer[16-18,38].

A C/G polymorphism (rs2910164) resides at position + 60 relative to the first nucleotide of *pre-miR-146a*, placing it in the passenger strand[22]. Compared with the major C allele, the minor G allele causes mispairing in the hairpin and a higher dG from -40.3 to -43.1 kcal/mol, suggesting

Table 2 The *pre-miR-146a* C/G genotype distribution in cases and controls and risk estimates for variant genotypes

	Cases (%)	Controls (%)[1]	Crude OR (95% CI)	*P* value	Adjusted OR[2] (95% CI)	*P* value
Genotype[3] CC	89 (29.28)	119 (39.14)	1.00		1.00	
CG	153 (50.33)	132 (43.42)	1.55 (1.08-2.22)	0.017	1.55 (1.07-2.22)	0.019
GG	62 (20.39)	53 (17.43)	1.56 (0.99-2.47)	0.056	1.62 (1.01-2.58)	0.045
GG + GC allele[4]	215 (70.72)	185 (60.85)	1.55 (1.11-2.18)	0.011	1.58 (1.12-2.22)	0.009
C-allele	331 (54.44)	370 (60.86)				
G-allele	277 (45.56)	238 (39.14)				

[1]The genotype distributions complied well with Hardy-Weinberg equilibrium in cases and controls (P = 0.7993, P = 0.1223, respectively); [2]Adjusted for age, sex, smoking status, hypertension, residence and diabetes; [3]Pearson χ^2 = 6.5786, P = 0.037; [4]Pearson χ^2 = 5.1232, P = 0.024.

Table 3 Stratified analyses for variant *pre-miR-146a* C/G genotypes in cases and controls

Variable	(GG + GC)/CC		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value
	Cases	Controls				
Age (median, yr)						
≤ 58	105/42	93/63	1.69 (1.05-2.74)	0.031	1.76 (1.08-2.87)	0.024
> 58	110/47	92/56	1.42 (0.88-2.29)	0.145	1.43 (0.88-2.33)	0.147
Sex						
Female	56/20	48/28	1.63 (0.82-3.26)	0.164	1.80 (0.88-3.67)	0.105
Male	159/69	137/91	1.53 (1.04-2.25)	0.031	1.53 (1.04-2.27)	0.033
Smoking status						
Smokers	52/21	34/21	1.53 (0.73-3.22)	0.263	1.49 (0.69-3.21)	0.310
Non-smokers	163/68	151/95	1.51 (1.03-2.20)	0.035	1.55 (1.06-2.28)	0.025
Residence						
Urban	114/44	103/62	1.56 (0.98-2.49)	0.063	1.58 (0.98-2.54)	0.058
Rural	101/45	81/57	1.58 (0.97-2.57)	0.066	1.58 (0.96-2.60)	0.074

[1]Adjusted for age, sex, smoking status, hypertension, residence and diabetes.

Table 4 Associations between variant *pre-miR-146a* C/G genotypes and clinicopathological characteristics of gastric cancer

Variable	GG + CG	CC	Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)[1]	*P* value
Tumor differentiation						
Well	26	15	1.00		1.00	
Moderate	123	42	1.69 (0.82-3.50)	0.157	1.60 (0.76-3.38)	0.216
Poor	60	31	1.12 (0.52-2.41)	0.779	1.06 (0.46-2.45)	0.894
Depth of tumor infiltration						
T1	37	15	1.00		1.00	
T2	25	11	0.92 (0.36-2.33)	0.863	0.90 (0.34-2.38)	0.839
T3	103	39	1.07 (0.53-2.16)	0.849	1.16 (0.57-2.39)	0.680
T4	44	23	0.78 (0.35-1.70)	0.525	0.78 (0.35-1.75)	0.547
Lymph node metastasis						
Negative	74	33	1.00		1.00	
Positive	135	55	1.09 (0.65-1.83)	0.732	1.09 (0.65-1.83)	0.746
Localization						
Cardia	61	24	1.00		1.00	
Non-cardia	154	65	0.93 (0.54-1.62)	0.804	0.89 (0.50-1.57)	0.683

Data of 7 plaintively treated cases was not obtained for the inoperable tumors. [1]Adjusted for age, sex, smoking status, hypertension, residence and diabetes.

a more stable secondary structure for the G allele[39]. The genetic variant might have an impact on processing the pre-miRNA into the mature microRNA. The mutation in *pre-miR-146a* gene has been reported to modulate cancer risks, although the results are inconsistent[22-26]. Xu *et al*[23] showed that male individuals with GG genotype were 2-fold more susceptible to HCC (OR = 2.016, 95% CI: 1.056-3.848, P = 0.034) compared to those with CC genotype. In a case-control study, Xu *et al*[24] suggested that CC homozygotes exhibited an association with prostate cancer with a 65% decreased risk (95% CI: 0.43-0.99, P = 0.03). Findings by Jazdzewski *et al*[22] demonstrated that the GC

heterozygous state was associated with an increased risk of acquiring PTC (OR = 1.62, P = 0.000007), and both homozygous states were protective with OR = 0.42 for the CC genotype and OR = 0.69 for the GG genotype. Although one study showed that this polymorphism did not appear to be linked with an increased risk of breast cancer[26], another indicated that those breast and ovarian cancer patients who had at least one C allele were diagnosed at an earlier age (median age: 45 *vs* 56, P = 0.029 for breast cancer; median age: 45 *vs* 50, P = 0.014 for ovarian cancer)[25]. We noted that, compared with the CC genotype, the G allele carriers had a 55% increased risk of gastric cancer. After adjustment for age, sex, smoking status, residence, hypertension and diabetes, the difference still existed, confirming that the polymorphism was related to the risk of gastric cancer in China. Although the precise mechanisms of miRNA expression regulation are largely unknown and the inconsistent effect of miRNA SNP for cancer diagnosis and prognosis is ambiguous, those results may imply that miRNA expression patterns are associated with the biological and clinical behavior of human cancers.

Our data showed that the polymorphism was associated with the increased risk of gastric cancer among subgroups of the subjects aged ≤ 58 years, whereas not among older subjects. Overwhelming accumulated exposure to environmental carcinogens and weak immune system in older individuals may account for the age difference we observed[28]. An old age is related to an increased risk for gastric cancer, which is more likely due to the age effect rather than the direct genetic effect. Therefore, the polymorphism in the *pre-miR-146a* may be more evident in early-onset gastric cancer, although this age-specific association needs to be further replicated.

Tobacco smoking is a known cause of gastric cancer[40]. The association between the polymorphism and gastric cancer risk may be masked by the relatively high-level exposure to tobacco carcinogens in smokers so that it is more influential in nonsmokers.

Subsequently, a positive correlation between *pre-miR-146a* genotype and sex was found. A significantly elevated risk of gastric cancer was observed for the GG + GC genotypes among males but not among females. Xu *et al*[23] also concluded that male individuals with GG genotype were more susceptible to HCC compared to those with CC genotype. Based on the findings, it would therefore be plausible to expect that the sex-specific effect of *pre-miR-146a* may be responsible for the different results between males and females, although the exact mechanism remains unclear.

Further stratified analyses by clinicopathologic parameters of gastric cancer showed no significant association between the *pre-miR-146a* genotypes and clinical pathological parameters of gastric cancer. Gastric cancer is considered to be a complex, multi-step and multi-factorial process. Although our data did not support a causal link between the variant genotypes and gastric cancer progression, we could not exclude the possibility that other factors might impact the interaction between them. Moreover, the size of cases in the subgroups was relatively small; our findings from the stratified analyses should be interpreted with caution before confirmed in further studies.

Several limitations should be addressed in the study. First, lack of information on *Helicobacter pylori* (*H. pylori*) infection status did not allow us to analyze the interaction between the polymorphism and *H. pylori* infection status, because it was unethical to do *H. pylori* test in every subject. Second, the sample size was relatively small. This may have been underpowered to detect small but real gene-environment associations. Finally, the study was conducted in Chinese population, data should be extrapolated to other ethnic groups cautiously. Nevertheless, our initial data presented valuable insights and interesting information to guide future studies in this area.

In summary, our results suggested that *pre-miR-146a* C/G polymorphism is associated with the increased risk of gastric cancer in Chinese population, especially in younger individuals, nonsmokers, and males. Further studies are required to elucidate the mechanism underlying the polymorphism and gastric cancer progression.

COMMENTS

Background

MiR-146a is a small non-coding regulatory RNA supposed to regulate innate immune, inflammatory response and antiviral pathway negatively. Polymorphism in human *pre-miR146a* has been recently implicated in human cancers.

Research frontiers

Using polymerase chain reaction-restriction fragment length polymorphism method in 608 individuals, this study explored the relationship between *pre-miR-146a* C/G polymorphism and gastric cancer risk.

Innovations and breakthroughs

The results suggest that the polymorphism is associated with the elevated risk of gastric cancer in Chinese population, especially in younger individuals, males and nonsmokers.

Applications

The results of this study could be helpful in further understanding the genetic determinants of gastric cancer.

Peer review

This manuscript describes the association of a single nucleotide polymorphism in the *hsa-miR-146a* with gastric cancer, which is novel for this particular type of cancer, but biologically plausible owing to the associations reported by other authors for different types of tumors.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3584

World J Gastroenterol 2010 July 28; 16(28): 3584-3591
ISSN 1007-9327 (print)

BRIEF ARTICLE

Enhancement of antitumor vaccine in ablated hepatocellular carcinoma by high-intensity focused ultrasound

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Author contributions: Wu F and Zhang Y designed the research; Zhang Y, Deng J, Feng J and Wu F performed the research; Zhang Y and Deng J analyzed the data; Wu F and Zhang Y wrote the paper.
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Received: December 8, 2009 Revised: April 6, 2010
Accepted: April 13, 2010
Published online: July 28, 2010

Abstract

AIM: To investigate whether tumor debris created by high-intensity focused ultrasound (HIFU) could trigger antitumor immunity in a mouse hepatocellular carcinoma model.

METHODS: Twenty C57BL/6J mice bearing H22 hepatocellular carcinoma were used to generate antitumor vaccines. Ten mice underwent HIFU ablation, and the remaining 10 mice received a sham-HIFU procedure with no ultrasound irradiation. Sixty normal mice were randomly divided into HIFU vaccine, tumor vaccine and control groups. These mice were immunized with HIFU-generated vaccine, tumor-generated vaccine, and saline, respectively. In addition, 20 mice bearing H22 tumors were successfully treated with HIFU ablation. The protective immunity of the vaccinated mice was investigated before and after a subsequent H22 tumor challenge. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, the cytotoxicity of splenic lymphocytes co-cultured with H22 cells was determined *in vitro* before the tumor challenge, and tumor volume and survival were measured *in vivo* after the challenge in each group. The mechanism was also explored by loading the vaccines with bone marrow-derived dendritic cells (DCs).

RESULTS: Compared to the control, HIFU therapy, tumor-generated and HIFU-generated vaccines significantly increased cytolytic activity against H22 cells in the splenocytes of the vaccinated mice ($P < 0.001$). The tumor volume was significantly smaller in the HIFU vaccine group than in the tumor vaccine group ($P < 0.05$) and control group ($P < 0.01$). However, there was no tumor growth after H22 rechallenge in the HIFU therapy group. Forty-eight-day survival rate was 100% in mice in the HIFU therapy group, 30% in both the HIFU vaccine and tumor vaccine groups, and 20% in the control group, indicating that the HIFU-treated mice displayed significantly longer survival than the vaccinated mice in the remaining three groups ($P < 0.001$). After bone marrow-derived DCs were incubated with HIFU-generated and tumor-generated vaccines, the number of mature DCs expressing MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$ molecules was significantly increased, and interleukin-12 and interferon-γ levels were significantly higher in the supernatants when compared with immature DCs incubated with mouse serum ($P < 0.001$). However, no differences of the number of mature DCs and cytokine levels were observed between the HIFU-generated and tumor-generated vaccines ($P > 0.05$).

CONCLUSION: Tumor debris remaining after HIFU can improve tumor immunogenicity. This debris releases tumor antigens as an effective vaccine to develop host antitumor immune response after HIFU ablation.

Key words: Hepatocellular carcinoma; High-intensity focused ultrasound; Immune response; Immunogenicity; Immunotherapy; Thermal ablation; Tumor vaccine

Peer reviewer: Tamara M Alempijevic, MD, PhD, Assistant Professor, Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Serbia

Zhang Y, Deng J, Feng J, Wu F. Enhancement of antitumor vaccine in ablated hepatocellular carcinoma by high-intensity focused ultrasound. *World J Gastroenterol* 2010; 16(28): 3584-3591 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3584.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3584

INTRODUCTION

As a non-invasive thermal ablation, high-intensity focused ultrasound (HIFU) therapy has received increasing attention for the clinical management of patients with solid tumors, including those of the prostate, liver, pancreas, breast, kidney, uterus, bone and soft tissue[1-3]. Under real-time imaging guidance, this technique uses ultrasound energy locally to ablate a targeted tumor at depth, with no damage to overlying tissues. In addition, it has been shown that HIFU ablation can enhance host antitumor immune response[4-13], which may be of benefit in local recurrence and metastasis control in cancer patients who have previous dysfunction of antitumor immunity.

Selective recognition and destruction of tumor cells by the host immune system is a major role of antitumor immunity, and tumor antigens expressed by tumor cells are essential to achieve this antitumor immune response specific to tumor cells. After HIFU ablation, large amounts of tumor debris remain *in situ,* and the host gradually reabsorbs them as a normal process of the healing response. Our previous findings revealed a variety of tumor antigen expressions on HIFU-ablated breast cancer cells[15]. Some tumor antigens disappeared completely, others remained in their entirety such as heat shock protein (HSP) 70, while most remained partially in the tumor debris after HIFU ablation. However, it is still unknown whether the remaining tumor debris may be a potential antigen source available for the induction of host antitumor immunity. To test HIFU effects on the inherent immunogenicity of the tumor debris, we performed HIFU to ablate *in vivo* hepatocellular carcinoma (HCC), and then used the tumor debris to inoculate naïve animals against subsequent tumor challenge. The purpose of this study was to investigate whether the remaining tumor debris created by *in situ* HIFU ablation could be strongly immunogenic as an effective tumor vaccine to stimulate host antitumor immunity, and to provide potential benefit in long-term survival in a murine tumor model.

MATERIALS AND METHODS

Animals

The animal study was approved by the Chongqing Experimental Animal Committee (Chongqing, China). Male and female C57BL/6J mice (6-8 wk old) were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China), and housed in microisolator cages in a laminar flow unit under ambient light in the same animal center. All animal experiments adhered to the Animal Welfare Committee guidelines.

Tumor and vaccine generation

The H22 HCC cell line was provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). 2×10^6 H22 cells (0.02 mL) were injected subcutaneously into the right flank of syngeneic C57BL/6J mice to establish a tumor model. Palpable tumors started to develop after 3 d, and reached the size of 5-7 mm after 7 d.

Twenty C57BL/6J mice bearing H22 tumors were used to generate antitumor vaccines at day 7 after tumor implantation. Under general anesthesia 10 mice underwent HIFU ablation, and the remaining 10 mice received a sham-HIFU procedure with no ultrasound irradiation. HIFU energy was produced by a 2-cm diameter transducer with a focal length of 8 mm, operating at a frequency of 9.5 MHz. Acoustic power was 5 W, and median exposure time was 220 s (range: 180-240 s). All mice were sacrificed 1 d after treatment, and tumor samples were harvested. The tissues were brought up to the same weight in both treated and untreated tumors, and they were respectively minced and homogenized. Repetitive freeze-thaw cycles were performed for the preparation of cellular lysate. Using the Bradford assay (Bio-Rad, Hercules CA, USA), the same protein concentration (0.5 mg/mL) was also achieved in both lysates, where the treated and untreated tumor tissues had the milligram per milliliter protein concentration in RPMI 1640.

Immunization

Sixty C57BL/6J mice were randomly divided into three groups: control group, tumor vaccine group and HIFU vaccine group. Each group had 20 mice, including 10 for cytotoxic T lymphocyte (CTL) assay 15 d after vaccination and 10 for long-term follow-up after tumor challenge. By using subcutaneous injection, the mice in the tumor group and HIFU group received either 10 μg untreated H22 vaccine or 10 μg HIFU-treated H22 vaccine in the left flank of each mouse. Those in the control group received only injection with same amount of saline solution. The vaccination times were 2 sessions, once a week for 2 consecutive weeks.

Cytotoxicity assay of CTL

Ten mice were sacrificed 7 d after the final vaccination in each group, and spleens were harvested to assess the activity of splenic CTL. Single cell suspensions were generated by passage through a metallic mesh. Erythrocytes were lysed with 0.87% ammonium chloride for 1 min, and macrophages were removed by exposure to plastic plates for 2 h. The nonadherent lymphocytic population was collected, washed, and resuspended at 2×10^6 cells/mL as CTL

effectors. H22 and B16 (a mouse melanoma cell line) cells were used as targeted cells, and the effector/target cell ratio was 10:1, which was the best cellular ratio in our pre-experimental study. The splenic lymphocytes were then co-cultured with either H22 or B16 cells in 96-well plates for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine the cytotoxicity of the CTLs in each group. This is a standard colorimetric assay for determining *in vitro* cytotoxicity of CTLs against tumor cells in experiments[16,17]. As negative control groups, an equal number of target cells were cultured alone and an equal number of effector cells were cultured without target cells in a total of 200 μL. Using a multiwell spectrophotometer reader (Molecular Devices, Menlo Park, California, USA), the optical intensity at 570 nm was measured. Each assay was performed in triplicate, and RPMI 1640 medium was used as a blank control. According to the optical intensity in each group, the cytotoxicity calculation was determined by the following equation: Cytotoxicity (%) = (Effector spontaneous + Target spontaneous - Experimental)/(Target spontaneous - Target maximal) × 100%.

Tumor challenge

Seven days after the final vaccination, 10 mice were challenged in each group by subcutaneous injection of 2×10^6 H22 cells (0.02 mL) in the right flank of each mouse. The tumor growth was detected every 3 d by measuring its diameter with a Vernier caliper. Tumor volume was calculated using the following formula: tumor volume (mm^3) = $d^2 \times D/2$, where d and D are the shortest and longest diameters of the measured tumor, respectively. All mice were followed up for 48 d to observe the survival data.

Immune response after HIFU

Twenty C57BL/6J mice bearing H22 tumors were treated with HIFU at day 7 after tumor implantation to determine whether specific antitumor reactivity could be detected directly after HIFU ablation. These mice were classified as the HIFU therapy group in this study, and HIFU therapeutic parameters were similar to those used in generating the HIFU-related tumor vaccine. Fifteen days after HIFU ablation, 10 mice were sacrificed for the assessment of *in vitro* CTL cytotoxicity as described above, and the remaining 10 were rechallenged with 2×10^6 H22 tumor cells to follow long-term survival.

Isolation and culture of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (DCs) were isolated from C57BL/6J mice, as described by Inaba and colleagues[18]. Briefly, DCs were obtained from bone marrow precursors by flushing mouse femur and tibia bones with cold PBS. After erythrocytes were lysed with ammonium chloride, erythrocyte-depleted bone marrow cells (4×10^6/2 mL per well) were cultured in 6-well plates (Nunc, Roskilde, Denmark) in complete medium (RPMI 1640 supplemented with 10% FBS, L-glutamine, 5 mmol/L 2-mercaptoethanol, and antibiotics) at 37℃ in a humidified atmosphere with 50 mL/L CO_2. The culture medium also contained 20 ng/mL mouse recombinant granulocyte macrophage colony-stimulating factor (mrGM-CSF; PeproTech, London, UK) and 20 ng/mL mouse recombinant interleukin-4 (mrIL-4; PeproTech, London, UK). The cultures were fed every 2 d with fresh medium containing 10 ng/mL mrGM-CSF and 10 ng/mL mrIL-4. On day 7, nonadherent and loosely adherent cells were collected, washed, and resuspended in PBS at 1×10^6/mL. These cells presented the typical morphological characteristics of immature DCs, and flow cytometry analysis showed that a majority (75%-80%) of them had positive expression of CD11c and CD205 molecules.

DCs loaded with vaccines

2×10^6/mL immature DCs were primed with either 5 μg H22 tumor vaccine (tumor vaccine group) or 5 μg HIFU-generated vaccine (HIFU vaccine group) in complete medium at 37℃. DCs co-cultured with the same amount of mouse serum alone were classified as the control group. These cells were incubated for 5 d in a humidified atmosphere with 50 mL/L CO_2. Using flow cytometry (FACSCalibur™ Flow Cytometer, BD Biosciences, San Jose, CA, USA), the cells in each group were then analyzed for the expression of MHC class Ⅱ, CD80, and CD86 molecules. Culture supernatants were harvested in each group, and cytokine production was determined in the supernatants by enzyme-linked immunosorbent assay using murine kits from R&D Systems (Minneapolis, MN, USA) for interleukin (IL)-12 and interferon (IFN)-γ, according to the manufacturer's recommendations. Each assay was performed in triplicate with separate DC preparations.

Statistical analysis

All observed data are displayed as mean ± SD. Statistical analysis was performed using the Student's *t* test. A cumulative survival rate was calculated by using the Kaplan-Meier method, and the statistical significance of any survival difference was evaluated by the log-rank test. Differences were considered statistically significant when the *P* value was less than 0.05.

RESULTS

HIFU-generated vaccine induces CTL cytotoxicity against H22 Cells

As shown in Figure 1 and Table 1, HIFU therapy, tumor- and HIFU-generated vaccines significantly increased cytolytic activity against H22 cells in the splenocytes of the vaccinated mice when compared with the activity in mice vaccinated with saline alone ($P < 0.001$). None of the vaccines elicited CTL cytotoxicity to control target B16 cells ($P > 0.05$), suggesting that HIFU therapy and the HIFU-generated vaccine could induce specific antitumor immunity. The splenocytes isolated from the HIFU-treat-

Table 1 Cytotoxicity rate of splenic lymphocytes against H22 and H16 tumor cells in the vaccinated and high-intensity focused ultrasound-treated mice (mean ± SD)

	Cytotoxicity rate (%)	
	H22 tumor cells	B16 tumor cells
Control group	15.9 ± 3.6	13.5 ± 2.3
Tumor vaccine group	30.7 ± 2.7[d]	14.1 ± 1.9
HIFU vaccine group	37.5 ± 4.5[a,d]	13.8 ± 2.2
HIFU therapy group	62.7 ± 6.5[d,f]	18.1 ± 3.9

[a]$P < 0.05$ *vs* the tumor vaccine group; [d]$P < 0.001$ *vs* the control group; [f]$P < 0.001$ *vs* the HIFU vaccine and tumor vaccine groups. HIFU: High-intensity focused ultrasound.

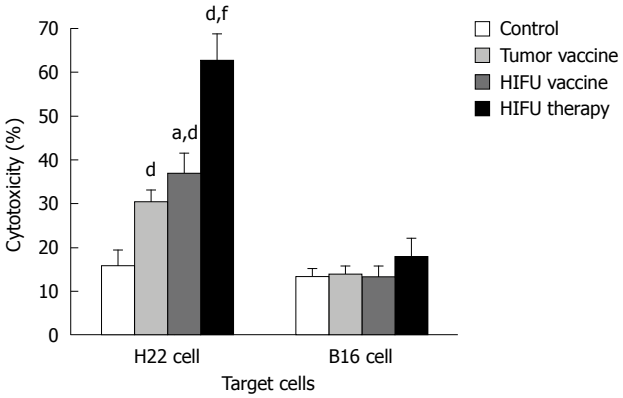

Figure 1 Cytotoxic activity of cytotoxic T lymphocytes against either H22 or B16 tumor cells *in vitro* at 10:1 effector:target ratio in the vaccinated and high-intensity focused ultrasound-treated mice. Naïve mice were vaccinated with high-intensity focused ultrasound (HIFU)-generated and tumor-generated vaccines, and saline alone once a week for 2 wk. The mice bearing H22 tumors were also treated with HIFU ablation. The vaccinated animals were sacrificed 7 d after the 2nd vaccination, and the HIFU-treated mice were sacrificed 15 d after HIFU therapy. The spleens were harvested, and single cell suspensions were generated. The splenic lymphocytes were then co-cultured with either H22 or B16 cells for 24 h. The cytotoxicity of the cytotoxic T lymphocytes was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in each group after 2 h co-incubation. [a]$P < 0.05$ *vs* the tumor-generated vaccine; [d]$P < 0.001$ *vs* the control; [f]$P < 0.001$ *vs* the HIFU- and tumor-generated vaccines.

ed mice produced significantly stronger anti-H22 CTL activity than that of either the HIFU vaccine or tumor vaccine group ($P < 0.001$). The HIFU-generated vaccine was significantly better than the tumor-generated vaccine at increasing the cytotoxicity of CTLs ($P = 0.013$).

HIFU-generated vaccine mediates inhibition of tumor growth

The tumors were monitored with the caliper for 4 wk until mean tumor size was too variable due to death of the mice. As shown in Figure 2 and Table 2, vaccination with HIFU-treated tumor had a marked inhibitory effect on tumor growth in the 3rd and 4th wk of the tumor challenge when compared with the control (saline) ($P < 0.01$) and tumor-generated groups ($P < 0.05$). The strongest inhibition was observed in the HIFU therapy group because after H22 rechallenge no tumor growth was detected during the follow-up period. An inhibition of tumor growth was

Table 2 Tumor volume of the vaccinated and high-intensity focused ultrasound-treated mice after a subsequent H22 tumor challenge (mean ± SD)

	Average tumor volume (mm^3) after H22 tumor challenge		
	2 wk	3 wk	4 wk
Control group	343.3 ± 129.5	829.3 ± 316.4	1953.0 ± 848.2
Tumor vaccine group	504.1 ± 173.7	733.4 ± 301.3	1760.2 ± 1075.1
HIFU vaccine group	279.7 ± 117.2	382.8 ± 170.6[a,d]	914.3 ± 474.2[a,d]
HIFU therapy group	0	0	0

[a]$P < 0.05$ *vs* the tumor vaccine group; [d]$P < 0.01$ *vs* the control group. HIFU: High-intensity focused ultrasound.

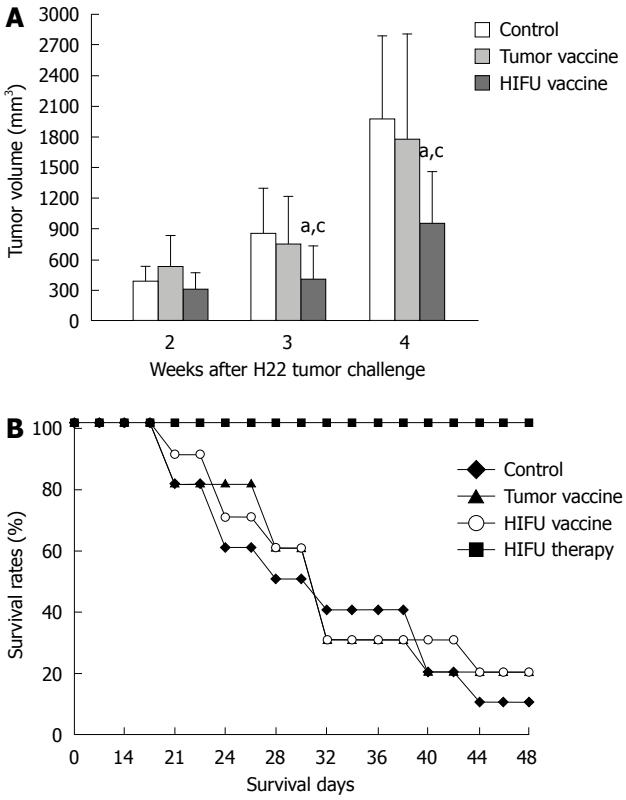

Figure 2 High-intensity focused ultrasound-generated vaccine inhibits tumor growth after a subsequent tumor challenge in a mouse H22 tumor model. Naïve mice were vaccinated with high-intensity focused ultrasound (HIFU)-generated and tumor-generated vaccines, and saline alone once a week for 2 wk. The mice bearing H22 tumors were also treated with HIFU ablation. 7 d after the 2nd vaccination, the vaccinated animals were challenged with 2×10^6 viable H22 cells, and the HIFU-treated mice received a second tumor challenge with the same number of H22 cells 15 d after HIFU therapy. Tumor diameters were measured for 3 wk, and the results were reported as the tumor volume. All mice were followed up for 48 d, and a cumulative survival rate was calculated in each group. A: The tumor volume, measured with a Vernier caliper, in the vaccinated and HIFU-treated mice after a subsequent tumor challenge. [a]$P < 0.05$ *vs* the control; [c]$P < 0.05$ *vs* the tumor-generated vaccine; B: Cumulative survival curves, calculated with the Kaplan-Meier method, in the vaccinated and HIFU-treated mice. Compared to the other groups, HIFU therapy shows a significant increase in survival ($P < 0.001$, the log-rank test).

observed in the tumor-generated vaccine group. However, there was no statistical difference between the control and tumor-generated vaccine groups ($P > 0.05$).

Table 3 CD86, CD80 and MHC-Ⅱ expression by bone marrow-derived dendritic cells after incubation with high-intensity focused ultrasound- and tumor-generated vaccines (mean ± SD, %)

	CD86	CD80	MHC-Ⅱ
Control group	2.05 ± 1.40	4.81 ± 1.56	3.04 ± 0.60
Tumor vaccine group	11.70 ± 0.85[b]	13.50 ± 0.14[b]	13.75 ± 0.59[b]
HIFU vaccine group	14.65 ± 0.49[b]	16.05 ± 0.50[b]	15.90 ± 0.28[b]

[b]$P < 0.001$ *vs* the control. HIFU: High-intensity focused ultrasound.

Table 4 Interleukin-12 and interferon-γ secretion by bone marrow-derived dendritic cells after incubation with high-intensity focused ultrasound- and tumor-generated vaccines (mean ± SD)

	IL-12 (pg/mL)	IFN-γ (pg/mL)
Control group	80.2 ± 4.6	58.0 ± 0.9
Tumor vaccine group	206.8 ± 5.3[b]	207.0 ± 3.4[b]
HIFU vaccine group	264.7 ± 2.0[b]	247.8 ± 9.0[b]

[b]$P < 0.001$ *vs* the control group. HIFU: High-intensity focused ultrasound; IL-12: Interleukin-12; IFN-γ: Interferon-γ.

HIFU therapy results in better survival than tumor or HIFU vaccines

Survival of the vaccinated mice and HIFU-treated mice was also recorded for up to 48 d after tumor challenge with H22 cells. Survival curves (Figure 2) showed that 100% of HIFU-treated mice, 30% of HIFU vaccinated mice, and 30% of tumor vaccinated mice survived for 48 d, whereas 20% of saline vaccinated mice (control) survived 48 d. The HIFU-treated mice displayed significantly longer survival than the vaccinated mice in the remaining three groups ($P < 0.001$). The mice inoculated with either the HIFU-generated vaccine or tumor-generated vaccine survived a little longer than the mice vaccinated with saline (control). However, no statistical differences were observed among them.

HIFU-generated vaccine activates immature DCs

The expression of MHC class Ⅱ, CD80 and CD86 molecules on DCs was determined by flow cytometry after incubation for 5 d. As shown in Figure 3 and Table 3, incubation with either the HIFU-generated vaccine or tumor-generated vaccine significantly increased the number of mature DCs (MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$) when compared with incubation with mouse serum ($P < 0.001$), suggesting both vaccines could induce phenotypic maturation of DCs. However, no differences in the expression of MHC-Ⅱ, CD80 and CD86 were observed between the HIFU- and tumor-generated vaccines ($P > 0.05$).

HIFU-generated vaccine induces IL-12 and IFN-γ secretion by DCs

As shown in Figure 3 and Table 4, after immature DCs were incubated with HIFU- and tumor-generated vaccines, IL-12 and IFN-γ levels were significantly higher in the supernatants compared to DCs incubated with mouse serum ($P < 0.001$). However, there was no statistical difference in IL-12 and IFN-γ secretion between the HIFU and tumor vaccine groups ($P > 0.05$). These results demonstrated that both HIFU- and tumor-generated vaccines could induce the functional maturation of DCs.

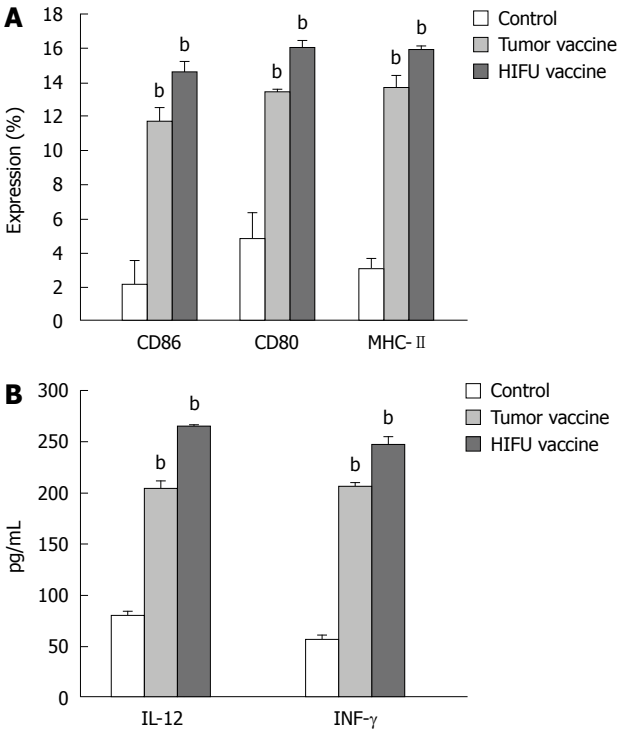

Figure 3 High-intensity focused ultrasound-generated and tumor-generated vaccines activate dendritic cells. Immature dendritic cells (DCs) were isolated from C57BL/6J bone marrow cultures, and then incubated for 5 d with the high-intensity focused ultrasound (HIFU)-generated vaccine, tumor-generated vaccine, and mouse serum alone. After incubation the cells were subjected to flow cytometry. Culture supernatants were harvested, and the enzyme-linked immunosorbent assay method was used to determine the production of interleukin (IL)-12 and interferon (IFN)-γ in the supernatants. [b]$P < 0.001$ *vs* the control. A: HIFU-generated vaccine induces the maturation of bone marrow-derived DCs. Results are reported as percentage of MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$ cells in the total population; B: HIFU-generated vaccine induces IL-12 and IFN-γ secretion by mature DCs.

DISCUSSION

The concept of HIFU as a noninvasive therapy for the local destruction of diseased tissue dates back more than 60 years. Much of the clinical application is recent, where clinical results are very promising in the treatment of solid malignancies[1-3]. It is obvious that large amounts of *in situ* tumor debris remain after HIFU ablation, however, little is known about whether this debris may be a potential antigen source for triggering host antitumor immune response. Using a newly developed tumor destruction model, we demonstrated that the remaining tumor debris can be immunogenic as an effective vaccine to elicit tumor-specific immune responses, including induction of CTL cytotoxic activity in the spleen, protection against a lethal tumor challenge in naïve mice, activation of immature DCs, and secretion of Th1-associated cytokines

by mature DCs for the development of cell-mediated immune response. To our knowledge, this is the first report of the use of HIFU ablation to generate an *in situ* tumor vaccine, and the first report of the use of this crude tumor vaccine which is functional in stimulating tumor-specific immunity in naïve animals in the absence of immune adjuvant. Our findings may contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response. However, with the use of flow cytometry, peptide MHC tetramers analysis is needed in the future to measure the number of antigen-specific CD8+ T lymphocytes.

Thermal and nonthermal effects are two major mechanisms related to HIFU-induced coagulation necrosis. During HIFU exposure, the absorption of ultrasound energy in a targeted tumor leads to a rapid temperature rise above 56℃ within the focal volume[19], and thus induces complete coagulation necrosis of the targeted cancer, with no direct evidence of apoptotic cells detected by the TUNEL method in the treated tumors[20].

It is postulated that antitumor immunity enhanced by the ultrasound thermal effect would be similar to those observed in other thermal therapies such as radiofrequency[21-23]. Cavitation is the most important nonthermal mechanism for HIFU-induced tissue destruction. It can cause membranous organelles, including mitochondria, endoplasmic reticulum, cell and nuclear membranes to collapse instantaneously, and thus lead to tumor cells breaking up into small pieces, on which the tumor antigens may remain intact[24]. Recent studies have revealed that acoustic cavitation can upregulate expression of tumor antigens such as heat shock proteins[4,5,8,14,15]. If heat shock proteins (HSPs) remain and upregulate as intracellular molecular chaperones in the tumor debris after HIFU ablation, they may bind tumor peptide antigens, and act as tumor vaccines to produce a potent cellular immune response[25,26]. However, as overexpression of HSPs may have deleterious effects on antitumor immunity after heat treatments such as hyperthermia, further studies are needed to evaluate whether HSPs could play an important role in the induction of host antitumor immune response after HIFU therapy.

Although the mechanism behind this enhanced immune response is still unknown, our findings reveal that it should be specific antitumor immunity. We have demonstrated that the HIFU-generated tumor vaccine can significantly elicit cytotoxicity of CTLs to H22 cells, whereas cytolytic activity against control target B16 cells was not observed in the splenocytes of vaccinated mice. Compared to tumor lysate, *in vitro* anti-H22 CTL activity was stronger in mice receiving the HIFU-generated tumor vaccine. Similar results were also observed in a mouse H22 tumor model after the vaccinated mice were challenged with H22 cells. Vaccination with HIFU-treated tumor caused a stronger inhibition of tumor growth than the control and tumor-generated vaccine groups, indicating the involvement of a tumor-specific immune response. However, this immune protection was still weak, because no significant survival benefit was observed after a lethal H22 challenge in the HIFU-generated vaccine group when compared with the control and tumor-generated vaccine groups.

We have found that the mice bearing H22 tumors, which were treated by HIFU previously, had the strongest protection against a second H22 cell challenge. The most potent cytotoxicity of CTLs against H22 cells *in vitro* was detected in mice who received previous HIFU ablation. Compared to the HIFU- and tumor-generated vaccine groups, *in situ* HIFU ablation of H22 tumors resulted in complete protection against a subsequent H22 tumor rechallenge. All mice survived during the follow-up period, with no evidence of tumor growth. These data suggest that once mice bearing H22 tumors are cured by HIFU treatment, a bona fide systemic memory response may be generated. In addition, tumor debris remaining after *in situ* ablation may continuously stimulate the host immune system during the reabsorption of dead tissue, leading to a stronger antitumor immune response. However, to support our speculation, further studies are needed to investigate the mechanisms behind this. Furthermore, as follow-up time was limited in this study, a longer period is necessary in future studies to observe the survival benefit and tumor development after tumor rechallenge in the HIFU-treated mice. Both the HIFU- and tumor-generated vaccine groups developed a relatively weak immune response, because the vaccination times were very limited, only performed once a week for 2 consecutive weeks. Therefore, further studies are necessary in this mouse H22 tumor model to optimize the vaccination method including the number of sessions, dosage and interval time. In order to induce a stronger immunological response, a longer vaccination time with the HIFU-treated tumor will be investigated, and immunoadjuvants will be used in combination with HIFU therapy.

Similar to other thermal therapies, a marked inflammatory reaction, with abundant leukocytic infiltration, has been histologically observed at the margins of coagulation necrosis in HIFU ablation[27-30]. We have recently found that HIFU ablation can significantly induce local infiltration of activated DCs within the marked inflammatory reaction in patients with breast cancer[31]. DCs are the most potent antigen-presenting cells for induction of adaptive immunity against cancer[32]. They infiltrate local tumors and present tumor antigens to naïve T lymphocytes in a MHC restricted fashion. Activating signals, delivered directly or indirectly by tumor cells including apoptotic and necrotic tumor cells, can induce the progression of infiltrating DCs from an immature to a mature stage[33]. During maturation DCs increase the expression of costimulatory molecules such as CD80 and CD86, and mature DCs secrete Th1-associated cytokines to induce cell-mediated immunity[34]. This study produced direct evidence that with no immune adjuvant, the remaining tumor debris can activate immature DCs, and thus induce secretion of IL-12 and IFN-γ by mature DCs for the development of cellular antitumor immune response. However, it is still

unknown whether the activated DCs could induce an *in situ* antitumor immune response by presenting tumor antigens directly to lymphocytes. Further studies are necessary to investigate the potential role of activated DCs in the induction of specific antitumor immunity *in vivo*.

Our findings indicate that a weak but tumor-specific immune response was produced by the HIFU-generated tumor vaccines after *in situ* tumor destruction. Therefore, active immunological stimulation such as immunoadjuvants, in combination with HIFU, could augment the efficacy of HIFU-induced antitumor immunity specifically against the targeted tumors, if the destruction of tumors releases tumor antigens or improves tumor immunogenicity.

HCC is one of the most common malignancies worldwide. Local tumor recurrence and metastasis are usually the cause of failure of multidisciplinary treatments of HCC in clinical practice. Using a mouse HCC model, we found that HIFU ablation can trigger host tumor-specific immune response. This may decrease or perhaps even eliminate residual and metastatic tumor cells in HCC patients who have had original antitumor immunity dysfunction.

In summary, this study demonstrated that tumor debris remaining after *in situ* HIFU ablation may improve tumor immunogenicity. This debris may release tumor antigens as an effective vaccine to elicit tumor-specific immune responses. However, further studies are needed to explore the nature of the "activation" factors in HIFU-generated tumor debris.

COMMENTS

Background

As a non-invasive thermal ablation, high-intensity focused ultrasound (HIFU) therapy has received increasing attention for the clinical management of patients with hepatocellular carcinoma (HCC). After HIFU ablation, large amounts of tumor debris remain *in situ*, and the host gradually reabsorbs them as a normal process of the healing response. However, it is still unknown whether the remaining tumor debris may be a potential antigen source available for the induction of host antitumor immunity.

Research frontiers

In the present study, the authors investigated whether tumor debris created by *in situ* HIFU could be an effective tumor vaccine to stimulate antitumor immunity in a mouse HCC model.

Innovations and breakthroughs

This study demonstrated that the remaining tumor debris after HIFU can be immunogenic as an effective vaccine to elicit tumor-specific immune responses, including induction of cytotoxic T lymphocyte cytotoxic activity in the spleen, protection against a lethal tumor challenge in naïve mice, activation of immature dendritic cells (DCs), and secretion of Th1-associated cytokines by mature DCs for the development of cell-mediated immune response. These findings may contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response.

Applications

These findings contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response. In addition, they suggest that HIFU ablation combined with subsequent immunotherapy such as immunoadjuvants may augment the efficacy of HIFU-induced antitumor immunity specifically against the targeted tumor, leading to a decrease of local recurrence and metastasis in HCC.

Peer review

It is a very interesting paper with adequately described and written all necessary parts of a manuscript.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3592

World J Gastroenterol 2010 July 28; 16(28): 3592-3596
ISSN 1007-9327 (print)

CASE REPORT

Sustained treatment response of metastatic hepatocellular carcinoma with bevacizumab and sorafenib

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Received: January 14, 2010 Revised: March 15, 2010
Accepted: March 22, 2010
Published online: July 28, 2010

Abstract

The overall survival for patients with advanced hepatocellular carcinoma (HCC) is still limited. Although the multi-kinase inhibitor sorafenib has recently been approved for this disease, response rates are still low and patients often face dose-limiting toxicities which lead to a reduction in prognosis and treatment success. We here report a patient with metastasized HCC who shows a sustained response for more than 30 mo to sorafenib therapy after failure of a first line therapy with gemcitabine, oxaliplatin and bevacizumab.

Key words: Hepatocellular carcinoma; Sorafenib; Bevacizumab; Angiogenesis

Peer reviewers: Chih-Chi Wang, MD, Department of Surgery, Chang Gung Memorial Hospital-Kaohsiung Medical Center, 123 Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan, China; Hitoshi Yoshiji, MD, PhD, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

Wich C, Agaimy A, Strobel D, Wissniowski TT, Hartmann A, Ocker M. Sustained treatment response of metastatic hepatocellular carcinoma with bevacizumab and sorafenib. *World J Gastroenterol* 2010; 16(28): 3592-3596 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3592.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3592

INTRODUCTION

Hepatocellular carcinoma (HCC) belongs to the most common malignancies worldwide and shows a rising incidence in Western countries due to high prevalence of chronic viral hepatitis, alcoholic liver damage and metabolic disorders associated with fatty liver degeneration[1-3]. Surgical and loco-regional approaches provide satisfying results for patient with early stage HCC, while advanced diseases with underlying cirrhosis have an unfavourable prognosis[4].

Angiogenesis and neo-vascularization are considered "hallmarks" of malignant tumors and several strategies have been established to interfere with the underlying signalling cascade[5], e.g. the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab or the small molecule tyrosine kinase inhibitor sorafenib. HCC represents a highly vascularised tumor[6] and various anti-angiogenic treatment strategies have therefore been applied to this disease[7]. So far, only the multi-kinase inhibitor sorafenib provided a significant patient benefit in this setting and has therefore been approved as the first-line therapy for advanced HCC in patients with good metabolic liver capacity (Child-Pugh A)[8,9]. Although sorafenib showed a good tolerability in the study populations, most patients in clinical practice suffer from underlying liver cirrhosis with impaired metabolic function and experience dose-limiting toxicities with the need to reduce the overall dose of sorafenib subsequently[10].

We herein report a case of advanced metastatic HCC which was treated with a reduced dose of sorafenib for more than 30 mo after failure of a first-line therapy with gemcitabine, oxaliplatine and bevacizumab and intermittent radiation therapy.

CASE REPORT

In January 2007, a 56-year-old man presented in our hospital for clinical diagnosis of elevated liver enzymes with suspicion of ethyltoxic liver cirrhosis due to regular alcohol consumption of 60-80 g/d. Blood tests and ultrasonic investigation confirmed liver cirrhosis of Child-Pugh B (score 7) with elevated bilirubin (2.3 mg/dL) and acquired coagulation deficiency (INT 1.39). Ascites or hepatic encephalopathy were not present. Other aetiologies, e.g. chronic viral or autoimmune hepatitis, haemochromatosis or α_1-antitrypsin deficiency were excluded by corresponding blood tests. Moreover, multilocular (n = 6), extended lesions (diameter > 1 cm) were detected by contrast enhanced ultrasound and magnetic resonance imaging scan (largest lesions in segments Ⅱ: 1.2 cm × 1.5 cm; Ⅳa: 4.9 cm × 3.7 cm × 5.6 cm; Ⅷ: 1.9 cm × 2.9 cm). Portal vein thrombosis or invasion were not identified. High levels of α-fetoprotein (1900 ng/mL) substantiated the diagnosis of HCC according to AASLD guidelines[4,11]. Biopsy proved a well differentiated HCC (Figure 1).

For treatment of multifocal HCC, based on AASLD practice guidelines, neither systemic chemotherapy nor therapy options for a better overall survival were established in spring 2007. Locoregional methods like high frequency thermotherapy (HFTT) or radiofrequency ablation as well as transarterial chemoembolization could not be applied in this patient due to the multifocal disease[12]. It was not possible to perform selective internal radiotherapy (SIRT) representing an experimental therapy option in multilocular hepatic cancer[13]. Extrahepatic tumor manifestation was detected by positron emission tomography/computed tomography (CT) presenting a single thoracic metastasis of the 2nd rib at the right side of 8.8 cm × 7.7 cm. Consequently, SIRT was not performed. No histological analysis of this lesion was obtained as osseous metastases are typical extrahepatic manifestations of advanced HCC[14-16]. According to the Barcelona Clinic Liver Cancer staging system, our patient was classified as stage C (advanced stage) with good ECOG performance (status 1-2)[17]. We therefore considered the patient for new agents or randomized controlled trials[6].

Off-label use of systemic drug therapy seemed to offer the best option to sustain this patient at this time. A review of the literature available in January 2007 disclosed a phase Ⅱ trial with moderate response rates in palliative HCC treatment by a combination of gemcitabine, oxaliplatin (GemOx) and the angiogenesis inhibitor bevacizumab[18]. After informed consent of the patient, an individualized chemotherapy with this regimen was started in May 2007. The patient received 10 mg/kg bevacizumab on day 1 together with 1000 mg/m^2 gemcitabine followed by oxaliplatin at 85 mg/m^2 on day 2. First staging was performed after four cycles (14 d/cycle) resulting in a moderate tumor progression on thoracic CT scan and ultrasound but showed a good overall tolerability and stable primary tumors in the liver. Therapy was continued based on a good overall tolerability, the strong wish of the patient for continuing and the fact that no other therapy option was available at this time. The ongoing systemic chemotherapy induced a moderate pancytopenia, slight renal dysfunction and weight loss (8 kg in 8 mo). Yet, discontinuation of the therapy regimen was not necessary until 2nd staging in September 2007. Here, further progression of the thoracic metastasis with stable disease of hepatic lesions was demonstrated by CT and ultrasound scans (Figure 2).

At this time, a significant benefit for advanced HCC was proven in a randomized controlled phase Ⅲ trial using the novel raf-kinase inhibitor sorafenib as a single agent[8] which lead to the approval of sorafenib for the first-line therapy of advanced HCC in December 2008[9]. Treatment was started in October 2007 with the recommended dose of 800 mg/d. Known side effects (e.g. diarrhea, hand-foot-reaction, oral ulceration) developed during the first 2 wk of treatment and were handled symptomatically (e.g. loperamide, oily creams, mucositis solutions) and with a concomitant dose-reduction of sorafenib to 400 mg/d. Staging after 12 mo revealed an extraordinary regression of both hepatic and thoracic tumor masses (Figure 3). To avoid potential tumor progression by drug resistance, the patient underwent local radiation of the osseous metastases with a total of 66 Gy in November 2008. In March 2009 and 18 mo after initiation of sorafenib therapy, a single remaining calcifying lesion of hepatic segment Ⅳa was ablated by ultrasound-controlled HFTT (Figure 4). Facial and cleavage erythema, dry cough and hoarseness remained under continued sorafenib therapy, but were at a tolerable level. Liver function was stabilized at Child-Pugh A after treatment of a temporarily acquired coagulation deficiency with vitamin K. Now, 36 mo after first diagnosis of advanced HCC, the patient still consults our outpatient ward for regular surveillance in excellent general condition.

Histological aspects

Histological examination of both the primary biopsy and the biopsy material following treatment revealed essentially similar findings (Figure 1). The liver parenchyma showed micronodular cirrhosis with moderate steatosis without significant necroinflammatory activity. The tumor was composed of large polyhedral tumor cells with centrally located vesicular nuclei and prominent nuclei, arranged in trabeculae and pseudo-acinar structures. The cytoplasm was eosinophilic to pale or clear. Immunohistochemistry revealed a prominent vascularisation. In addition, tumor cells at the periphery expressed the VEGFR1, but were negative for VEGFR2/3. The proliferative index (Ki67) was well below 5%. The cytological features were consistent with a well differentiated

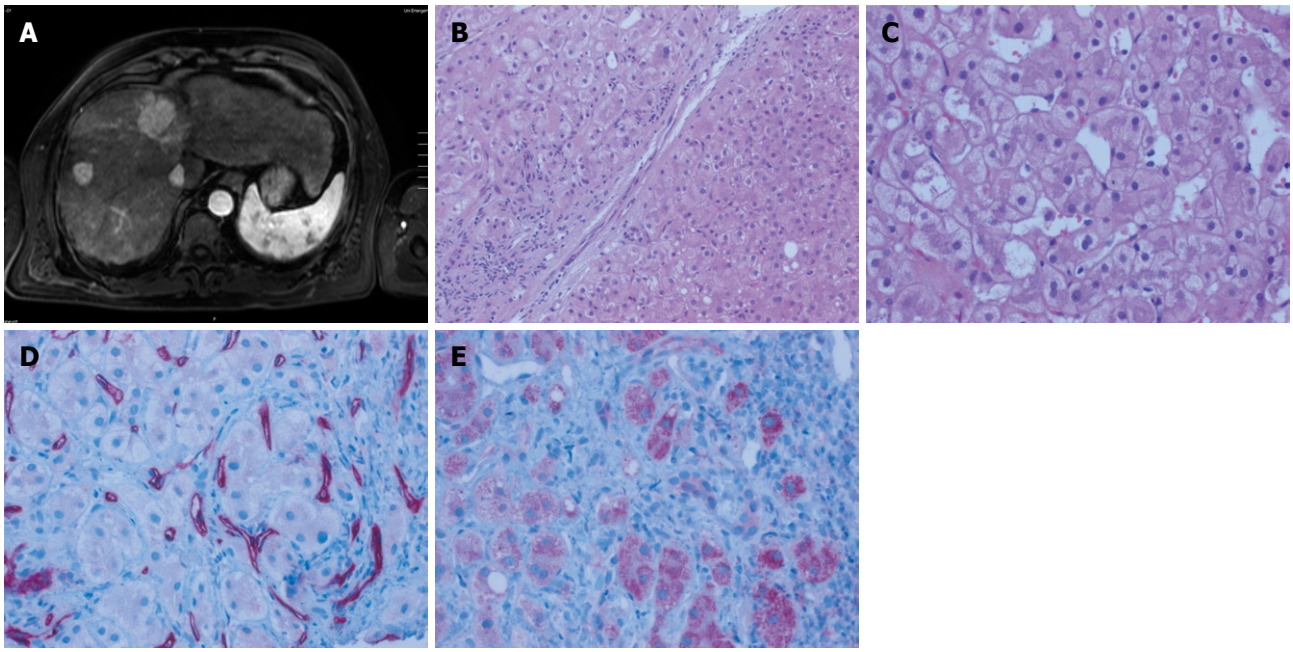

Figure 1 Baseline radiologic and pathologic assessment of the patient in late 2006 before initiation of therapy. A: Initial magnetic resonance imaging (MRI) scan revealing multiple hepatocellular carcinoma (HCC) nodules; B: Regenerative nodule (lower right) juxtaposed to well circumscribed HCC (upper left) separated by a fibrous septum with a sparse lymphocytic infiltrate (magnification, × 100); C: Representative high power magnification of the HCC showed large tumor cells with occasional vacuoles and microvesicular fatty degeneration of hepatocytes (magnification, × 200); D: Abnormally increased microvessel density within the HCC as demonstrated by CD34 immunohistochemistry (magnification, × 200); E: Tumor cells express VEGFR1 at the tumor front (right) contrasting with weak expression towards to the tumor centre (left; magnification, × 200).

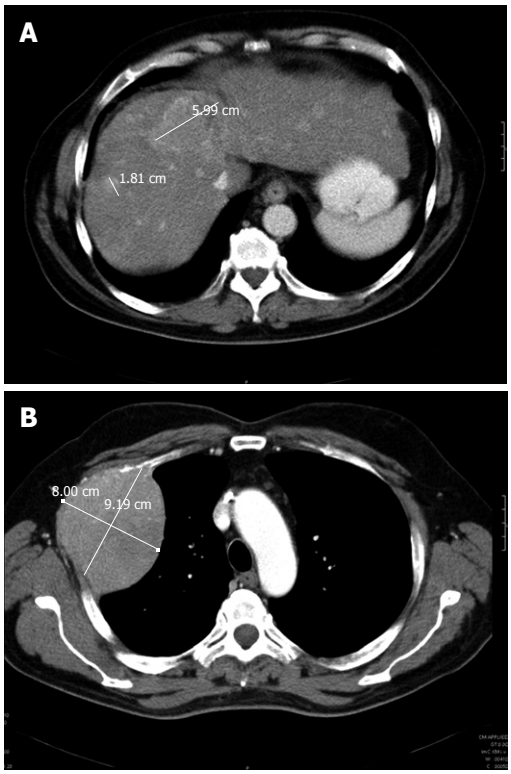

Figure 2 Radiologic assessment of the primary liver tumors and the thoracic metastasis before initiation of sorafenib therapy in September 2007. A: Multiple hepatocellular carcinoma lesions (maximum diameter 6 cm) in computed tomography scan of the liver; B: Thoracic metastasis of the 2nd rib (maximum diameter 8.00 cm × 9.19 cm).

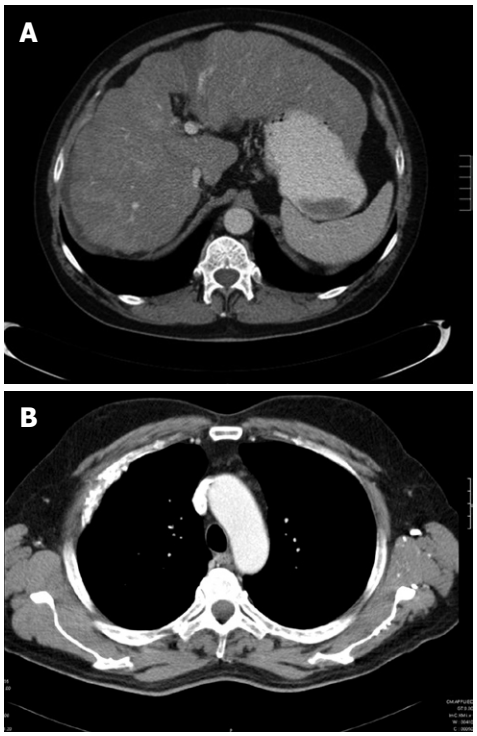

Figure 3 Computed tomography scans of liver (A) and thorax (B) taken in September 2008, 12 mo after initiation of sorafenib therapy showing a dramatic regression of previously described tumor lesions.

HCC in a background of cirrhosis.

DISCUSSION

We here report the extraordinary success in treating metastatic HCC by a sequential therapy with gemcitabine,

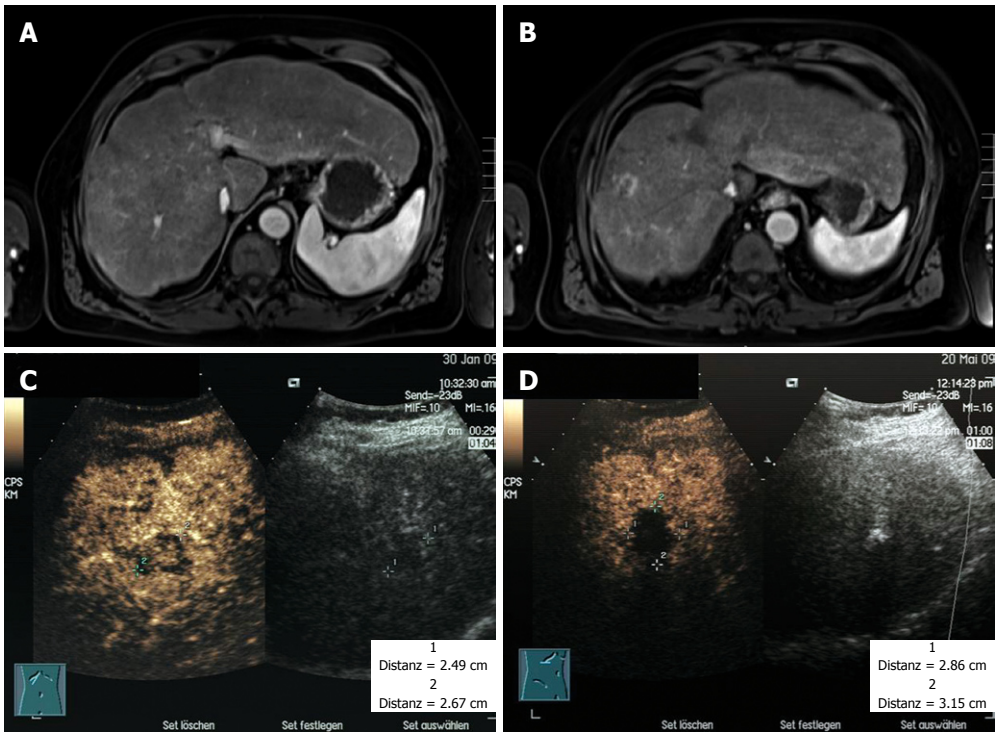

Figure 4 Magnetic resonance imaging scans of the liver in spring 2009, 18 mo after initiation of sorafenib therapy (A, B) and the contrast-enhanced ultrasound investigation of a single calcifying lesion of segment IVa before (C) and after (D) ablation by ultrasound controlled high frequency thermotherapy in spring 2009.

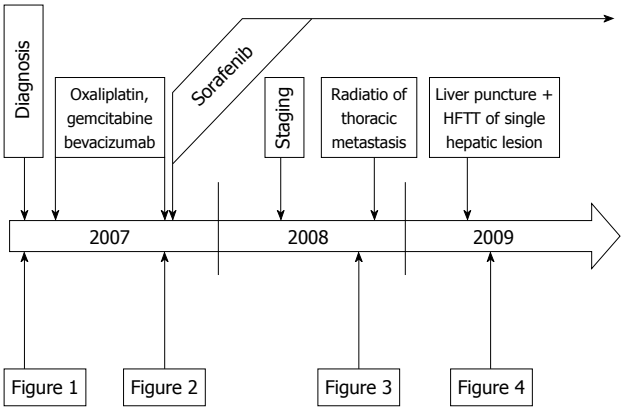

Figure 5 Schematic course of the disease from diagnosis in early 2007 until late 2009 indicating the time points of therapy and staging examinations. Representative examples are shown in Figures 1 to 4 as indicated here. HFTT: High frequency thermotherapy.

oxaliplatin, bevacizumab, internal radiation and treatment with the novel multi-kinase inhibitor sorafenib in a single patient with a survival time of more than 40 mo since the initial time of diagnosis and more than 30 mo since beginning sorafenib therapy (Figure 5).

Although sorafenib was established as first-line therapy for advanced HCC and approximately 70% of patients showed a stable disease in the initial trials[8,9], the overall response rate is below 3% and it does not therefore provide a significant survival benefit compared to other recently established experimental therapies[7]. Interestingly, the initial report on GemOx and bevacizumab in advanced HCC showed an overall response rate of 20% as well as progression free survival and overall survival rates comparable to the SHARP trial[7,9,18]. The tumor of our patient was well differentiated which indicates a good metabolic and detoxifying capacity of the tumor cells. In addition, the proliferation index was below 5% which indicates that drugs with a strong S-phase effect like pyrimidine-analogues are ineffective in this case.

Several recent studies highlighted the importance of anti-angiogenesis in HCC[19]. As angiogenesis-inhibitory effects represent the main mechanisms of action for both sorafenib and bevacizumab, additive effects are therefore conceivable. This concept has been proven successfully by Azad *et al*[20] in a phase I trial with advanced solid tumors and in a phase II trial in epithelial ovarian cancer[21], although enhanced toxicities were observed. In contrast to this study where both agents were applied simultaneously, we used a sequential approach of GemOx and bevacizumab before applying sorafenib, which may explain the good tolerability of this regime in our case. We started sorafenib therapy 26 d after the last application of bevacizumab, which is still in the range of bevacizumab's half-life in humans (approx 20 d, range 11-50 d)[22,23].

Preclinical data suggest that radiation therapy ameliorates the effect of anti-angiogenic therapeutics in rectal cancer, non-small cell lung cancer or malignant glioma[24]. Several mechanisms are discussed for this effect, e.g. increasing vascular permeability with improved delivery of cytotoxic agents to the tumor, and further investigations on determining the optimal biological dose as well as drug sequencing are therefore urgently needed. Yet, several studies showed that radiation therapy can sensitize human tumors, including HCC, for sequential sorafenib therapy[25-27].

Our patient experienced a rapid need for dose reduction of sorafenib which was accompanied by a good response of the tumor lesions. As dose reductions for sorafenib are usually not associated with the here observed rapid response, we conclude that the combination therapy and not sorafenib monotherapy is responsible for the observed therapeutic effect.

Based on these findings from the literature and our own experience, we assume that the sequential therapy with bevacizumab and sorafenib inhibits angiogenetic signalling pathways from both upstream at the receptor level and downstream at the level of signalling kinases and that this dual effect is further supported by the influence of radiation therapy. This strategy should therefore be validated in a larger series of patients with advanced HCC and other solid malignancies.

ACKNOWLEDGMENTS

We thank Dr. Ernst Fellner, Bad Windsheim, Germany, for providing CT scans and radiologic assessments shown in Figures 2 and 3.

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S- Editor Wang JL **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3597

World J Gastroenterol 2010 July 28; 16(28): 3597-3600
ISSN 1007-9327 (print)

CASE REPORT

Placement of removable metal biliary stent in post-orthotopic liver transplantation anastomotic stricture

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Received: January 19, 2010 Revised: February 3, 2010
Accepted: February 10, 2010
Published online: July 28, 2010

Abstract

Postoperative biliary strictures are the most common cause of benign biliary stricture in Western countries, secondary to either operative injury or bile duct anastomotic stricture following orthotopic liver transplantation (OLT). Surgery or endoscopic interventions are the mainstay of treatment for benign biliary strictures. We aim to report the outcome of 2 patients with refractory anastomotic biliary stricture post-OLT, who had successful temporary placement of a prototype removable covered self-expandable metal stent (RCSEMS). These 2 patients (both men, aged 44 and 53 years) were given temporary placement of a prototype RCSEMS (8.5 Fr gauge delivery system, 8 mm × 40 mm stent dimensions) in the common bile duct across the biliary stricture. There was no morbidity associated with stent placement and removal in these 2 cases. Clinical parameters improved after the RCSEMS placement. Long-term biliary patency was achieved in both the patients. No further biliary intervention was required within 14 and 18 mo follow-up after stent removal.

Key words: Anastomosis; Biliary stent; Biliary stricture; Orthotopic liver transplantation; Niti-S stent

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Tee HP, James MW, Kaffes AJ. Placement of removable metal biliary stent in post-orthotopic liver transplantation anastomotic stricture. *World J Gastroenterol* 2010; 16(28): 3597-3600 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i28/3597.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i28.3597

INTRODUCTION

Endoscopic intervention has emerged to become the first line treatment for benign biliary strictures (BBS) following orthotopic liver transplantation (OLT)[1,2]. The current endoscopic approach involves repetitive dilatation of the stricture and placement of multiple large-diameter parallel plastic stents with frequent stent exchange to prevent cholangitis.

Self-expandable metal stents (SEMS) have been used for malignant biliary strictures, however SEMS placement for BBS has not been widely accepted owing to tissue ingrowth leading to long-term complications[3-5]. A removable covered SEMS (RCSEMS) has not been extensively studied in the context of BBS but shows promise in early reports[6]. We report our preliminary experience with a prototype RCSEMS in 2 patients with refractory benign post-OLT anastomotic biliary strictures in Royal Prince Alfred Hospital, Sydney.

CASE REPORT

Two patients in our department with post-OLT anas-

Table 1 Patient characteristics

Case No.	Age (yr)	Gender	Etiology	Location of stricture	Interventions prior to RCSEMS placement	Duration of RCSEMS placement (d)
1	53	Male	Post-OLT anastomotic stricture Hepatitis C cirrhosis	Mid-CBD	3 ERCPs with balloon dilatation and multiple stent insertions	70
2	44	Male	Post-OLT anastomotic stricture Hepatitis C and alcoholic cirrhosis	Mid-CBD	5 ERCPs with balloon dilatation and multiple stent insertions One session of PTC with biliary dilatation and stent placement	42

RCSEMS: Removable covered self-expandable metal stent; OLT: Orthotopic liver transplantation; CBD: Common bile duct; PTC: Percutaneous transhepatic cholangiography; ERCP: Endoscopic retrograde cholangiopancreatography.

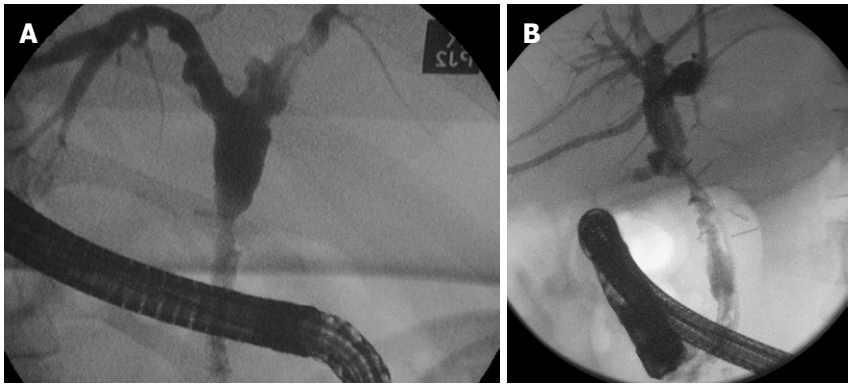

Figure 1 Stricture at mid-common bile duct. A: Case 1; B: Case 2.

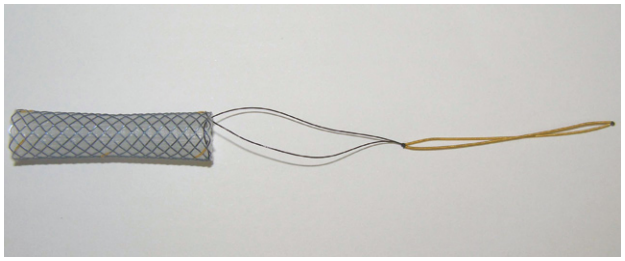

Figure 2 Removable covered self-expandable metal stent with characteristic waist at mid-portion and a radio-opaque string attached to its distal end.

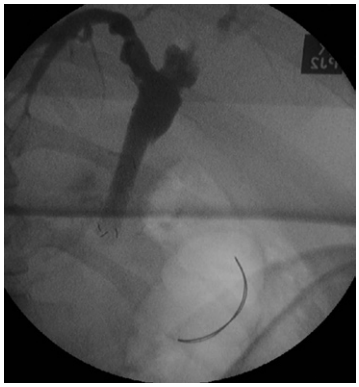

Figure 3 Deployed removable covered self-expandable metal stent with radio-opaque string extending into the duodenum (Case 1).

tomotic stricture refractory to conventional endoscopic and/or surgical interventions were treated with RCSEMS and prospectively followed. They both had similar clinical scenarios: recurrent cholangitis secondary to post-OLT anastomotic stricture, and failure of conventional placement of plastic stent in the common bile duct (CBD) to alleviate biliary stricture.

The clinical presentations of these 2 patients are described in Table 1. Their cholangiograms prior to stenting are shown in Figure 1A and B.

The prototype RCSEMS is a Niti-S biliary stent (Figure 2) which consists of an implantable metal stent and a flexible introducer system (Taewoong Medical Co Ltd., South Korea). The stent is a semi-rigid, flexible and expandable tubular device made of nitinol (nickel titanium alloy) wire. Upon deployment, RCSEMS imparts an outward radial force on the luminal surface of the biliary duct to establish patency. RCSEMS is available in one diameter (8 mm mid-portion and 10 mm at either ends) and one length (40 mm). There are 2 characteristic features of this prototype stent. Firstly, a 10 cm radio-opaque nylon string is incorporated into the distal end of the stent to facilitate endoscopic retrieval. Secondly, it has a waist which is 2 mm narrower than each end. RCSEMS is approved by Australian Therapeutic Goods Administration for use in biliary strictures.

Side-viewing duodenoscopes (TJF-Q160R, and TJF-160VR; Olympus, Japan) were used for all procedures. All procedures were performed under general anesthesia. Sphincterotomy was performed in both cases prior to RCSEMS insertion. Over a guidewire and with fluoroscopic control, the RCSEMS was placed in the CBD across the stricture. Once the outer sheath was retracted and the stent deployed the catheter sheath was further retracted to release the 10 cm-long removal string. Figure 3 shows the cholangiogram with the RCSEMS *in situ*.

The removal of the RCSEMS was performed using a standard endoscopy biopsy forceps by grasping the

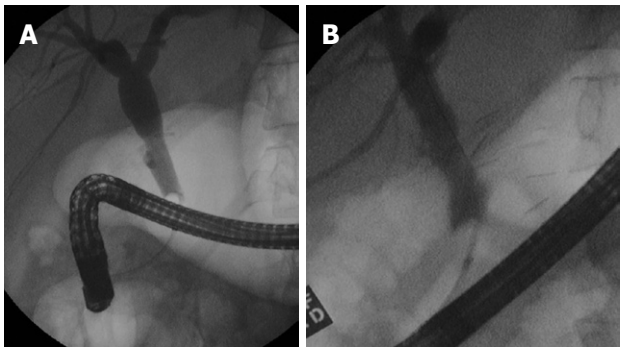

Figure 4 Common bile ducts of cases 1 (A) and 2 (B) after removal of the stent.

string attached at the distal end of the RCSEMS and then pulling it *via* the working channel of the duodenoscope. A cholangiogram was performed at the end of the procedure to confirm the patency of CBD after RCSEMS removal (Figure 4A and B). All procedures were performed by a single dedicated endoscopist.

An RCSEMS was successfully placed in both patients as outpatient day procedures. The immediate post-RCSEMS placement period was uneventful. Liver biochemistry improved. The stent remained *in situ* for 42 and 70 d and no stent migration was observed.

RCSEMS removal was performed as an outpatient day procedure and was straightforward. Endoscopic retrograde cholangiopancreatography images of CBD post-RCSEMS removal demonstrated good patency (Figure 4A and B) with an improvement in diameter of more than 50% at the strictured area.

Patients were followed up after the RCSEMS removal for 14 and 18 mo. During follow-up, liver function tests remained stable. No further episode of jaundice and cholangitis and no further procedure was indicated.

DISCUSSION

In Western countries, postoperative BBS are usually secondary to intraoperative injury, most commonly during laparoscopic cholecystectomy. BBS following OLT can be anastomotic secondary to local ischemia or non-anastomotic, which are usually related to hepatic artery ischemia and often result in complex hilar structuring.

Historically, surgery was considered the treatment of choice for refractory BBS, offering 70% to 90% long term success[7,8]. However, endoscopic intervention has proven to be as effective as surgical intervention in the management of BBS in recent years[9-12]. This was best shown by treatment protocols usually consisting of insertion of increasing numbers of parallel plastic stents with or without biliary dilatation. Despite these endoscopic approaches, significant failure rates in stricture resolution have been encountered. In a study by Alazmi *et al*[13] on 143 post-OLT patients who were followed for 28 mo, the recurrence rate of stricture following endoscopic treatment was estimated to be 18%.

The use of SEMSs in malignant biliary stricture has been studied by many large, well-designed, randomized, controlled trials[14,15] and is accepted as standard practice. Vakil *et al*[16] examined human tissue responses to endoluminal metallic stents, and observed that the presence of foreign stent material could evoke granulation tissue formation and extensive fibrosis. A systemic appraisal of 400 cases of BSS treated with uncovered SEMSs with a median follow-up of 31 mo revealed a 35% rate of stent occlusion[17]. These authors therefore cautioned against long-term use of SEMSs in BBS.

Several groups have studied the temporary placement of covered SEMSs with planned retrieval and obtained promising results. Gwon *et al*[18] studied a total of 36 temporary bile duct stent-grafts and found a primary patency rate of 90.6% but migration in 11%. One large series of 79 cases using covered SEMSs in BBS was published by Kahaleh *et al*[6] in 2008, who reported 90% resolution of BBS and stent migration in 14%. These studies offered some promise but clearly significant problems remained.

In designing the ideal metal stent for a post-OLT stricture, there are several important considerations. The actual stricture length is usually short (0.5-1.0 cm) and the rest of the bile duct is usually of normal caliber. Using a long SEMS across the stricture and extending into the duodenum in this situation would impart pressure over a large area of normal duct, potentially risking pressure necrosis and fibrosis. Conversely, using a short SEMS would result in the stent being high up the CBD, making removal challenging. Stent migration is another well-recognized problem with covered SEMS and occurred in 5.8% to 25% of cases[19,20]. Having a stent of uniform caliber and covered with teflon or a similar material are 2 factors which may predispose to stent migration.

To address these problems, this prototype RCSEMS was designed with 2 key features. Firstly, a long removal string was attached to the distal end of RCSEMS. Removal of RCSEMS is possible by grasping this string with standard or "rat-tooth" forceps introduced *via* the working channel of the scope. Also, the RCSEMS has a waist diameter of 8 mm with 10 mm at both ends. These design features allow the radial force of the metallic stent to be directed maximally to the center hence inhibiting stent migration.

In conclusion, our initial experience in 2 cases treated with a prototype RCSEMS has shown no migration, easy insertability and removability with excellent stricture resolution at the end of the treatment and during medium-term follow-up. A pilot randomized, controlled trial is currently underway at our institution to verify the effectiveness and safety of this new device.

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S- Editor Wang JL **L- Editor** Cant MR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i28.3601

World J Gastroenterol 2010 July 28; 16(28): 3601-3602
ISSN 1007-9327 (print)

Role of endoscopic ultrasound/SpyScope in diagnosis and treatment of choledocholithiasis in pregnancy

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Received: April 17, 2010 Revised: May 27, 2010
Accepted: June 4, 2010
Published online: July 28, 2010

Abstract

Cholelithiasis and choledocholithiasis occur frequently in pregnancy and their management can be complicated. Traditional endoscopic retrograde cholangiopancreatography (ERCP) is the first line treatment for choledocholithiasis, but in addition to its baseline risks, fluoroscopy poses an additional radiation risk to the fetus. Endoscopic ultrasound (EUS) is an accurate modality for detecting common bile duct stones, but its role has not been defined in pregnancy. We describe an alternative management strategy to conventional ERCP in a pregnant patient with choledocholithiasis and cholangitis detected using EUS and choledochoscopy.

Key words: SpyScope; Endoscopic ultrasound; Choledocholithiasis; Management; Pregnancy

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TO THE EDITOR

We read with great interest the experiences of Fishman *et al*[1] in managing choledocholithiasis with choledochoscopy. Endoscopic ultrasound (EUS) is an accurate modality for detecting common bile duct (CBD) stones, but its role has not been defined in pregnancy. We describe an alternative management strategy to conventional endoscopic retrograde cholangiopancreatography (ERCP) in a pregnant patient with choledocholithiasis and cholangitis detected using EUS and choledochoscopy.

A 26-year old pregnant female at 28-wk gestation presented with right upper quadrant pain, progressive jaundice, and low-grade fever. Her past medical history was significant for sickle cell disease, preeclampsia and choledocholithiasis. She underwent a previous ERCP for stone removal after her last pregnancy. Upon presentation to the hospital, her total bilirubin was 6.8 mg/dL, aspartate transaminase 139 U/L, alanine transaminase 113 U/L, alkaline phosphatase 128 U/L and white blood cells count was 16 500/mm^3. Transabdominal ultrasound revealed a 9-mm common bile duct with a possible filling defect. In order to avoid radiation exposure to fetus by fluoroscopy, EUS and choledochoscopy were used to examine the bile duct and achieve its complete clearance. The linear echoendoscope revealed multiple hyperechoic well-rounded stones throughout the common bile duct (Figure 1A). A prior sphincterotomy was performed, so deep cannulation of the bile duct using the SpyGlass choledochoscope (Boston Scientific) was easily achieved. The SpyScope was useful in determining the exact location of the stones. A guidewire was advanced through the SpyScope into the left intrahepatic system. A 10-mm extraction balloon was used to extract multiple yellow pigment stones from the CBD. The SpyScope was then reinserted and complete clearance of the CBD was confirmed (Figure 1B). A 10-Fr

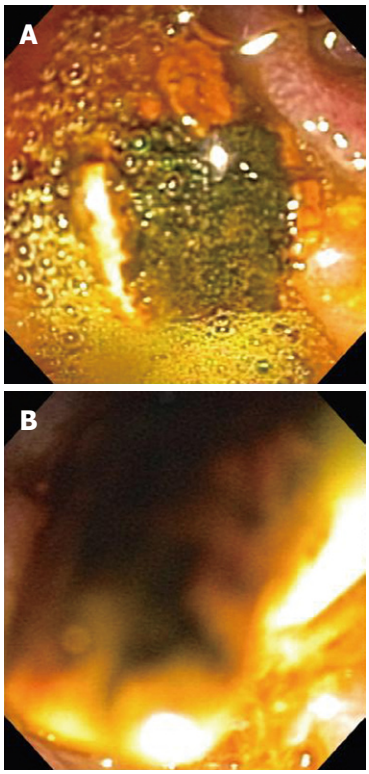

Figure 1 Multiple hyperechoic well rounded stones throughout the common bile duct (A), visualized and retrieved with Spyscope (B).

5-cm plastic biliary stent was placed to ensure drainage and prevent recurrent cholangitis during the remainder of her pregnancy. The linear echoendoscope was used to confirm adequate placement of the biliary stent. Subsequently, the patient had complete resolution of symptoms and improvement in her liver enzymes.

The incidence of gallstones during pregnancy is estimated to be 3%-12%[2]. Symptomatic gallstone disease is the second most common abdominal emergency in pregnant women and may require surgical intervention. In addition to the baseline risks of ERCP, fluoroscopy poses an additional radiation risk to the fetus. Various publications have been reported on using ERCP during pregnancy without fluoroscopy by using bile aspiration for confirmation of CBD cannulation, sphincterotomy and balloon extraction of stones[3,4]. All these approaches are not perfect with regard to ensuring complete CBD clearance. This case highlights how choledochoscopy and endoscopic ultrasound are safe alternatives to fluoroscopy for the evaluation of biliary disorders during pregnancy. If choledochoscopy is not available, an alternative approach is to use EUS-guided extraction balloon sweeps to achieve clearance of ductal stones.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Lin YP

World Journal of Gastroenterology

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wjg@wjgnet.com
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World J Gastroenterol 2010 July 28; 16(28): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 July 28; 16(28): I
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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
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World J Gastroenterol 2010 July 28; 16(28): I-IV
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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

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

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Write as mean ± SD or mean ± SE.

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ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 29
August 7, 2010
World J Gastroenterol
2010 August 7; 16(29): 3603-3730

Online Submissions
www.wjgnet.com/1007-9327office
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 29 August 7, 2010

EDITORIAL

3603 Non-viral causes of hepatocellular carcinoma

Blonski W, Kotlyar DS, Forde KA

3616 Primary biliary cirrhosis: What do autoantibodies tell us?

Hu CJ, Zhang FC, Li YZ, Zhang X

TOPIC HIGHLIGHT

3630 Colonoscopic polypectomy and associated techniques

Fyock CJ, Draganov PV

GUIDELINES FOR BASIC SCIENCE

3638 Interleukin-21 triggers effector cell responses in the gut

De Nitto D, Sarra M, Pallone F, Monteleone G

ORIGINAL ARTICLE

3642 Non-peptidyl low molecular weight radical scavenger IAC attenuates DSS-induced colitis in rats

Vasina V, Broccoli M, Ursino MG, Canistro D, Valgimigli L, Soleti A, Paolini M, De Ponti F

3651 Effects of protein deprivation and re-feeding on $P2X_2$ receptors in enteric neurons

Misawa R, Girotti PA, Mizuno MS, Liberti EA, Furness JB, Castelucci P

3664 Proteomic analysis reveals molecular biological details in varioliform gastritis without *Helicobacter pylori* infection

Zhang L, Hou YH, Wu K, Zhai JS, Lin N

3674 Magnetically labeled mesenchymal stem cells after autologous transplantation into acutely injured liver

Shi XL, Gu JY, Han B, Xu HY, Fang L, Ding YT

BRIEF ARTICLE

3680 ICC density predicts bacterial overgrowth in a rat model of post-infectious IBS

Jee SR, Morales W, Low K, Chang C, Zhu A, Pokkunuri V, Chatterjee S, Soffer E, Conklin JL, Pimentel M

3687 Is chronic hepatitis C virus infection a risk factor for breast cancer?
Larrey D, Bozonnat MC, Kain I, Pageaux GP, Assenat E

3692 Nasogastric or nasointestinal feeding in severe acute pancreatitis
Piciucchi M, Merola E, Marignani M, Signoretti M, Valente R, Cocomello L, Baccini F, Panzuto F, Capurso G, Delle Fave G

3697 Approach to early-onset colorectal cancer: Clinicopathological, familial, molecular and immunohistochemical characteristics
Perea J, Alvaro E, Rodríguez Y, Gravalos C, Sánchez-Tomé E, Rivera B, Colina F, Carbonell P, González-Sarmiento R, Hidalgo M, Urioste M

3704 Is autoimmune hepatitis a frequent finding among HCV patients with intense interface hepatitis?
Badiani RG, Becker V, Perez RM, Matos CAL, Lemos LB, Lanzoni VP, Andrade LEC, Dellavance A, Silva AEB, Ferraz MLG

3709 Capecitabine with radiation is an effective adjuvant therapy in gastric cancers
Tham CK, Choo SP, Poon DYH, Toh HC, Ong SYK, Tan SH, Wang MLC, Foo KF

3716 Stent-grafts placement for treatment of massive hemorrhage from ruptured hepatic artery after pancreaticoduodenectomy
Wang MQ, Liu FY, Duan F, Wang ZJ, Song P, Fan QS

CASE REPORT

3723 Right anterior segmental hepatic duct emptying directly into the cystic duct in a living donor
Ishiguro Y, Hyodo M, Fujiwara T, Sakuma Y, Hojo N, Mizuta K, Kawarasaki H, Lefor AT, Yasuda Y

3727 Ultrasonography of sclerosing angiomatoid nodular transformation in the spleen
Cao JY, Zhang H, Wang WP

World Journal of Gastroenterology
Volume 16 Number 29 August 7, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
August 7, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
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World Journal of Gastroenterology
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3603

World J Gastroenterol 2010 August 7; 16(29): 3603-3615
ISSN 1007-9327 (print)

EDITORIAL

Non-viral causes of hepatocellular carcinoma

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Received: February 3, 2010 Revised: April 30, 2010
Accepted: May 7, 2010
Published online: August 7, 2010

Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and represents an international public health concern as one of the most deadly cancers worldwide. The main etiology of HCC is chronic infection with hepatitis B and hepatitis C viruses. However, there are other important factors that contribute to the international burden of HCC. Among these are obesity, diabetes, non-alcoholic steatohepatitis and dietary exposures. Emerging evidence suggests that the etiology of many cases of HCC is in fact multifactorial, encompassing infectious etiologies, comorbid conditions and environmental exposures. Clarification of relevant non-viral causes of HCC will aid in preventative efforts to curb the rising incidence of this disease.

Key words: Hepatocellular carcinoma; Etiology; Non-alcoholic steatohepatitis; Obesity; Diabetes; Alcohol; Tobacco; Oral contraceptive

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Blonski W, Kotlyar DS, Forde KA. Non-viral causes of hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(29): 3603-3615
Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3603.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3603

INTRODUCTION

Hepatocellular carcinoma (HCC) represents an international public health concern as one of the most common and deadly cancers worldwide. Globally, HCC accounts for 85%-90% of primary liver cancers[1] and its lethality is underscored by the fact that it is the third most frequent cause of cancer-related mortality[2]. In those patients who are not transplant candidates, HCC is particularly lethal, with a 5-year survival of less than 5%[3]. In the United States, the incidence of HCC appears to be increasing, with a more than twofold increase observed from 1976 to 2002 (Figure 1)[1,3,4]. A significant proportion of this increase is accounted for by the growing prevalence of hepatitis C virus (HCV) infection[5]. However, other potential causes of HCC are garnering close attention.

Increased body mass index and diabetes with subsequent development of non-alcoholic steatohepatitis (NASH) represent significant risk factors for HCC. This is especially concerning in light of the growing epidemic of obesity in adults and children over the past 25 years[1,5-8]. Other non-viral causes of HCC include iron overload syndromes, alcohol use, tobacco use, oral contraceptive use, aflatoxin exposure and betel quid chewing, a prevalent habit in the developing world. Emerging evidence suggests that the etiology of many cases of HCC is in fact multifactorial, including both viral infections and non-viral environmental and dietary exposures. This review focuses on the non-viral causes of HCC.

HEREDITARY HEMOCHROMATOSIS AND IRON OVERLOAD SYNDROMES

Hereditary hemochromatosis, a condition characterized by excess iron absorption, is caused by mutations in the

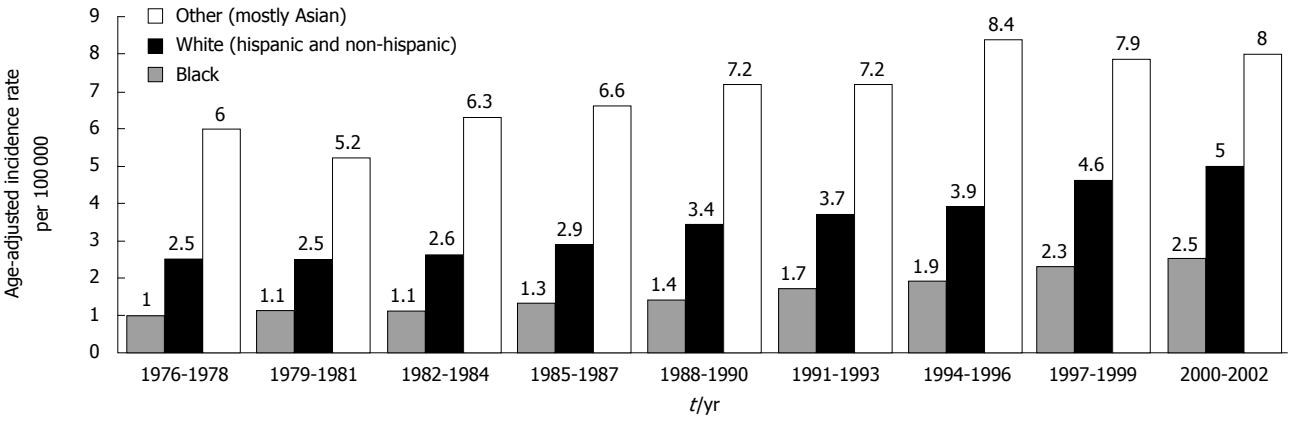

Figure 1 Average yearly, age-adjusted incidence rates for hepatocellular carcinoma men and women in the United States shown for 3-year intervals between 1976 and 2002. Whites included approximately 25% Hispanics, whereas other race was predominantly (88%) Asian. Source: Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence-SEER 13 Regs Public-Use, Nov 2004 Sub (1973-2002 varying), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2005, based on the November 2004 submission. Reprinted from El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; 132: 2557-2576, Copyright (2007), with permission from Elsevier[1].

HFE gene and/or other mutations in the iron metabolism machinery. This condition represents one of the most common autosomal recessive genetic disorders, affecting as many as 1 in 200 people of Northern European descent[9-11]. The *HFE* gene is required for efficient *in vivo* iron metabolism and two mutations within the *HFE* gene product, C282Y and H63D, have been well described in patients with hereditary hemochromatosis[10]. The C282Y mutation, which results in a base pair substitution in which tyrosine is substituted for cysteine at amino acid 282, is found in the homozygous state in up to 83% of patients with hereditary hemochromatosis[10]. The H63D mutation, characterized by substitution of histidine with aspartic acid at codon 63, is present in a minority of cases of hereditary hemochromatosis either in a homozygous state or with one copy of the C282Y mutation, a state referred to as a compound heterozygote[10]. The clinical significance of this latter mutation within the *HFE* gene, however, continues to be controversial.

The altered iron metabolism seen in hereditary hemochromatosis leads to excess iron storage in the liver and the subsequent development of liver dysfunction. Although other organs systems are also susceptible to iron overload, the liver bears the majority of malignant disease, with those patients with hereditary hemochromatosis being 20 times more likely to develop liver cancer than all other cancers combined[12].

Several population-based and case-control studies have shown that the diagnosis of hereditary hemochromatosis confers a consistent and markedly elevated risk for the development of HCC[12-17]. A sentinel study from the US National Center for Health Statistics found that patients who were diagnosed with hereditary hemochromatosis and who died were 23-fold more likely to have liver cancer compared to those without a diagnosis of hemochromatosis [Proportionate Mortality Ratio (PMR) 22.5, 95% CI: 20.6-24.6)[13]. In addition, the relationship between hereditary hemochromatosis and HCC is modified by diabetes, sex and genetics. Subjects with liver

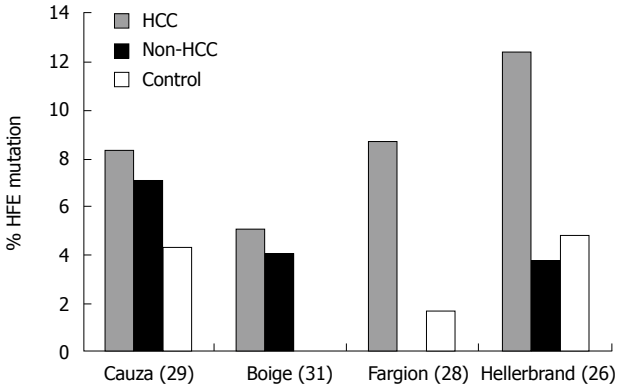

Figure 2 Prevalence of HFE mutations among patients with hepatocellular carcinoma, cirrhosis without hepatocellular carcinoma (non-hepatocellular carcinoma), and normal controls. Reprinted from Kowdley KV. Iron, hemochromatosis, and hepatocellular carcinoma. *Gastroenterology* 2004; 127: S79-S86, Copyright (2004), with permission from Elsevier [24]. HCC: Hepatocellular carcinoma.

cancer and concomitant diabetes mellitus were 82 times more likely to have a diagnosis of hemochromatosis[13]. Furthermore, a population-based study from Scandinavia found that men with hemochromatosis had a 29-fold increase in risk of liver cancer, whereas women with hemochromatosis had a sevenfold increase in risk[12]. Lastly, highlighting the genetic predisposition of disease and its consequences, an analysis of 5973 first degree relatives of patients with hemochromatosis found that these subjects had a nearly twofold increase in risk of HCC[12].

The presence of a single copy of the C282Y HFE gene mutation, although not diagnostic for hereditary hemochromatosis, has been studied to determine its prevalence and clinical significance in patients with HCC (Figure 2)[18-23]. Researchers comparing 81 patients with cirrhosis and HCC to 128 normal controls observed a significantly higher prevalence of the C282Y mutation in patients with HCC[18]. Another group observed that patients with HCC had a higher frequency of the C282Y mutation when compared to cirrhotic controls without HCC and healthy controls[19].

Additionally, they demonstrated that those subjects with the C282Y mutation had higher levels of serum ferritin, transferrin saturation, and hepatic iron deposition when compared to those without the C282Y mutation[19]. These studies suggest that increased iron load in HCC patients with a C282Y mutation exerts a cause and effect relationship in hepatocarcinogenesis[18,19]. This risk of HCC in patients with the C282Y mutation may not be equally conferred to all however. A recent prospective cohort study from France found that the C282Y mutation and iron overload were associated with a significantly increased risk of HCC among patients with alcoholic cirrhosis but not among those with HCV-related cirrhosis[23].

Contrary to the data presented above, two well-executed European studies did not find a significant difference in the prevalence of the C282Y mutation between patients with and without HCC[21,22]. Researchers from France observed comparable proportions of the C282Y heterozygous state in 133 cirrhotic patients with HCC and 100 without[21]. Likewise, in another cohort of 162 consecutive patients with HCC, the majority with cirrhosis, the frequency of the C282Y mutation did not differ from historical healthy controls or patients with HCV[22]. Concrete conclusions from these studies might be elusive, however, because of the small sample sizes, differences in the prevalence of the C282Y mutation in the respective populations, and referral bias to tertiary care centers[24].

More studies are therefore needed to determine correctly, in larger populations, the prevalence and effect of a single copy of the C282Y mutation. Additionally, on an individual basis, further study is needed to better characterize the comorbid, demographic and genetic factors that play a role in the risk of HCC in those with a single copy of the C282Y mutation.

It has also been proposed that the H63D mutation is not directly associated with hemochromatosis[10,25]. Certainly, none of the aforementioned studies observed a significant difference in the prevalence of the H63D mutation between patients with and without HCC[18-22]. Future studies are needed to assess further the relationship between this *HFE* gene mutation and the development of HCC.

Hereditary hemochromatosis is only one of the iron overload syndromes that leads to excessive iron deposition in the liver and other tissues. In fact, those patients with excess total body iron secondary to other etiologies have been shown to have a higher risk of HCC in the absence of genetic hemochromatosis[26-28]. Studies have suggested that conditions such as β thalassemia or iron overload in people of African descent might be associated with an increased risk of HCC[27,29,30]. One such study found that African iron loaded subjects had a 10-fold increase in the risk of developing HCC after adjusting for viral hepatitis, alcohol use and environmental exposures, such as aflatoxin[27]. Regardless of etiology, iron overload is not a benign condition and when recognized, surveillance for HCC should be undertaken.

NON-ALCOHOLIC FATTY LIVER DISEASE

Several case reports and subsequent observational studies have proposed that non-alcoholic fatty liver disease (NALFD), and more specifically, NASH, confers an elevated risk of developing HCC (Figure 3)[31]. NAFLD is a spectrum of clinical disease that ranges from benign or bland steatosis to NASH. The latter stage of this disease, through a process of chronic inflammation and subsequent hepatic fibrosis, can lead to cirrhosis[32]. The presence of cirrhosis itself is an independent risk factor for the development of HCC[33]. To characterize the natural history of NALFD, 420 patients identified in Olmstead County, MN, USA with the disorder were followed for an average of 7 years to determine overall mortality as well as liver related morbidity and mortality. In this population-based study, NAFLD was associated with a 34% increase in mortality and a significant increase in the risk of HCC, with two cases or 0.5% being diagnosed over the period of follow-up[34]. In subjects with NASH-related cirrhosis, however, the rate of HCC approached 10%[34]. These findings are well aligned with a series of studies from Japan. In one report, among 82 NASH patients treated from 1990 through 2001, six patients with HCC were identified over 11 years of follow-up[35]. All six patients developed HCC

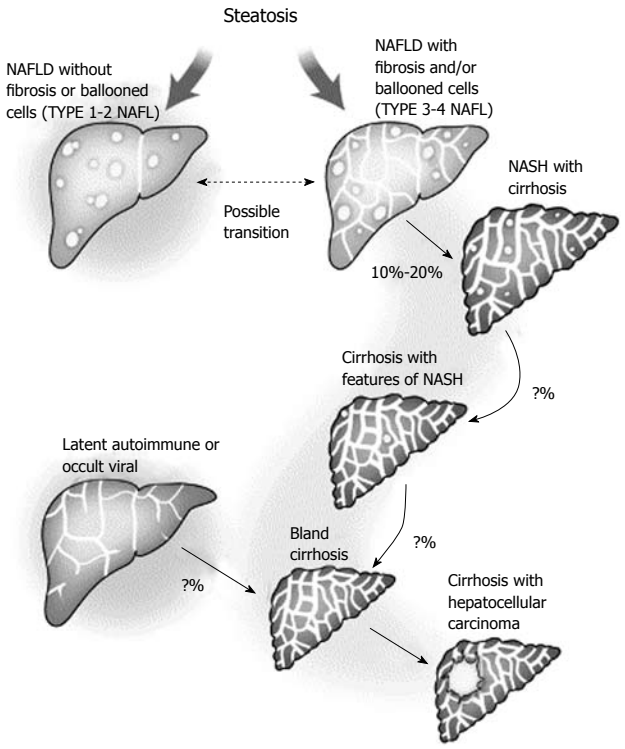

Figure 3 Progression of non-alcoholic fatty liver disease to cryptogenic cirrhosis. The explanation for the disappearance of steatosis remains uncertain but it is likely to be multifactorial and to involve changes in blood flow and exposure to fat-promoting hormones, as well as possible changes in the intracellular metabolism as a result of long-standing exposure to lipid peroxidation. Theoretically, this could represent a form of lipoatrophy that occurs within the fat-storing hepatocytes. Other forms of chronic liver disease may also present with a well-established bland cirrhosis. Efforts are needed to define better residual markers of past silent disease to improve our understanding of cryptogenic cirrhosis. Reprinted from Caldwell SH, Crespo DM. The spectrum expanded: cryptogenic cirrhosis and the natural history of non-alcoholic fatty liver disease. *J Hepatol* 2004; 40: 578-584, Copyright (2004), with permission from Elsevier[31]. NALFD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Table 1 Characteristics of cohort studies included in the meta-analysis

Study	Country	No. of cases (men/women)	Study participants	Assessment of exposure	Adjustments
Møller *et al* (1994)	Denmark	22/36	Men: 14531 Women: 29434	Discharge diagnosis of obesity	Age
Wolk *et al* (2001)	Sweden	15/13	Men: 8165 Women: 19964	Discharge diagnosis of obesity	Age, calendar year
Nair *et al* (2002)	USA	659[1]	Men and women: 19271[1]	Measured	Age, sex, race, diabetes
Calle *et al* (2003)	USA	620/345	Men: 404576 Women: 495477	Self-reported	Age, race, education, marital status, smoking, physical activity, aspirin use, estrogen-replacement therapy (women), alcohol, dietary factors
Samanic *et al* (2004)	USA	322 whites/38 blacks	White men: 3668486 Black men: 832214	Discharge diagnosis of obesity	Age, calendar year
Kuriyama *et al* (2005)	Japan	69/31	Men: 12485 Women: 15054	Self-reported	Age, type of health insurance, smoking, intakes of alcohol, meat, fish, fruits, vegetables, bean-paste soup[2]
Batty *et al* (2005)	UK	51	Men: 18403	Measured	Age, employment grade, marital status, physical activity, smoking, other[3]
Oh *et al* (2005)	Korea	3347	Men: 781283	Measured	Age, area of residence, family history of cancer, smoking, exercise, alcohol
Rapp *et al* (2005)	Austria	57	Men: 67447	Measured	Age, occupational group, smoking
N'Kontchou *et al* (2006)	France	220[1]	Men and women: 771[1]	Measured	Age, sex, cirrhosis cause, diabetes
Samanic *et al* (2006)	Sweden	297	Men: 362552	Measured	Age, smoking

[1]Patients with cirrhosis; [2]ORs for women were further adjusted for age at menarche, age at end of first pregnancy, and menopausal status; [3]Other factors adjusted for include disease at entry, weight loss in the last year, height-adjusted FEV1, triceps skinfold thickness, blood pressure-lowering medication, blood pressure, plasma cholesterol, glucose intolerance, and diabetes. Reprinted by permission from Macmillan Publishers Ltd: [Br J Cancer]. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer* 2007; 97: 1005-1008, copyright (2007)[42].

in the setting of NASH-related cirrhosis[35]. In an update to this original observation, over a 17-year period, the authors found that among 382 patients with NASH, HCC was diagnosed in 34, 9% of the cohort, with 11 patients diagnosed during a 40-mo mean follow-up[36]. Comparing those NASH patients with and without HCC, multivariate logistic regression analysis identified older age (OR: 1.1, 95% CI: 1.03-1.2) and advanced hepatic fibrosis (OR: 4.2, 95% CI: 1.8-9.7) as independent predictors for the development of HCC[36]. In a prospective study of 118 patients with NASH and advanced liver fibrosis from the same cohort, the observed 5-year cumulative incidence of HCC was 7.6%, with HCC accounting for 46% of all fatalities[36]. In summary, these data highlight an association between NASH cirrhosis and an increase in the incidence of HCC over that of the general population. Therefore, regular HCC surveillance is imperative in patients with NASH cirrhosis.

The impact of NASH on the incidence of HCC may well be underestimated. In advanced fibrosis, an absence of steatosis may be appreciated, a finding which can obscure identification of the underlying etiology of liver injury in these patients. In this case, patients might be classified as having cryptogenic cirrhosis. In a United States study that examined 105 consecutive patients with HCC, after HCV, cryptogenic cirrhosis was the most common etiology of liver injury[37]. Among patients presenting with cryptogenic cirrhosis, 58% had a body mass index (BMI) $\geqslant$ 30, 47% had diabetes, and 50% had a prior histological diagnosis of NASH or clinical characteristics consistent with NAFLD. Furthermore, only 23% of subjects with cryptogenic cirrhosis were undergoing surveillance for HCC in comparison to 61% of subjects who had a history of HCV-related liver disease[37]. Clearly, these observations emphasize the importance of HCC surveillance in this group of patients and the failure thus far to appropriately screen for HCC in this disease process.

OBESITY

The prevalence of obesity has increased to epidemic proportions over the last three decades. Excess body mass is classified as overweight if the BMI is > 25 kg/m^2 and < 30 kg/m^2, or obese if the BMI is $\geqslant$ 30 kg/m^2. In addition to the increase in an array of disease processes observed with being overweight or obese, both classifications of excess body mass are associated with a higher risk of developing all cancers, including liver cancer[38]. In one population-based study from Sweden, 28 cases of HCC were diagnosed in 28129 patients from 1965 to 1993, thus conferring an almost threefold higher risk of HCC in obese patients[39]. A recent European case-control study observed a significantly increased risk of HCC among obese (OR: 3.5, 95% CI: 1.3-9.2) or diabetic (OR: 3.5, 95% CI: 1.6-7.7) patients without viral hepatitis. This risk of HCC was even greater if both obesity and diabetes were present comorbid conditions (OR: 11.8, 95% CI: 2.7-51.9)[40]. A Danish study further confirmed these results, finding a twofold increase in liver cancer incidence in obese subjects compared to non-obese subjects[41].

A meta-analysis of 11 cohort studies that evaluated the association between being overweight or obese and liver

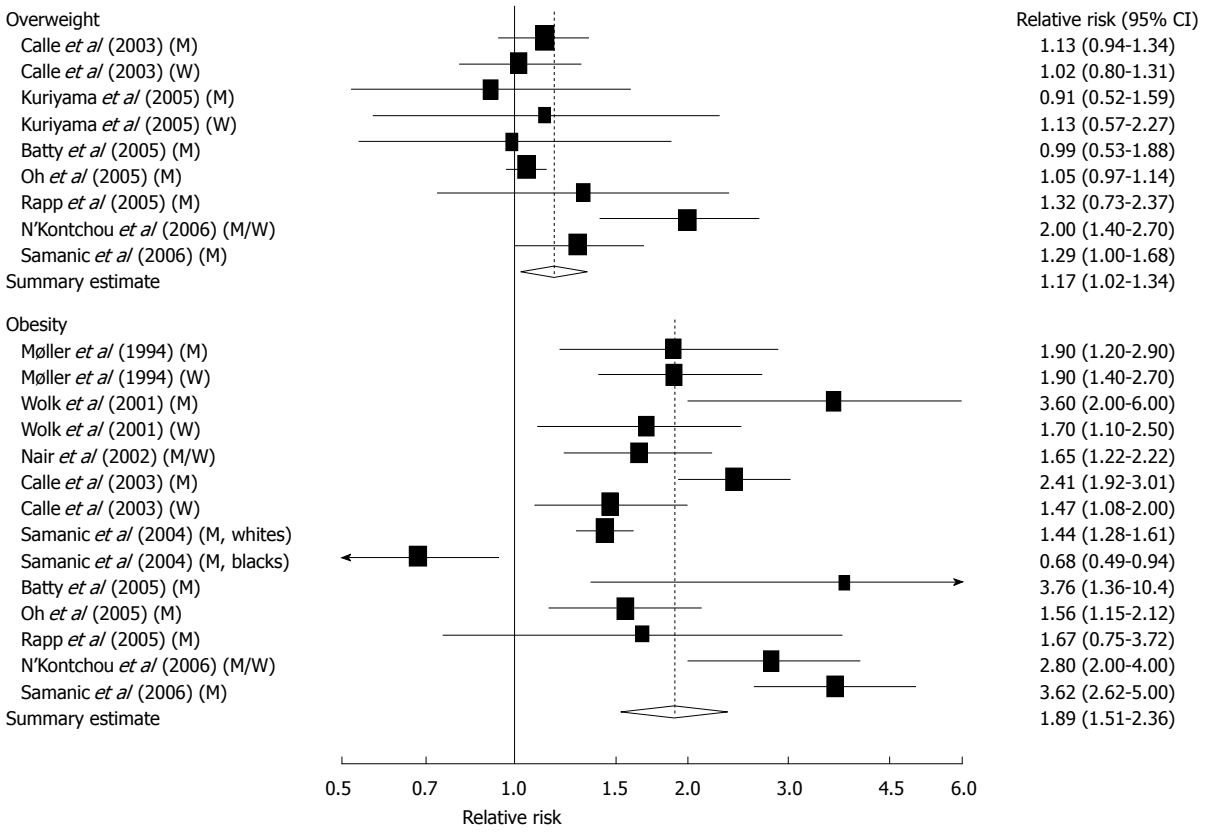

Figure 4 Relative risks of liver cancer associated with overweight and obesity. Relative risk estimates are for overweight and obese persons compared with normal weight persons. Tests for heterogeneity: overweight, Q = 16.83, P = 0.03, I^2 = 52.5%; obesity, Q = 88.03, P < 0.001, I^2 = 86.4%. M: Men; W: Women[42]. Reprinted by permission from Macmillan Publishers Ltd: [Br J Cancer]. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer* 2007; 97: 1005-1008, copyright (2007)[42].

cancer was published in 2007, and clarified the risk of development of HCC (Table 1)[42]. Of the included studies, seven examined a total of 5037 overweight patients and 10 examined 6042 obese patients[42]. Patients who were overweight had a 17% increase in risk of developing HCC, whereas obese patients had an 89% increase in risk (Figure 4)[42]. Based on the prevalence of HCC, it was estimated that 28% of HCC cases in men and 27% in women were due to being overweight or obese[42].

In addition to an increased risk of developing HCC, overweight or obese patients appear to be at increased risk for HCC-related mortality. In a population-based study of cancer mortality and BMI, men with a BMI of 30-34.9 were found to have a twofold increase in the risk of death from HCC, with a 4.5-fold increase noted in men with BMI > 35[38].

Lastly, *via* the pathway of the metabolic syndrome with resultant NASH cirrhosis, obese patients have been found to be at an increased risk for HCC occurrence. Many lines of evidence point to the role of cirrhosis as a mediator in these patients. Firstly, patients presenting with cryptogenic cirrhosis were found to have a significantly higher prevalence of obesity than patients with cirrhosis from non-alcoholic hepatitis C or autoimmune liver disease, but a similar prevalence of obesity when compared to patients with documented NASH[43]. These data are supported by a case-control study in which 49 patients with cryptogenic cirrhosis were compared to 98 matched controls with an established cause of cirrhosis. In that study, obesity was significantly more prevalent in the cryptogenic cirrhosis patients[44]. Additionally, a retrospective analysis of 19 271 American patients who had undergone liver transplantation found that there were 653 cases of HCC, and those with a diagnosis of cryptogenic cirrhosis had an 11-fold increase in the risk of having HCC[45]. Therefore, being overweight and obesity, secondary to cryptogenic cirrhosis, or more likely undiagnosed NASH cirrhosis, can increase the risk of developing HCC. Clearly, these data suggest that screening is important for diagnosis of asymptomatic HCC and highlight the need for surveillance in this population.

DIABETES

Diabetes has been found to increase the risk of developing chronic liver disease and HCC[46]. Studies that have compared patients with cryptogenic cirrhosis to patients with a known etiology of their cirrhosis have shown a significantly higher prevalence of diabetes among the latter group[43,44]. Again, as noted with the overweight and obese, a similar prevalence of diabetes has been observed among patients with cryptogenic and NASH cirrhosis[43].

In addition to increasing the prevalence of chronic liver disease, diabetes has also been shown to be an independent risk factor for the development of HCC. In a recent systematic review of 13 case control studies, 11

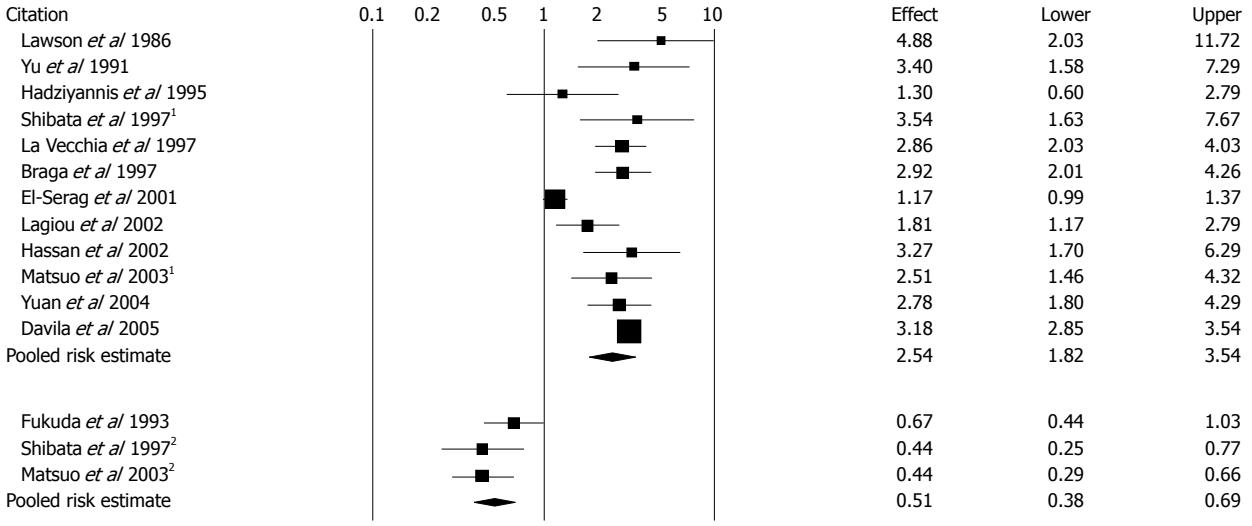

Figure 5 Unadjusted ORs and 95% CIs for the association between diabetes and hepatocellular carcinoma in 13 case-control studies. [1]Population-based control groups; [2]Hospital-based control groups. Black diamonds indicate weighted average of all studies; Black boxes indicate point estimates for ORs[47] Reprinted with El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; 4: 369-380, Copyright (2006), with permission from Elsevier[47].

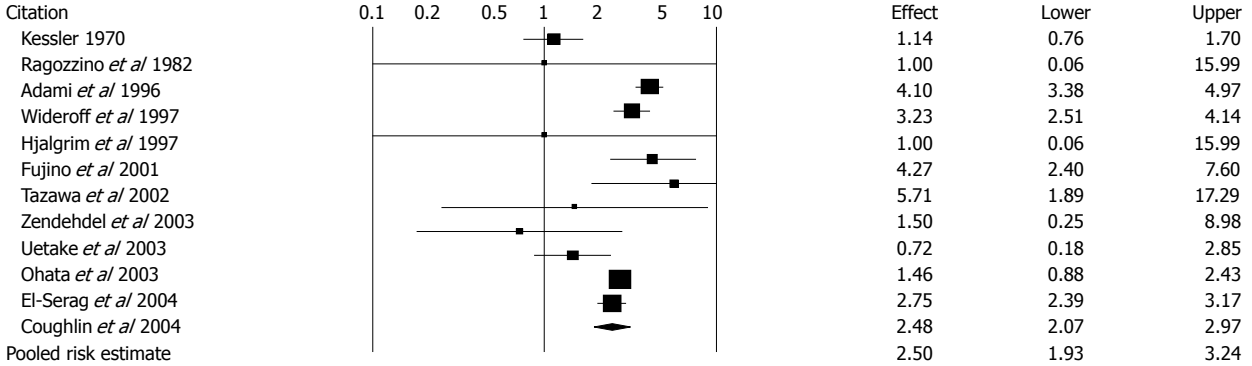

Figure 6 Unadjusted risk ratios and 95% CIs for the association between diabetes and hepatocellular carcinoma in 12 cohort studies. Black diamond indicates weighted average of all studies; Black boxes indicate the point estimates for risk ratios[47]. Reprinted from El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; 4: 369-380, Copyright (2006), with permission from Elsevier[47].

supported an association between diabetes and the development of HCC[47]. Among the 13 case-control studies, subjects with diabetes were found to have a twofold increase in the risk of HCC; an association that was further strengthened by excluding studies with significant heterogeneity (Figure 5)[47]. This association was also appreciated amongst 12 cohort studies evaluated (Figure 6). The presence of diabetes remained an independent risk factor for HCC after adjustment for alcohol use or viral hepatitis in the studies that evaluated these factors[47]. However, as dictated by the limitations of the studies available in the literature, further well-defined studies are required to account for dietary factors and obesity.

DIET

Several studies have examined whether alterations in diet have an effect on the risk of HCC. A trial from Italy has examined a broad range of dietary habits among 185 patients with HCC and 412 patients without cancer[48,49]. Those with HCC were more likely to consume a large amount of calories, were five times more likely to be former drinkers, and were 30 times more likely to be infected with either HCV or hepatitis B virus (HBV). Among dietary compounds, consumption of iron and thiamine were associated with a significant threefold and twofold increase in risk of HCC, respectively. Conversely, β-carotene and linoleic acid consumption was associated with a reduced risk of HCC[48]. An association between intake of iron was also evaluated according to the presence or absence of viral hepatitis[48]. When compared to appropriate controls, consumption of iron among patients without viral hepatitis was associated with a significantly increased risk of HCC[48]. This increase in risk was not conferred to those with HCV or HBV. In a similar study, those subjects with consumption in the highest quartile for yogurt and milk, white meat and eggs had a significantly lower likelihood of developing HCC[50]. This effect was observed in patients with and without viral hepatitis[50].

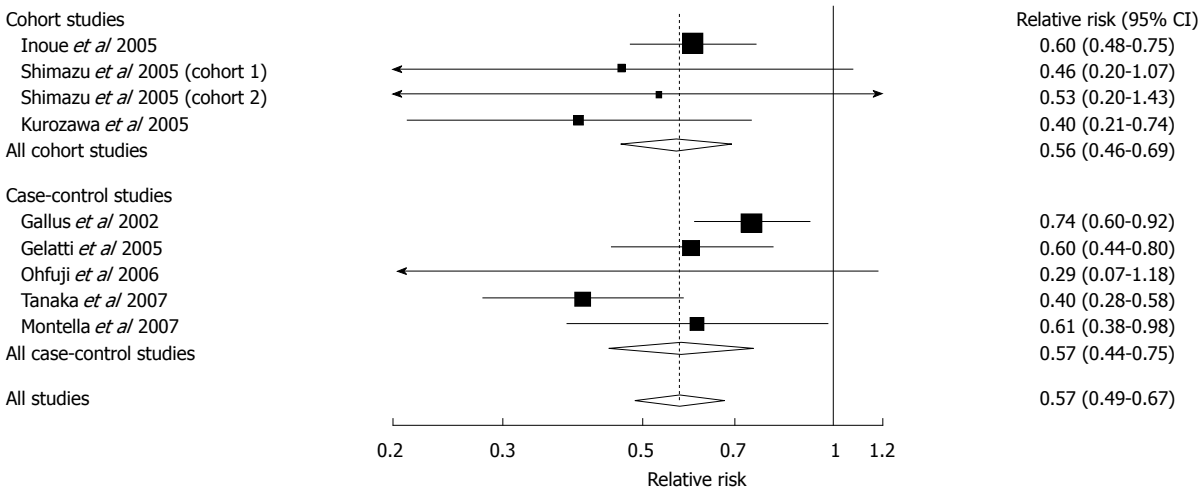

Figure 7 Relative risks of liver cancer associated with coffee consumption (per 2 cups/d increment). Squares represent study-specific relative risk estimates (size of the square reflects the study-specific statistical weight, that is, the inverse of the variance); horizontal lines represent 95% CIs; diamonds represent summary relative risk estimates with corresponding 95% CIs. Tests for heterogeneity: all studies, Q = 11.56, P = 0.17, I^2 = 30.8%; cohort studies, Q = 1.74, P = 0.63, I^2 = 0%; case-control studies, Q = 9.28, P = 0.05, I^2 = 36.9%. Reprinted from Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a meta-analysis. *Gastroenterology* 2007; 132: 1740-1745, Copyright (2007), with permission from Elsevier[66].

Other studies from Japan and Europe have found those who consume a large amount of green vegetables have a significantly lower likelihood of developing HCC[51-53]. One study has shown that eating green vegetables daily, as compared with consumption fewer times per week, had a protective effect against the development of HCC (OR: 0.75, 95% CI: 0.60-0.95)[51]. On the contrary, a Greek study has found no association between vegetable intake and reduction in the risk of developing HCC[54].

In summary, there is evidence to suggest that consumption of yogurt and milk as well as vitamin supplements offers a protective effect against HCC. The enthusiasm for these findings however should be tempered by the fact that the majority of these studies were retrospective in nature.

COFFEE

In addition to its reported association with reductions in bladder cancer and colorectal cancer, coffee consumption has also been extensively studied and appears to have a potentially favorable effect on the prevention of liver diseases, including HCC[55,56]. There are several hypotheses that could explain why consuming coffee attenuates the risk of developing HCC. One hypothesis argues that coffee intake lowers serum levels of γ-glutamyl transferase (GGT), which is associated with a lower incidence of HCC[56-59]. Coffee consumption has also been linked to a lower incidence of cirrhosis, which is a major risk factor for the development of HCC[56].

An analysis of two large prospective studies of > 70 000 participants in Japan has shown that those who drank one or more cups coffee daily had a significantly lower risk of developing HCC[60]. A case-control study of 2746 people has found that those who drank three or more cups of coffee were 40% less likely to develop HCC[56]. Similar results have also been found in an array of studies conducted in Europe and Japan[60-65].

Additionally, two meta-analyses that have examined the association between coffee drinking and the risk of developing HCC have recently been published. The first was inclusive of four cohort studies and five case-control studies[66]. In the pooled analysis, a 43% lower risk of developing HCC was found for those who drank more than two cups of coffee per day (RR: 0.57, 95% CI: 0.49-0.67) (Figure 7)[66]. The second meta-analysis examined four cohort studies from Japan and six from Japan and Southern Europe[67]. There were differing definitions of low and high coffee consumption, however, the results of the studies were consistent. In summary, those who drank any coffee compared to non-drinkers had a significantly lower risk of HCC (RR: 0.59, 95% CI: 0.49-0.72). The greater the coffee consumption, the greater the attenuation in HCC risk. Low coffee consumption was associated with a 30% reduction in risk and high consumption with a 55% reduction in HCC risk (Figure 8)[67].

Although these results are impressive and consistent, one must consider that the findings of an inverse relationship between coffee consumption and the risk of HCC might be influenced by bias. Coffee metabolism is impaired in cirrhotic livers as compared to the normal liver. This altered metabolism generates an increase in the untoward side effects of the beverage. Therefore, the presence of liver disease might lead affected patients to consume less coffee. This could result in a falsely negative association. Therefore, the potential bias of this association in the liver disease patient cannot be discounted.

ALCOHOL

The mechanism by which alcohol consumption increases the risk of HCC is primarily through the development of cirrhosis. It has been suggested that heavy alcohol consumption of > 80 g/d ethanol for at least 5 years increases the risk of HCC by nearly fivefold[68]. The risk appears to be proportional to the amount of alcohol consumed.

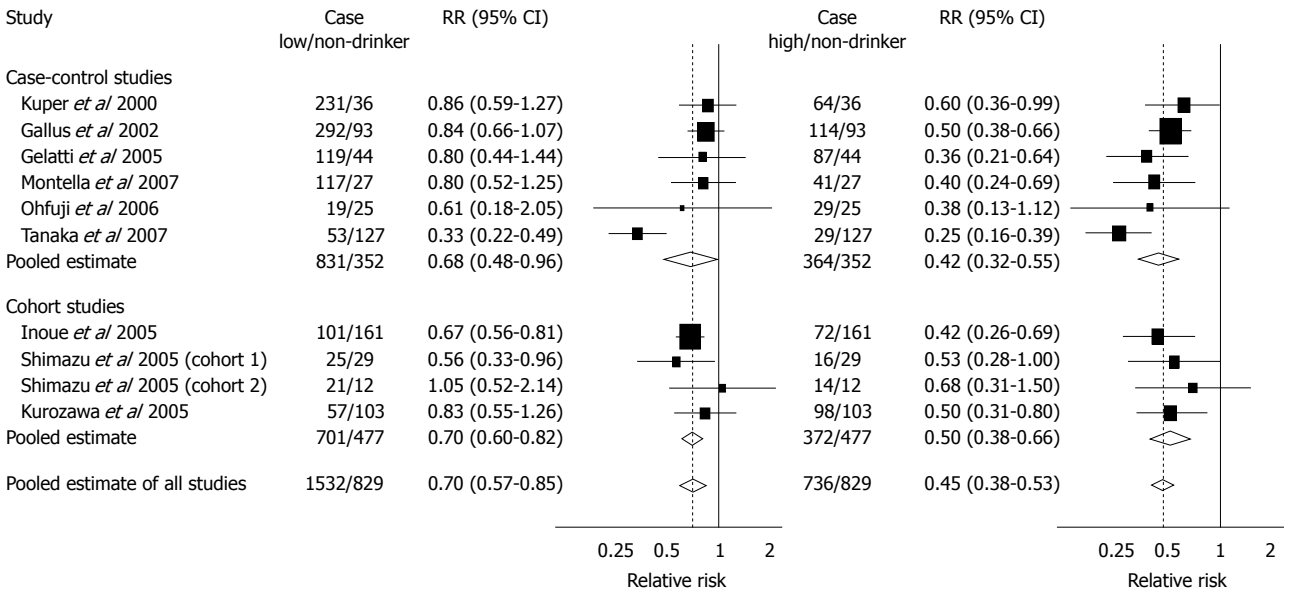

Figure 8 Summary RRs of hepatocellular carcinoma for low or moderate and high coffee drinkers *vs* non-drinkers from case-control and cohort studies. Low or moderate consumption was defined as < 3 cups per day for Gallus *et al*, Gelatti *et al*, Inoue *et al* and Montella *et al* and as < 1 cup per day for Ohfuji *et al*, Tanaka *et al*, Kurozawa *et al*, and Shimazu *et al*; High consumption was defined as ⩾ 3 cups per day for Gallus *et al*, Gelatti *et al*, Inoue *et al*, and Montella *et al* and as ⩾ 1 cup per day for Ohfuji *et al*, Tanaka *et al*, Kurozawa *et al*, and Shimazu *et al*. Bravi F, Bosetti C, Tavani A, Bagnardi V, Gallus S, Negri E, Franceschi S, La Vecchia C. Coffee drinking and hepatocellular carcinoma risk: a meta-analysis. *Hepatology* 2007; 46: 430-435[67]. Copyright 2007, John Wiley & Sons, Inc. Reproduced with permission of John Wiley & Sons, Inc.

As characterized by a meta-analysis, relative risks of 1.19 (95% CI: 1.12-1.27), 1.40 (95% CI: 1.25-1.56), and 1.81 (95% CI: 1.50-2.19) were associated with the consumption of 25, 50 and 100 g/d alcohol, respectively[69].

In addition to a daily dose response, persistent alcohol consumption appears to have a long-term effect on the risk of HCC occurrence. A prospective case-control study from Japan has observed that heavy alcohol drinkers, defined as > 600 L of alcohol during a lifetime, had a fivefold increase in the risk of HCC in comparison to non-drinkers or those who consumed < 600 L of alcohol (OR: 5.19, 95% CI: 2.53-10.64)[70]. However, the risk of HCC among those who consume low or moderate levels of alcohol remains unknown[1].

An association between genetic polymorphisms of the enzymes participating in the metabolic pathway of ethanol and the increased risk of HCC in heavy alcohol drinkers has been also proposed as a mechanism by which HCC develops. The frequency of aldehyde dehydrogenase 2 (*ALDH2*) genotype polymorphisms is significantly associated with increased risk of HCC in heavy alcohol drinkers (OR: 2.53, 95% CI: 1.63-58.60)[70]. A study from Italy has observed that, among subjects who consumed > 100 g/d of ethanol and were bearers of the gluthatione S-transferase M1 (GSTM1) null genotype had twice the risk of HCC compared with bearers of the GSTM1 non-null genotype (OR: 8.5, 95% CI: 3.9-18.6 *vs* OR: 4.5, 95% CI: 2.0-10.0)[71].

SMOKING

Several studies have evaluated the association between smoking and development of primary liver cancer. A prospective cohort study including 4050 men aged ⩾ 40 years who were followed-up for an average length of 9 years observed that those who smoked had a threefold increased risk of primary liver cancer when compared to never smokers (RR: 3.3, 95% CI: 1.2-9.5)[72]. Additionally, a study from Korea has found a 50% increase in the risk of primary liver cancer for current male smokers compared to never smokers[73]. In contrast however, a recent population-based case-control study from the United States did not observe a significantly increased risk of primary liver cancer among current male smokers[74]. Male ex-smokers, however, had a significant increase in risk of primary liver cancer, which suggests that there is perhaps a dose or duration response underlying this association[72-74]. Such responses were further explored in the Korean Cancer Prevention study that included 1 283 112 subjects[75]. Although the amount of smoking did not alter the risk of HCC, the duration of smoking significantly increased the risk of HCC for subjects who had smoked for > 20 years when compared to those who had smoked for < 10 years[75].

The association between tobacco and liver cancer and its reliance on host factors such as genetics, sex, and an underlying history of viral hepatitis has also been explored. With respect to the role of genetics, a small study from Japan has evaluated 78 patients with HCC and genetic polymorphisms of tobacco and alcohol-related metabolizing enzymes and 138 hospital controls without cancer. They have demonstrated that cigarette smokers did not have a significantly increased risk of HCC when compared with non-smokers[70]. To analyze the effect of sex, a prospective cohort study that included 83 885 patients followed up for 8 years observed a positive association between smoking and HCC in women who smoked > 10 cigarettes per day (RR: 4.2, 95% CI: 1.3-13.8)[76]. However, no significant increase in the risk of HCC was

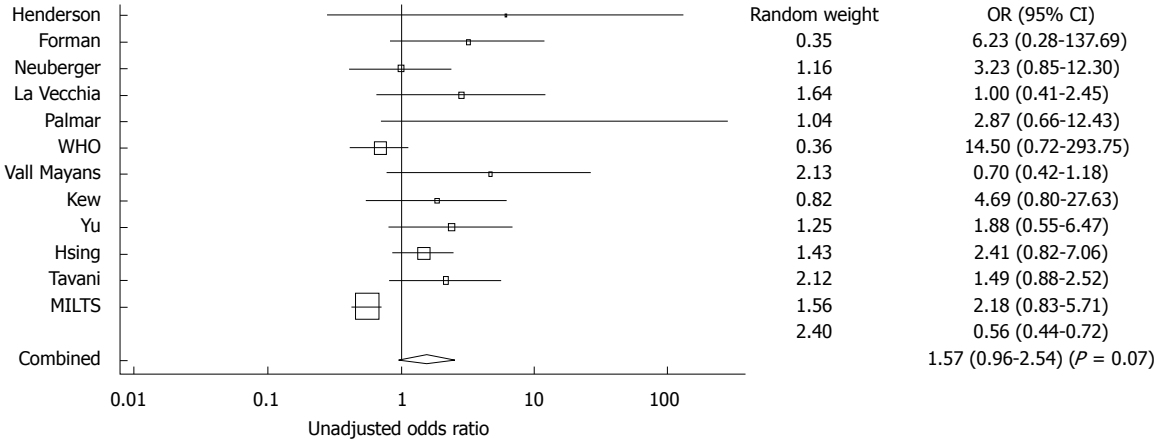

Figure 9 Forest plot showing adjusted OR and 95% CI for the association between oral contraceptive and hepatocellular carcinoma for the eight studies that included adjusted ORs. The diamond symbol indicates the weighted random pooled OR of all studies included in the analysis. Reprinted from Maheshwari S, Sarraj A, Kramer J, El-Serag HB. Oral contraception and the risk of hepatocellular carcinoma. *J Hepatol* 2007; 47: 506-513, Copyright (2007), with permission from Elsevier[95].

demonstrated among male smokers[76]. Additionally, to determine the effect of viral hepatitis on the association between HCC and tobacco, a prospective study of 12 008 men observed that smoking significantly increased the risk of HCC only in anti-HCV-positive patients but not in those who were anti-HCV-negative when compared to anti-HCV-negative nonsmoking individuals[77].

In addition to an increase in the risk of developing HCC, it is also suggested in the literature that smoking increases the risk of death in HCC. In the Korean Cancer Prevention cohort study, men who were current smokers had an increased risk of death from HCC[75]. Women who were current smokers did not have the same elevation in risk of HCC-related death as that observed in men[75]. These findings were further replicated in the Japan Collaborative Cohort (JACC) Study that analyzed 65 528 subjects aged 40-79 years[78]. In this cohort, an increased risk of death due to HCC was shown among current and ex-smokers[78]. Further analyses from the JACC cohort demonstrated that cigarette smoking significantly increased the risk of death from HCC in individuals positive for anti-HCV antibody[79].

ORAL CONTRACEPTIVES

Prior to the widespread use of oral contraceptives (OCs), benign liver tumors in young women were rarely observed. In the current case report literature, however, therapy with oral contraceptives appears to be associated with the development of benign liver tumors such as hepatic hemangioma, hepatocellular adenoma or focal nodular hyperplasia[80,81]. Although not well researched, it has been proposed that OCs might also be associated with malignant liver tumors including HCC[82,83]. Rarely, malignant transformation can occur within the context of hepatic adenomas. It is unclear, however, whether the use of OCs influences the likelihood of developing adenoma and that these benign tumors transform.

Within the literature, there have been 14 cases of hepatic adenoma with focal malignant transformation to HCC in women taking OCs[83-93]. The mean age of these patients at the time of diagnosis of malignant transformation was 36 years (range: 23-57 years) and the mean duration of OC use was 11 years (range: 1 mo-20 years)[83-93]. Although difficult to obtain from the literature, the frequency of HCC among hepatic adenomas appears to vary from 5% to 18%[89,92-94].

To evaluate further the risk of HCC in the setting of OC use, several observational studies have been conducted. A recent meta-analysis of 12 case-control studies, including 739 cases and 5223 controls, which evaluated the risk of HCC among women using OCs indicated that there was no increase in risk of HCC with short-term use; defined as < 5 years of exposure[95]. An analysis of all studies in the aforementioned meta-analysis yielded a pooled unadjusted OR of 1.57 (95% CI: 0.96-2.54)[95]. An adjusted analysis, which accounted for variables such as age, race and parity, did not yield significant findings (Figure 9)[95]. On the contrary, six studies have observed a significantly increased risk of HCC among women taking OCs for > 5 years; an increase in risk of 2-20-fold[95]. However, given the variable periods of duration used in each of the studies, a pooled estimate of risk could not be generated[95]. Based on these results, further studies are required to evaluate the association between OCs and the risk of HCC and how such risk is modified by duration of OC use. Additionally, it should be noted that an association between new-generation OCs with lower doses of hormones and the risk of HCC has not yet been explored.

BETEL QUID

The chewing of betel quid is woven into the cultural fabric of up to 20% of the world population. Betel quid consists of the nut of the *Areca catechu* palm (areca nut), betel leaf or fruit from *Piper betle* and red slaked paste[96]. These ingredients have been shown to have genotoxic, mutagenic and tumorigenic properties[97-102]. A case-con-

trol study from Taiwan has shown that betel quid chewing was an independent risk factor for liver cirrhosis (OR: 3.56, 95% CI: 1.41-8.96)[103].

Recently, two prospective case-control studies from Asia also have observed a significant association between betel quid chewing and the incidence of HCC. One such study included 263 pairs of age- and sex-matched patients with HCC and healthy controls and observed that betel quid chewing was an independent risk factor for HCC, with a threefold risk noted (OR: 3.49, 95% CI: 1.74-6.96). The aggregate risk increased with increasing duration and/or quantity of consumption[96]. These data were further supported by a study from Taiwan, including 420 age- and sex-matched patients with HCC and liver cirrhosis, liver cirrhosis only and healthy controls. In this study, a nearly sixfold and nearly twofold increased risk of HCC was observed in patients with HCC compared with healthy controls and patients with liver cirrhosis, respectively[104]. Additionally, they found an additive interaction between betel quid chewing and chronic HBV and/or HCV infection.

AFLATOXIN

Aflatoxin B1 (AFB1) is the major metabolite of the molds *Aspergillus fumigatus* and *Aspergillus parasiticus*. These molds grow on a variety of food products that are stored in warm and damp conditions or are cultivated in countries with hot and humid climates[1,105]. AFB1 induces a single nucleotide substitution in codon 249 in the *p53* tumor suppressor gene, which results in the change of the amino acid arginine to serine[106,107]. This mutation is present in up to 50% of patients with HCC who are indigenous to geographic regions with high exposure to AFB1[108-111]. On the other hand, this mutation is absent in patients with HCC from regions with low exposure to AFB1[112,113]. Moreover, it has been recently demonstrated that AFB1-albumin adducts in patients with HCC correlate significantly with the presence of plasma DNA hypermethylation and mutations in the *p16* and *p53* tumor suppressor genes[114].

Several studies have evaluated an association between the risk of HCC and exposure to AFB1. A prospective case-control study from China which included 18 244 middle-aged men showed that individuals with the presence of urinary aflatoxin biomarkers had a significantly increased risk of HCC after adjusting for HBV surface antigen seropositivity and cigarette smoking[115]. These data were further supported by a community-based cohort study from Taiwan which found that elevated AFB1 exposure measured by detectable AFB1-albumin adducts was an independent risk factor for HCC after adjustment for important confounders (OR: 5.5, 95% CI: 1.2-24.5)[116].

It should be stressed that areas with high exposure to AFB1 are also characterized by a high prevalence of HBV infection. AFB1 is independent of the risk conferred by HBV, however concomitant exposure to both HBV and AFB1 markedly increases the risk of HCC. When compared to those without HBV infection and absence of urinary AFB1 markers, the risk of HCC was 60 times higher in patients with HBV infection and a concomitant elevation of urinary AFB1 markers (RR: 59.4, 95% CI: 16.6-212.0)[115]. Patients with HBV infection and normal urinary AFB1 markers had sevenfold increase in risk of HCC when compared to appropriate controls[115].

CONCLUSION

Multiple non-viral factors have been implicated in the development of HCC. Hemochromatosis and iron overload syndromes have consistently been shown to dramatically increase the rate of HCC. Additionally, factors such as obesity and diabetes, which operate *via* NASH cirrhosis or perhaps independently, have also been demonstrated to increase the risk of HCC. This phenomenon has closely mirrored the epidemic of obesity over the last 15-25 years.

With respect to other exposures, although alcohol and tobacco clearly increase the risk of HCC development and mortality, other exposures such as coffee and high levels of vegetable consumption may be protective against this condition. Further studies are urgently needed to determine the pathogenesis that underlies the occurrence of HCC in the setting of these exposures, as well as the way in which such risk is modified by environmental and host characteristics such as genetics.

Clarification of relevant non-viral causes of HCC will help to focus clinicians on those risk factors that are modifiable. With more information, future surveillance efforts will be more appropriately targeted toward populations at greatest risk. This multilevel preventative approach will hopefully lead to a reduction in incidence of non-viral HCC, and a decrease in the patient morbidity and mortality as well as the societal economic burden associated with HCC.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3616

World J Gastroenterol 2010 August 7; 16(29): 3616-3629
ISSN 1007-9327 (print)

EDITORIAL

Primary biliary cirrhosis: What do autoantibodies tell us?

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Received: March 15, 2010 Revised: April 28, 2010
Accepted: May 5, 2010
Published online: August 7, 2010

Abstract

Primary biliary cirrhosis (PBC) is a chronic, progressive, cholestatic, organ-specific autoimmune disease of unknown etiology. It predominantly affects middle-aged women, and is characterized by autoimmune-mediated destruction of small- and medium-size intrahepatic bile ducts, portal inflammation and progressive scarring, which without proper treatment can ultimately lead to fibrosis and hepatic failure. Serum autoantibodies are crucial tools for differential diagnosis of PBC. While it is currently accepted that antimitochondrial antibodies are the most important serological markers of PBC, during the last five decades more than sixty autoantibodies have been explored in these patients, some of which had previously been thought to be specific for other autoimmune diseases.

Key words: Primary biliary cirrhosis; Autoimmune disease; Autoantibody; Anti-mitochondrial antibody; Anti-gp210 antibody; Anti-sp100 antibody; Anti-centromere antibodies

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Hu CJ, Zhang FC, Li YZ, Zhang X. Primary biliary cirrhosis: What do autoantibodies tell us? *World J Gastroenterol* 2010; 16(29): 3616-3629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3616.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3616

INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease characterized by infiltration of lymphocytes in portal tracts, progressive destruction of intrahepatic small bile ducts and the presence of serum antimitochondrial antibodies (AMA)[1,2]. As is the case for the majority of autoimmune diseases, PBC affects predominantly women. Recent investigations have suggested that PBC, sometimes asymptomatic, is not a rare disease. During the last several years advanced biochemical assays, further delineation of specific liver histological findings, more effective serum autoantibody detection methods and improved diagnostic abilities have led to higher prevalence estimates worldwide[3-5]. Currently it is believed that PBC is likely to be triggered by a combination of environmental factors including infection in a genetically susceptible individual. This hypothesis is supported by the high concordance rate of PBC among first-degree relatives and in homozygous twins (approximately 60%)[6,7]. Specific immunologic damage to biliary epithelium and a mechanism of tissue destruction in PBC has been elucidated[8,9]. In addition, epitopes of T cells and B cells targeting mitochondrial autoantigens have been identified[10-12]. Furthermore, a number of autoantibodies previously thought to be spe-

cific markers for another autoimmune disease have been detected in patients with PBC.

Disease progression and clinical manifestations in PBC varies. The fact that a variety of autoantibodies have been detected in PBC suggests the disease has a complicated pathogenesis. In this review, the properties of these autoantibodies and their autoantigen characteristics, as well as their pathogenetic and clinical significance were discussed.

AMA

The presence of AMA is pathognomonic for PBC[13], and it is generally accepted that AMA can be detected in serum years before the advent of any clinical manifestation or biochemical abnormality[14-16]. AMA were first described in 1958[17] in sera from patients with chronic liver disorders and then detected by Walker *et al*[18] in 1965 using an immunofluorescence test. In the past 40 years an enormous number of experimental studies have focused on AMA, and numerous rewarding discoveries have been made. There are nine subtypes of AMA, four of which have been involved in PBC, including anti-M2, anti-M4, anti-M8 and anti-M9. It has been demonstrated that the autoantigens recognized by anti-M2 are located in the inner membranes of mitochondria, whereas those recognized by anti-M4, anti-M8 and anti-M9 are located in the outer mitochondrial membranes. Anti-M9 can be detected in both anti-M2-positive and -negative PBC patients, while anti-M4 is only positive in the presence of anti-M2. All four of these AMA subtypes are relatively specific for the diagnosis of PBC.

Anti-M2

M2 has been found to contain five antigenic determinants, with molecular weights of 70 kDa (a), 56 kDa (b), 51 kDa (c) 45 kDa (d) and 36 kDa (e), all of which were identified subsequently as members of the 2-oxoacid dehydrogenase complex of enzymes within the mitochondrial respiratory chain, including the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), the E2 subunit of the branched-chain 2-oxoacid dehydrogenase complex, the E2 subunit of the 2-oxoglutarate dehydrogenase complex, E1t alfa subunits of PDC and E3 binding protein (protein X)[19,20]. The exact molecular weight of the M2 band differs among laboratories according to mitochondria species being used and specifics of techniques for antigen preparation and detection. In patients with PBC, approximately 90%-95% of serum samples react against PDC-E2, making this the most important autoantigen in the disease. Anti-M2 is the most important subtype used in routine diagnostic tests for PBC. Its level in affected sera is high and it also exists in other body fluids such as saliva and bile[21-23]. As AMA is considered to be the hallmark of PBC, a positive test is potentially diagnostic, or at least indicative that the individual is at increased risk for future development of PBC[15].

Anti-M4

The anti-M4 antibody was originally detected in patients with chronic cholestatic liver disease (mixed form) associated with two different types of complement-fixing AMA[24]. M4 is a single antigen with molecular weight of 52 kDa. It can be detected by a complement fixation test but not immunoblotting. Unlike M2, the M4 antigen is trypsin-insensitive and its band at sucrose densities is 1.08 to 1.14. Anti-M4 is found predominantly in patients with histological features of chronic active hepatitis and PBC. Recent studies have identified the major proteins in the M4 fraction which is related to the PDC-E1 subunits and sulphite oxidase[25,26].

Anti-M8

The M8 antigen is also trypsin-sensitive with a band at sucrose densities of 1.16 to 1.24. Anti-M8 has been found only in coexistence with anti-M2, the presence of anti-M8 indicates progressive disease activity. On the other hand, not all anti-M2-positive patients have anti-M8. Like M4, the M8 antigen also locates in the outer mitochondrial membranes[27].

Anti-M9

Anti-M9 antibody was accidentally found when testing anti-M2-positive sera against trypsinized submitochondrial particles from rat liver shown to be devoid of anti-M2[28]. Anti-M9 antibody is detected predominantly in patients with asymptomatic and early PBC, and it also can be positive in anti-M2-negative PBC patients. Unlike anti-M4 and anti-M8, which seem to reflect disease activity, anti-M9 antibody occurs early in PBC. Patients with only anti-M9 have all the typical biochemical features found in classic anti-M2-positive patients, but seem to have slower disease progression and benign outcome, whereas patients having complement-fixing antibodies against anti-M2, anti-M4, and anti-M8 seem to have more active disease and worse outcome[29-31], though this finding wasn't supported by a blinded study on Dutch PBC patients conducted by Vleggaar *et al*[32].

ROLE OF AMA IN PBC PATHOGENESIS

Although AMA serve as highly sensitive markers for the diagnosis of PBC, autoantibodies against various mitochondrial enzymes can frequently be detected in patients with other diseases, such as primary Sjögren's syndrome (pSS), scleroderma, autoimmune hepatitis[33,34] and some infectious diseases like tuberculosis and viral hepatitis[35-38]. It is very interesting that the prevalence of AMA in first-degree relatives of PBC probands is as high as 13.1%, whereas in gender, age, race, and residence-matched controls the prevalence is only 1%, suggesting that environmental risks and genetic determinants are likely implicated in the etiology of PBC[7].

As no clinical correlation can be found, and animal models with serum AMA do not consistently have PBC-like liver lesions, the exact role played by AMA in the immunopathology and pathogenesis of PBC remains elusive. However, current data indicate that the destruction

of biliary cells is mediated by liver-infiltrating autoreactive T cells specific for the dominant PDC-E2 autoantigen[39]. The dominant epitopes of autoreactive T and B cells have been identified. The $CD4^+$ T cell epitope appears to localize to peptides 163-176, the $CD8^+$ T cell epitope appears to localize to peptides 159-167, while the B cell epitope appears to localize to peptides 167-186[39-43]. Furthermore, the most prominent immune features of autoreactive $CD4^+$ and $CD8^+$ T cells can be detected in peripheral blood from patients with PBC. The disease-related AMA-specific $CD8^+$ T cells are enriched up to about 10-fold and the $CD4^+$ T cells are enriched up to more than 100-fold in liver compared to peripheral blood samples[42,44]. Presently the data suggest that B and T cells in PBC patients respond simultaneously to the same autoantigen, and that both are involved in the pathogenesis of PBC.

Study of stored sera of well-characterized PBC patients followed for 7-28 years indicate that AMA levels are not associated with disease severity and progression. Most studies except the one conducted by Poupon *et al*[45] did not support that AMA levels could be affected by treatments during the observation period[45-47]. In fact, low levels of AMA persisted for up to 11 years following liver transplantation[47]. AMA are non-organ- and non-species-specific, and contain IgA, IgG and IgM subclasses. Data from PBC patients demonstrate that the presence of AMA IgA in sera or saliva might be associated with disease progression[23] and some studies suggested that greater concentrations of AMA IgA in biliary and mucosal secretions, constant transcytosis, would render the exposed cells more susceptible to apoptosis resulting in subsequent bile duct damage[48], while others proposed the hypothesis that AMA IgA can be transported to the vascular side of the bile duct cell where it can induce apoptosis by reacting with PDC-E2-like molecules located on the luminal surface cell membrane[49]. Many studies have demonstrated that the different AMA IgG subclasses have different clinical significance. PBC patients positive for IgG3 AMA had histologically more advanced disease and were more frequently cirrhotic than those who were negative. Furthermore, there was a positive correlation between AMA IgG3 titers and Mayo risk scores: this subclass is associated with poor prognosis, possibly reflecting the peculiar ability of this isotype to engage mediators of immunological damage[50].

Currently it is believed that a positive AMA titer is virtually pathognomonic of current PBC or risk for future development of the disorder, although the mechanisms leading to the generation of AMA have not been elucidated. Several possible mechanisms have been suggested regarding the generation of AMA, such as oxidative damage, molecular mimicry and changed biliary epithelial cell (BEC) apoptosis[51,52]. The fact that high levels of AMA can be detected in patients with acute liver failure supports the hypothesis that oxidative stress-induced liver damage may lead to induction of AMA[53]. But it is also surprisingly true that the AMA in these patients disappear rapidly, suggesting the pathogenesis of PBC is multifactorial. It has been demonstrated that molecular mimicry between bacterial or viral antigens and mitochondrial antigens can trigger the generation of AMA in PBC[54,55]. Modification of the inner lipoyl domain of E2 with halide or ethyl halide results in increased reactivity of AMA from PBC patients, suggesting that xenobiotics might make cellular components antigenic[56].

There is growing evidence showing that the onset of PBC may be the result of inefficient removal of apoptotic cells. It is of interest to note that a recent report proposed that PDC-E2 in patients with PBC is released without caspase cleavage from apoptotic BEC, supported by the fact that glutathionylation of the lysine lipoic acid moiety on the PDC-E2 is sometimes, though not commonly, decreased by serum AMA *via* Bcl-2[57]. Other studies show that apoptotic cells are phagocytosed by BECs, a function mediated by anti-CD16, and so consequently act as an exogenous source of autoantigens in cholangiocytes[9,58]. Defects in the elimination of apoptotic cells can lead to secondary necrosis accompanied by subsequent release of intracellular components, which might explain the generation of autoantibodies against intracellular antigens like AMA[59].

Further studies in the field of AMA in PBC have led to speculation about the existence of an AMA-negative PBC subgroup. It is not clear whether there is indeed such a subgroup, having distinct features, or if this is an artifact due to technical limitations of current AMA detection methods leading to false-negative results in some PBC patients[60]. Present data indicate that there is no discernable difference between AMA-positive and -negative PBC in terms of clinical manifestations, liver biochemistry and histopathology findings, disease course, as well as response to treatment[61-63]. As more sensitive and specific serologic tests are applied, many patients initially believed to be AMA-negative are subsequently found to be AMA-positive[64,65]. These findings cast doubt on the existence of a true AMA-negative PBC subgroup.

ANTINUCLEAR ANTIBODIES IN PBC

Antinuclear dot antibodies (SP100, PML, NDP52 and SP140)

PBC patients often have autoantibodies with nuclear dot (ND) stain patterns in the indirect immunofluorescence (IIF) assay. The major antigens associated with ND are as follows: sp100 proteins, which are transcription-activating proteins autoantigenic primarily in patients with PBC and occasionally in rheumatic disorders[66,67]; promyelocytic leukemia (PML) protein, a transformation and cell-growth suppressing protein aberrantly expressed in PML cells that was discovered in studies of the development of acute PML; NDP52, a protein of the myosin VI binding partners which was previously shown to contribute to innate immunity[68,69]; and sp140 proteins, which are identified as autoantigenic proteins in PBC recently. Sp100 and PML were discovered in the context of leukemic transformation and as autoantigens in PBC[70].

They are reported to be co-autoimmunogenic, often in patients with PBC[71]. The sp100 antigen was described by Szostecki *et al*[66] as a peptide of 480 amino acids with an aberrant electrophoretic mobility to 100 kDa, and a calculated molecular weight of 53 kDa. It was subsequently characterized by complementary DNA cloning, and the deduced amino acid sequence was found to contain sequence similarities with HIV-1 nef proteins[72]. The prevalence of anti-sp100 antibodies in PBC is about 25%, and it appears to be highly specific for a diagnosis of PBC, but only when other diseases can be excluded and the typical clinical context is present[73,74]. The presence of anti-sp100 antibodies serves as a serologic marker of PBC, which could be useful in clinics to confirm the diagnosis, especially in AMA-negative PBC patients[75,76]. Recent data indicate that as reports of AMA-negative PBC decrease due to development of more sensitive and specific serologic tests for serum AMA, anti-sp100 antibodies appear to be more common in AMA-positive PBC patients than in those who are AMA-negative[77,78]. Also, anti-sp100 antibodies are increasingly found to be present in many clinical conditions, such as systemic lupus erythematosus (SLE) and pSS. It is of interest to note that among female patients with urinary tract infections but no liver disease, 80% of the AMA-positive, but none of the AMA-negative patients were also positive for anti-sp100 antibodies. It is also well established that among PBC patients, about 74% of patients with urinary tract infections were positive for anti-sp100, whereas the positivity was only 4.8% in PBC patients without urinary tract infections[79]. Given the high specificity of anti-sp100 as an immunoserological hallmark of PBC, these findings support the hypothesis that some infections such as *Escherichia coli* are involved in the induction of PBC-specific autoimmunity.

PML protein was discovered in cells of patients with acute PML as a protein fused with the retinoic acid receptor-a (RAR)[80,81]. PML protein functions as a nuclear hormone receptor transcriptional coactivator[82]. Subsequently it was shown to form ND patterns when tested by immunofluorescence microscopy with serum anti-PML antibodies from PBC patients. Anti-PML antibodies often coexist with anti-sp100 antibodies in individuals with PBC[71], and are present in about 19% of PBC patients[83]. Current study indicates that anti-PML antibodies are highly specific for PBC even when autoantibodies against mitochondrial antigens are not found[84].

Anti-sp140 antibodies were recently identified for the first time in patients with PBC by Granito *et al*[85]. They are present in about 15% of PBC patients and are highly specific for PBC. Anti-sp140 antibodies coexist with anti-sp100 and anti-PML antibodies. No association was found between anti-sp140 and any particular clinical feature of PBC.

Antinuclear pore antibodies (gp210 and p62)

In addition to AMA, a number of nuclear antigens have been recognized as targets of antinuclear antibodies (ANA) in patients with PBC, including several components of the nuclear pore complex (NPC), such as the gp210 and p62 proteins. These antibodies have a nuclear periphery fluorescence pattern in the IIF assay, as first reported by Ruffatti *et al*[86] in 1985. Several reports revealed that the frequency of PBC-specific nuclear envelope antibodies ranged from 16% to 30%[76,87], and that the frequency increased greatly when fluorescent-labeled specific antiserum of the IgG subclass was applied[88,89]. In 1990 a study by Lassoued *et al*[90] showed that autoantibodies from patients with PBC having a punctate fluorescence pattern in IIF react with a protein of molecular mass approximately 200 kDa, which was identified as the NPC membrane protein gp210[91]. Gp210 is an integral glycoprotein of the nuclear pore consisting of three main domains: a large glycosylated luminal domain, a single hydrophobic transmembrane segment and a short cytoplasmic tail. Gp210 is recognized by antibodies in approximately 25% of patients with PBC[92]. The gp210 epitope recognized by most of the autoantibodies is a 15 amino acid stretch in the cytoplasmic, carboxyl-terminal domain of the protein. In the ANA category, these anti-gp210 antibodies are particularly significant since they are highly specific for PBC[93,94]. In addition, several reports link the presence of anti-gp210 antibodies in PBC patients with disease severity and poor prognosis. Since the presence of anti-gp210 antibodies correlates with an unfavorable disease course and more rapid progression, it is useful for monitoring the effect of ursodeoxycholic acid and for the early identification of patients at high risk for end-stage hepatic failure, and so may potentially become an important prognostic marker in PBC patients[95,96]. Findings to date clearly indicate that anti-gp210 antibodies having the best predictive value regarding progression to end-stage hepatic failure. The proposed mechanism for this predictive role is based on the following hypothesis that the breakdown of immunological tolerance to mitochondrial antigens such as PDC-E2 is not enough for the progression to hepatic failure, whereas the breakdown of immunological tolerance to nuclear antigens such as gp210, in which molecular mimicry is involved as well as increased and aberrant expression of gp210 in small bile ducts, may play a crucial role[97].

A few years after the discovery of anti-gp210 antibodies in PBC, reactivity of PBC sera with a 60 kDa component of NPC was reported. Anti-p62 antibodies, which also generate a perinuclear pattern in IIF, were first described in 1987[98-100]. They occur as frequently as the anti-gp210 glycoprotein autoantibodies[101], and with a specificity for PBC of up to 97%. Anti-p62 antibodies reacting with the 60 kDa component localize to the NPC. The frequency of anti-p62 antibodies in PBC is about 30%-55%. Their presence in PBC is not associated with AMA, but is associated with disease progression. Data from a multicenter study indicated that anti-p62 complex antibodies might be related to the progressive or advanced stage of PBC[99,102], that their prevalence is higher in symptomatic patients and that they are associated with more severe

Table 1 Autoantibodies in primary biliary cirrhosis that are closely related to other autoimmune diseases

No.	Autoantibody	Autoantigen properties	Prevalence in PBC (%)	Clinical associations	Ref.
ANA					
1	Anti-chromatin	Chromatin	8.9-25.0	Anti-chromatin antibodies are reported to be associated with disease activity in AIH, but their roles in PBC remains to be investigated	[99,127-129]
2	Anti-dsDNA	Double-stranded deoxyribonucleic acid	17.0-22.0	Anti-dsDNA antibodies are one of important criteria for the diagnosis of SLE. Co-existence of AMA and anti-dsDNA autoantibodies can be considered the serological profile of AIH/PBC overlap syndrome	[76,127,128,130,131]
3	Anti-ssDNA	Single-stranded deoxyribonucleic acid	59.0-71.0	Anti-ssDNA antibodies can be detected in many diseases	[132,133]
4	Anti-histone	Histone	43.6	Anti-histone antibodies are generally considered to be related to drug-induced lupus, though it can be detected in many autoimmune diseases including PBC	[132,134]
5	Anti-scl-70	Topoisomerase-1	3.0-24.0	Anti-scl-70 antibody serve as a specific maker for diffuse SSc and presents in 30%-60% of subjects with diffuse SSc	[127,132]
6	Anti-Sm	Proteins of 28/29, 16, 16.5, 18, and 12, 11, 6 kDa which participate in pre-messenger RNA processing into spliced mature mRNA	7.0-34.0	Anti-Sm autoantibodies are highly specific for SLE	[76,127,132,135]
7	Anti-SSA	Intracellular ribonucleoproteins of 60 and 52 kDa that are associated with small RNAs	5.0-30.0	Anti-Ro(SS-A) and anti-La(SS-B) antibodies are more frequently seen in SS and SLE. Their presence in PBC suggests that PBC often overlaps with SS	[76,127,132,135,136]
8	Anti-SSB	An intracellular ribonucleoprotein of 47 kDa that are associated with small RNAs	2.0-21.0		
9	Anti-RNP	Ribonucleoprotein	5.0	More frequently seen in SLE	[76,127]
10	Anti-Jo-1	Histidyl tRNA synthetase	26.0	Anti-Jo-1 antibodies are predominantly detected in patients with myositis	[135]
11	Anti-U1RNP	U1snRNPs that contain specific proteins of 70, 33 and 20 kDa	3.1-5.0	Anti-U1snRNP antibodies predominantly present in SLE, and can be detected in PBC patients. The clinical significance of anti-U1snRNP antibodies in PBC is unknown	[137,138]
Other liver diseases-associated autoantibodies					
12	Anti-SMA	A variety of target antigens including F-actin, G-actin, myosin, tropomyosin, troponin, desmin, vimentin, keratin, *etc.*	8.0-25.0	Anti-SMAs present mainly in AIH-Ⅰ, and can also be detected in chronic active hepatitis. The presences of anti-SMAs in PBC are potential indicators of AIH/PBC overlap syndrome	[131,139]
13	Anti-SLA	SLA and liver and pancreas antigen	2.0-3.9	Anti-SLAs are autoantibodies seen in AIH-Ⅲ.The presences of SLA autoantibodies in PBC indicate secondary autoimmune hepatitis	[9,140,141]
14	Anti-LKM	Liver kidney microsomal antigen	0.7	Anti-LKM antibodies occur preferentially in AIH-Ⅱ. Anti-LKM autoantibodies can be seen in 21.4% of HCV-infected PBC patients, which suggests a close association between LKM and HCV-infected PBC	[142]
15	Anti-ASGPR	Asialoglycoprotein receptor	22.0-23.0	Anti-ASGPR antibodies mainly present in AIH and PBC. The autoimmune responses against ASGPR have been implicated in the development of AIH and PBC	[143-146]
16	Anti-LCM	Liver cell membrane specific antigen	42.0	Anti-LCM antibodies are detected predominantly in patients with HBsAg-negative chronic active hepatitis, but are also found in other liver diseases such as PBC	[147-149]
17	Anti-LSP	Liver specific protein	48.5	Anti-LSP antibodies present in viral hepatitis and autoimmune liver disease, and are found to correlate with severity of periportal inflammation and piecemeal necrosis in PBC	[144,150]
18	Anti-calreticulin	Calreticulin	20.0	Anti-calreticulin antibodies present in autoimmune liver disorder and IBD. They are not specific for PBC	[151,152]
19	Anti-FH	Fumarate hydratase	19.4	Anti-FH antibodies are found to be present predominantly in AIH. It can also be detected in PBC and other liver disease. The prevalence and clinic significance of anti-FH in PBC need further study	[153]
20	Anti-PGAM-B	Phosphoglycerate mutase isozyme B	16.7	Anti-PGAM-B antibodies are found to be present in 70.0% of AIH and 16.7% of PBC. It is also present in about 10% of viral hepatitis and 3.7% of healthy control. The clinical significance of anti-FH needs further study	[154]

21	Anti-p97/VCP	P97/valosin-containing protein	12.5	Anti-p97/VCP antibodies predominantly present in PBC, and can be detected in about 9.7% of AIH. The presence of anti-p97/VCP antibodies in PBC suggests less progressive disease course and benign prognosis	[155-157]
22	Anti-GSTA1-1	Glutathione S-transferase	10.0	Anti-GST autoantibodies are detected in 16.0% of AIH and 10.0% of PBC. Patients of AIH with positive anti-GST have severe diseases and poor prognosis	[158]
23	Anti-ASL	Argininosuccinate lyase	23.0	Anti-argininosuccinate lyase is a newly identified autoantibody in liver disease and its clinical relevance remains unknown	[159]
24	Anti-calmodulin	Calmodulin	IgM 50.0 IgA 42.9	Anti-calmodulin autoantibodies neither associate with anti-SMA, ANA and AMA, nor with hyperglobulinemia. The clinic significant of anti-calmodulin is unclear	[160]
Gastroenteropathy-associated autoantibodies					
25	ASCA	Baker's yeast saccharomyces cerevisiae	24.2	ASCA serves as a serological marker of Crohn's disease, and has also been detected in other autoimmune disorders and in 5%-6.3% of blood donors. The prevalence of ASCA in AIH is 20%-30%, in AMA-negative PBC 44%. ASCA is common in PBC patients and correlates with higher level of circulating IgA. The prevalence of ASCA in PBC may be an indirect sign of enhanced mucosal immunity, but does not necessarily indicate concomitant inflammatory bowel disease	[161-163]
26	Anti-Galectin-3	Galectin-3, a member of -galactoside-binding lectins	30.0	Anti-Galectin-3 autoantibodies are primarily associated with Crohn's disease, and correlate negatively with disease activity. The significance of anti-Galectin-3 IgG autoantibodies in patients with PBC is unknown	[164]
27	Anti-tTG	Tissue transglutaminase	10.0-26.7	Anti-tTG autoantibody is mainly found in celiac disease. The prevalence of anti-tTG in PBC varies due to different types of substrate utilized in detection	[127,165,166]
28	AGA	Gliadin	16.0-21.0	Anti-gliadin antibodies are considered as the most reliable serological markers for celiac disease. They are also frequently seen in PBC, and IgA subclass of anti-gliadin antibodies are more pronounced in patients with Scheuer's stage Ⅲ-Ⅳ disease	[166,167]
Vasculitis-associated autoantibodies					
29	ANCA	Antigens including proteinase 3, myeloperoxidase, bactericidal/permeability-increasing protein, lactoferrin,human leukocyte elastase, cathepsin G, lysozyme, azurocidin, *etc.*	2-26	ANCAs are primarily associated with systemic vasculitides such as Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome	[76,131,168]
30	Anti-MPO	Myeloperoxidase	9.0	Predominantly in microscopic polyangiitis, necrotizing and crescentic glomerulonephritis, Churg-Strauss syndrome	[169]
31	Anti-PR3	Proteinase 3	3.0	Predominantly in Wegener's granulomatosis, and also detectable in microscopic polyangiitis, necrotizing and crescentic glomerulonephritis	[127]
32	Anti-LF	Lactoferrin	25.0-35.7	Are detected in several autoimmune disorders, such as Crohn's disease, SLE, systemic vasculitides. They are not specific markers for PBC	[170,171]
Thrombophilia-associated autoantibodies					
33	Anti-β2GP Ⅰ	β(2)-glycoprotein Ⅰ	IgG 2-15	Represent specific features of patients with antiphospholipid syndrome. Their presence in PBC often indicates severe disease and worse prognosis	[127,172,173]
34	Acl	Cardiolipin	IgG 27.3		
35	Anti-PS	Phosphatidylserine	IgM 75		
36	Anti-PT	Prothrombin	IgG 7		
37	Anti-PE	Phosphatydilethanolamine	IgG 5		
Diabetes mellitus-associated autoantibodies					
38	Anti-GAD	Glutamic acid decarboxylase	5.5	Anti-GAD occurs preferentially in the patients with type 1 diabetes. Clinical significance of Anti-GAD in PBC is unclear	[174]
39	Anti-SOX13	Transcriptional factor SOX13	18.0	SOX13 was initially identified in type 1 diabetes. The present of anti-SOX13 in PBC may merely indicate an immune response to products of damage to parenchymal tissue	[175]
Autoimmune thyroid diseases-associated autoantibodies					
40	Anti-TG	Thyroglobulin	54.5	Anti-TG, anti-TPO and anti-TR are markers of autoimmune thyroid diseases. Their significances in PBC are unknown	[176]
41	Anti-TPO	Thyroid peroxidase	45.5		
42	Anti-TR	TSH receptor	9.1		

Others autoantibodies					
43	Anti-CCP	Cyclic citrullinated peptide	2.7-4.0	Anti-CCP antibodies are highly specific for RA with sensitivity of 60%-70%. Presence of anti-CCP antibodies in PBC patients suggests RA overlap	[125,126,177]
44	Anti-ClpP	Microbial caseinolytic proteases P	30-47	ClpP is highly conserved among bacteria. Anti-ClpP in PBC suggests infection factors and molecular mimicry involved in the pathogenesis	[178,179]
45	Anti-β-subunit of bacterial RNA polymerase	β-subunit of bacterial RNA-polymerase	32.8	These autoantibodies in PBC, suggest bacterial triggers of PBC	[180]
46	Anti-EPO	Eosinophil peroxidase	52.5	PBC patients with positive anti-EPO antibodies have less peripheral eosinophils	[181]
47	Anti-p53	Nuclear protein of 53 kDa that regulates cell proliferation and apoptosis	8.0	Anti-p53 autoantibodies are commonly seen in malignancies and organ-specific autoimmune diseases such as type 1 diabetes, thyroid diseases, PBC and AIH	[182]
48	Anti-acetylcholine receptor	Nicotinic acetylcholine receptor	58.8-74.0	Anti-acetylcholine receptor antibodies are primarily associated with myasthenia gravis, though PBC patients with positive anti-acetylcholine receptor antibodies do not have clinical symptoms of myasthenia	[169,183,184]
49	Anti-CA Ⅱ	Carbonic anhydrase Ⅱ	18-31	Anti-CA Ⅱ antibody is likely a nonspecific marker of autoimmunity. It has been detected in a variety of autoimmune diseases, including Graves' disease, type 1 diabetes, SS, SLE, AIH and PBC. In cases of PBC, no significant correlation has been found between anti-CA Ⅱ antibody and AMA	[185-189]
50	Anti-α enolase	α-enolase	28.6	Anti-α-enolase antibodies present in a variety of inflammatory and autoimmune disorders, such as SLE, IBD, RA and AIH, and are not likely to be specific markers for any disease. They might be involved in destruction of biliary epithelium and are associated with hepatic failure	[190-195]
51	Anti-HSP	Heat shock proteins	45.7	Enhanced biliary expression of heat shock protein is found in PBC. Anti-HSPs are common in PBC, and are related to titers of AMA. They might cross-react with the main mitochondrial antigens in PBC	[196-199]
52	Anti-FKBP12	FK506 binding protein 12	44.4	The significance of anti-FKBP12 antibodies in PBC is unclear	[200]

SLA: Soluble liver antigen; PBC: Primary biliary cirrhosis; AMA: Antimitochondrial antibodies; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis; ANA: Antinuclear antibodies.

disease, defined as the presence of cirrhosis or its complications. In addition, it has been reported that anti-p62-positive patients have higher levels of serum bilirubin and more marked inflammatory infiltrates on liver biopsy[87].

Antinuclear envelope antibodies (Lamin and Lamin B receptor)

The nuclear envelope is a bilayered membranous structure that can be divided into five distinct components: the inner nuclear membrane, having a distinct set of integral membrane proteins; the outer nuclear membrane; a perinuclear space, which is continuous with the lumen of the endoplasmic reticulum; the pore domains, regions where the inner nuclear membrane and outer nuclear membrane come together and fuse; and an underlying nuclear lamina, containing the nuclear lamins[103]. A smooth membrane fluorescence pattern is characteristic of the presence of antibodies to nuclear lamins in IIF using sera from PBC patients. Three subtypes of anti-lamin antibodies have been described: anti-lamin A, B and C[102,104-106]. Anti-lamin antibodies do not seem to be disease-specific as they are found in patients with several different autoimmune disorders, such as SLE, chronic fatigue syndrome, and PBC[107-110]. Anti-lamin A, B and C antibodies are detected with frequencies of 6%-8% in sera from patients with PBC. The usual scenario is to find anti-lamin A and C together, and less frequently either anti-lamin B alone or all three in the same patient[111].

Lamin B receptor (LBR) is a protein integral to the inner nuclear membrane with a nucleoplasmic, amino-terminal domain of 208 amino acids, followed by a carboxyl-terminal domain with eight putative transmembrane segments. Anti-LBR antibodies from PBC patients recognize the nucleoplasmic, amino-terminal domain but not the carboxyl-terminal domain. Anti-LBR antibodies appear to be highly specific for PBC, but their clinical significance is unclear. The prevalence of anti-LBR antibodies in PBC is approximately 2%-6%[76,102,112,113].

Anti-centromere antibodies

Anti-centromere antibodies (ACA) are important diagnostic markers of systemic sclerosis (SSc), found in about 25% of these patients[114]. In patients with CREST syndrome or limited cutaneous SSc, the positivity rises to 50%-90%. ACA in SSc are usually associated with a good prognosis, though they are not specific for SSc. ACA can be detected in patients with other rheumatic diseases including pSS, SLE and PBC (about 30%)[115-120]. It is of interest to note that several subtypes of ACA have been identified, including anti-CENP-A, anti-CENP-B, anti-CENP-C and anti-CENP-O antibodies[121]. Research during the past several years has found that prevalence of the ACA subtypes

differs among various autoimmune diseases[122]. Recent studies have demonstrated that ACA positivity in patients with PBC is of significant predictive value for progression to portal hypertension[123,124].

OTHER AUTOANTIBODIES DETECTED IN PBC

Although extensive research has focused on AMA, it is of interest to note that, to date, more than sixty different autoantibodies have been found in PBC patients. Some target at nuclear or cytoplasmic molecules and cell membranes, while others react with lipid components. Some, like AMA, occur frequently and almost universally in PBC, while others, like anti-lamin and anti-LBR, are present in only a few patients. It should be noted that among these autoantibodies, some are not specific for any disease, and some are thought to be more closely related to other autoimmune diseases, such as anti-CCP which is relatively specific for rheumatoid arthritis[125,126]. Prevalence and properties of these autoantibodies in PBC are summarized in Table 1.

CONCLUSION

The presence of serum autoantibodies is characteristic of PBC, and is useful in the clinical diagnostic process in combination with histology and imaging studies. Numerous autoantibodies are found in sera from patients with PBC. This suggests that the development of PBC is a multi-factorial process. With growing numbers of clinical studies of autoimmune diseases and extensive application of more sensitive testing methods for antibodies, it has gradually been realized that the association between an individual autoantibody and autoimmune disease is not as specific as previously thought. AMA is very sensitive and anti-gp210 and anti-sp100 are highly specific for PBC. Other antibodies found in PBC, such as ACA, ASCA, ANCA and anti-sm, could also be found in other autoimmune diseases[131,161,162,168]. Although some autoantibodies are believed to be associated with the pathogenesis of PBC, these associations are likely to be extremely complicated and surely exert complex effects in many different ways. It is hard to understand these delicate associations based on our current knowledge of PBC, and further advanced studies are required to elucidate the pathogenesis of this autoimmune disease.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3630

World J Gastroenterol 2010 August 7; 16(29): 3630-3637
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Peter V Draganov, Dr., MD, Associate Professor, *Series Editor*

Colonoscopic polypectomy and associated techniques

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Received: April 4, 2010 Revised: May 24, 2010
Accepted: May 31, 2010
Published online: August 7, 2010

Abstract

Polypectomy of colonic polyps has been shown to reduce the risk of colon cancer development and is considered a fundamental skill for all endoscopists who perform colonoscopy. A variety of polypectomy techniques and devices are available, and their use can vary greatly based on local availability and preferences. In general, cold forceps and cold snare have been the polypectomy methods of choice for smaller polyps, and hot snare has been the method of choice for larger polyps. The use of hot forceps has mostly fallen out of favor. Polypectomy for difficult to remove polyps may require the use of special devices and advanced techniques and has continued to evolve. As a result, the vast majority of polyps today can be removed endoscopically. Since electrocautery is frequently used for polypectomy, endoscopists should be thoroughly familiar with the basic principles of electrosurgery as it pertains to polypectomy. Tattooing of a polypectomy site is an important adjunct to polypectomy and can greatly facilitate future surgery or endoscopic surveillance. The two most common post-polypectomy complications are bleeding and perforation. Their incidence can be decreased with the use of meticulous polypectomy techniques and the application of some prophylactic maneuvers. This review will examine the technique of polypectomy and its complications from the perspective of the practicing gastroenterologist.

Key words: Colonic polyp; Polypectomy; Colonoscopy; Polypectomy technique; Complications

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Fyock CJ, Draganov PV. Colonoscopic polypectomy and associated techniques. *World J Gastroenterol* 2010; 16(29): 3630-3637 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3630.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3630

INTRODUCTION

Polypectomy is a fundamental skill utilized by all endoscopists who perform colonoscopy. Mastery of polypectomy is difficult and requires both significant experience and study. It is clear that polypectomy is efficacious in reducing the risk of colon cancer development by interrupting the adenoma to carcinoma progression[1,2]. Endoscopic techniques used in colonoscopic polypectomy continue to evolve, and it is important for all endoscopists to be familiar with these concepts.

Decision making about how to perform polypectomy is often made during colonoscopy when a polyp is detected. A general rule is that all potential adenomas should be removed. The endoscopic appearance of a polyp is often not necessarily a good indicator of its histologic nature. While as many as 70% of diminutive polyps (less

than 5 mm) may be adenomas, the risk of any particular polyp containing malignancy increases with the size of the polyp[3-6]. The method chosen for polypectomy is often related to the appearance and size of the polyp. Polyps are usually described as being pedunculated, sessile or flat. The risk of a polyp 2 cm in size or larger being malignant is greater than 10%[7]. Some polyps blur the lines though, by not falling into these strict categories. Nevertheless, consideration of polyp characteristics is helpful in determining the best approach to polypectomy[8].

COLD FORCEPS POLYPECTOMY

The simplest method for polypectomy is cold forceps removal. A survey of common practices among gastroenterologists found that cold forceps polypectomy was the technique of choice for small polyps, particularly polyps 1 to 3 mm in size[9]. In slightly larger polyps, jumbo forceps could be considered. Cold forceps can easily grasp small polyps that otherwise might be too small to snare.

After passing the forceps through the channel, the forceps and the scope can be manipulated in order to grasp as much polyp tissue as possible. Turning the scope to bring the polyp to the five to seven o'clock position can be useful since that is the position at which the forceps exit the endoscope channel. After closing the forceps on the polyp, a gentle pull on the wire removes the bite of polyp from the colon mucosa. The area is examined to determine if further bites are necessary to complete polyp excision.

Advantages to cold forceps polypectomy include avoiding risk associated with electrocautery and an almost negligible risk of colonic perforation[10]. One challenge associated with cold forceps polypectomy is that after the initial bite, minor bleeding can obscure the polypectomy field increasing the risk of leaving residual polyp behind[11].

HOT FORCEPS POLYPECTOMY

Hot forceps polypectomy is another option for small polyps. Hot forceps polypectomy is similar to cold forceps except it uses electrocautery to try to destroy residual polyp tissue intentionally left behind[12]. In hot forceps polypectomy, only the tip of the polyp is grabbed in the forceps. The small polyp is pulled into the colon lumen to create a tent-like effect and electrocautery is applied to destroy the polyp base while preserving the polyp tissue inside the forceps as a histological specimen[13].

Over the years, the use of hot forceps has fallen out of favor. One randomized study by Ellis looked at 72 polyps 6 mm or less in size and found that hot forceps still left residual polyp tissue behind 22% of the time compared to only 5%-14% of the time with either cold or hot snare[14]. Another study by Peluso retrospectively looked at 62 hot forceps polypectomies for polyps 3-6 mm in size and found that 17% of the time residual polyp tissue remained on follow-up endoscopic exam 1 to 2 wk later[15]. Cold forceps and snare polypectomy have been described as having a 16% residual polyp rate which suggests that hot forceps are either no better or even worse than other accepted methods of polyp removal. Hot forceps may still be useful though for small polyps that have a tip easily grasped with forceps but a polyp base that is hard to reach yet could still be destroyed with application of electrocautery.

SNARE POLYPECTOMY

Snare polypectomy was found to be the preferred method for removal of polyps 1 cm or greater in size in a survey of common gastroenterology practices[9]. A snare is a self-contained metal ring that is opened over the polyp and then closed entrapping polyp tissue for resection by closing the ring. Before pulling the snare out of the scope, the polyp should be brought to the six o'clock position. Sometimes advancing the snare proximal to (beyond) the polyp is useful if the polyp is behind a fold or inclined to flop out of the opened snare. The snare can also be used to position a pedunculated polyp in such a position as is more amenable to capturing once the snare is opened. Once the polyp is captured in the snare, the snare plastic sheath should be advanced moving the polyp away from the scope tip if electrocautery is to be used to avoid electrical damage to the scope. When snaring a pedunculated polyp, the snare should be placed about half way up the stalk, so that after cutting, a stalk remnant is left which can be grabbed or clipped if hemorrhage occurs. The polyp is pulled away from its base into the lumen tenting the colon wall to avoid burning the adjacent deep colon layers[11].

A snare can be either hot or cold in that it can be supplemented with electrocautery or not. During hot snaring, the endoscopist's assistant should close the snare slowly and gently. If the snare is too tight prior to electrocautery application, it could result in inadvertent cold cutting the polyp, resulting in bleeding from the stalk or in the snare becoming entrapped into coagulated tissue in the stalk[16]. Once the snare is in position, a few seconds of electrocautery can be applied if opted for, and then the endoscopist instructs the assistant to cut through the polyp.

There are many different types of snares each with specific advantages which can be chosen depending on the situation. Oval and hexagonal snares are most commonly used. We suggest using a barbed snare for hard to grab tissue as can be the case in flat or sessile polyps or when the snare slipping off the polyp seems to be a problem. Crescent snares are often used in EMR. A rotatable snare is useful when initially the snare comes out of the scope in such a way that is not optimal for snaring the polyp and it is desirable to rotate the snare to an angle that is better for capturing the polyp. A mini-snare can be used for cold snaring smaller polyps or to remove a small amount of residual tissue after piecemeal polypectomy[17]. There is a combination snare-injection needle which allows for quick injection prior to opening the snare and avoids having to change out an injection needle wire for

the snare (i-Snare system, US endoscopy, Mentor, Ohio, USA)[18].

ELECTROCAUTERY

The purpose of electrocautery in polypectomy is to either provide extra power in cutting tissue or to prevent bleeding by coagulation of tissue. The basic principle in electrocautery is that if enough electrical current is delivered, heat will be generated to cause cellular bursting leading to tissue cutting. If somewhat less heat is generated then cell shrinkage leading to tissue coagulation occurs. Even pure cut current causes some coagulation, and pure coagulation current has some cutting property. Snares and hot forceps use monopolar electrocautery, which means that the electrical circuit runs through the patient body to a grounding pad placed on the patient. Cautery probes can also use bipolar electrocautery, which means that the electrical circuit runs between two electrodes both located on the tip of the probe. Energy deliverance is also proportional to the time it is applied, so the length of time the endoscopist keeps their foot on the pedal is very important[16]. The use of coagulation current has been associated with more delayed post-polypectomy hemorrhage, whereas the use of cutting and blended current have been associated with more immediate hemorrhage[19]. A review of electrocautery by Morris suggests using coagulation at a setting of 20 Watts for hot snaring. Since cut has also been associated with a higher risk of perforation, we suggest first using coagulation for standard colonoscopic snare polypectomy. Then after using coagulation, the endoscopist can consider using some cut function next if the polyp has a thick stock and coagulation alone is unable to cut through it or in the case that the snare becomes entrapped on the polyp stock. For hot forceps electrocautery coagulation at 10-20 Watts can be used[16]. Most modern electrosurgical units have preset polypectomy settings.

LARGE POLYPS

In the past, large polyps often required surgery for removal, but now many can be managed endoscopically[20]. Endoscopic Mucosal Resection (EMR) can be performed on sessile polyps 2 cm in size or larger. EMR involves submucosal injection (often of saline) creating a cushion for the polyp and then hot snaring the polyp either *en bloc* (all together) or piecemeal (multiple snarings). EMR can provide resection down to the muscularis propria[21-23]. There is no official distinction between saline assisted piece meal polypectomy and EMR but typically the term polypectomy is reserved for removal of flat lesions measuring less than 2 cm and the term EMR is used for larger lesions[4,24]. Endoscopic Submucosal Dissection aims to remove all dysplastic tissue en-block as one piece rather than the piecemeal technique that is used with saline assisted polypectomy and EMR[25]. Large polyps are often adenomatous, therefore complete resection is the goal even though it is often time-consuming. Iishi found that 55% of polyps resected in piecemeal fashion required further resection on a repeat colonoscopy, but complete resection was possible in 83% of polyps after up to three repeat colonoscopies[26]. Flat and sessile polyps can be challenging to snare as they are often level with the colon floor. The first piece of tissue snared can leave divots or ledges in the remaining polyp that can make it more easily grabbed in subsequent snares. If residual polyp tissue is left after piecemeal polypectomy, argon plasma coagulation (APC) can be used to tryw to destroy the residual tissue[27]. After any piecemeal polypectomy, the site should be re-examined in 2 to 6 mo to evaluate for any residual polyp tissue[7,8].

POLYP RETRIEVAL

Once polyp tissue is snared, actually retrieving it can be challenging. Many endoscopists periodically experience the frustration of successfully snaring a large adenoma-appearing polyp only for it to fall out of view or get lost in the colon somewhere[28]. However, even experienced endoscopists may fail to retrieve polyp tissue up to 16% of the time[8]. Possibly the most common way to retrieve a polyp once it is snared is to drive the scope up to the polyp in the six o'clock position and then to suction the polyp through the scope into a trap, using a back flush if needed. If the polyp is too big to be suctioned into the scope, the snare can be used to cut the large polyp into pieces small enough to fit through the suction channel. Polyp tissue can also be grabbed with forceps while the entire colonoscope is withdrawn. In these cases the forceps can be advanced out a few centimeters so that simultaneous examination of the remaining colon can be performed while the specimen is kept in view. A Roth net can be used to remove large polyps or several polyp fragments at once. Also, an overtube can be used for easy repeated colonoscopic intubation to the polypectomy site with repeated removal of polyp fragments[29,30].

RESIDUAL POLYP TISSUE

Leaving residual polyp tissue behind leaves behind cells that may continue to progress through the adenoma to carcinoma sequence, therefore the purpose of polypectomy is to break that sequence. Risk of residual polyp tissue is often the outcome measured in studies comparing different methods of polypectomy, such as snare *vs* forceps. In an observational study, Tappero *et al*[17] found that a snare never left behind residual polyp tissue but cold forceps often did. Zlatanic found that for treating residual tissue, piecemeal polypectomy left behind residual tissue 46% of the time, APC destruction still left residual tissue 50% of the time, and doing nothing left behind residual polyp obviously 100% of the time[24].

THE CHALLENGING POLYPECTOMY

Some polyps provide distinct challenges that call for utilizing other approaches than just standard polypectomy techniques. Endoscopists periodically find polyps that

are very difficult to remove. These can include polyps that are located behind colon folds, polyps that are very large, polyps that are just out of reach, and flat, carpeted, or thick polyps. For polyps hiding behind folds and large pedunculated polyps, Valentine *et al*[31] described a technique using a double channel therapeutic endoscope. A tripronged grasper is advanced *via* one of the channels to pull the polyp into better view and into the snare, while a snare for polypectomy is inserted through the other channel. A standard upper endoscope can also be considered for difficult to reach polyps as it has a tip with a tighter bending angle than a colonoscope[32]. A side viewing scope can be used for polyps that are behind folds or on a side of the colon wall unable to be reached by a standard colonoscope. Friedland[33] described either retroflexing the colonoscope or injecting a large amount of saline proximal to the lesion as options to try to reach polyps on the inside wall of tight turns. Even two different scopes manipulated by two endoscopists can be attempted with one scope grabbing the polyp and pulling it into a convenient location while the other scope performs polypectomy has been described[8]. Colon spasms can present a challenge by constantly moving the polyp in and out of view, and glucagon can be given intravenously to decrease these spasms[8]. Some polyps may not be amenable to endoscopic polypectomy and are better served with surgery. If a large polyp is in the cecum, extends into the ileocecal valve, or extends into the appendix, surgery may need to be considered. Also polyps that involve more than 30% of the colon circumference are often impossible to remove endoscopically[11].

Injection

An important related tool to consider for polypectomy is injection with either saline or epinephrine (1:10000) into the polyp base or stalk. The submucosa is the target location for fluid deposit, so the endoscopist should try not to penetrate the colon wall with the needle. Injected fluid can diffuse fast, so sometimes repeat injections are needed. Injection is suggested in the literature for larger polyps specifically. Most studies looking at resection of large or giant polyps include epinephrine injection in their polypectomy protocol. Injection can lift up flatter polyps rendering them more polypoid and more amenable to snare polypectomy and complete resection[34]. The injected fluid may also serve as a safety cushion by increasing the distance between the mucosa and the muscle layer and serosa, thereby at least theoretically decreasing risk of perforation[21,35]. If a polyp does not lift with an appropriate injection technique it may be caused by an underlying cancer extending to deeper colon layers. Pedunculated polyps with large stalks are more inclined to bleeding. Injecting these large stalks before snare polypectomy may provide prophylactic hemostasis and reduce the risk of a post-polypectomy bleed. Epinephrine is a potent vasoconstrictor, and both saline and epinephrine can exert a tamponade effect on blood vessels[36]. A study by Dobrowolski randomized 100 polyps to either epinephrine injection or no injection and found one post-polypectomy bleed in the injection group compared to 8 bleeds in the no injection group[37].

Endoloops

In addition to injection, another option for prevention of post-polypectomy bleed is an endoloop[38]. The endoloop is a detachable oval-shaped nylon snare. It is deployed in the same way as a standard snare but then tightened and released around the stalk or base of the polyp prior to polypectomy. A gastroenterology survey showed that 38% of endoscopists report using endoloops[9]. A trial done in Greece by Kouklakis randomized 64 patients with polyps greater than 2 cm in size to get either epinephrine injection or a combination of endoloop and endoclip placement. The combination endoloop and clip group did significantly better with only 3% post-polypectomy hemorrhages compared to the epinephrine group which had a 12% rate of post-polypectomy hemorrhage[39]. The Di Giorgio study found a lower rate of post-polypectomy bleed at 1.8% with a detachable snare compared to 3% for epinephrine injection and 8% for no prevention[40]. In 152 snare polypectomies, Paspatis *et al*[41] found that combination epinephrine injection with endoloop placement was associated with only a 1% rate of delayed bleeding whereas epinephrine used alone was associated with an 11% rate of delayed bleeding. However many problems with endoloops such as slipping off the polyp stalk, inadequate tightening, and persistence of bleeding despite endoloop placement were described in a retrospective study by Matsushita *et al*[42].

Tattooing

Large or polyps suspicious for invasive cancer should be considered for tattooing for easier future localization either by a surgeon during colectomy or by an endoscopist during future surveillance colonoscopy[43,44]. Endoclip placement and inter-operative colonoscopy are other ways to re-identify a lesion, however the endoclips can slip off prior to surgery, and inter-operative colonoscopy can be cumbersome and time-consuming. India ink is the preferred identification agent for tattooing polyps[45] because the ink is phagocytosed by macrophages giving the site an almost permanent easily detected marking. Other dyes like indigo carmine and methylene blue are too rapidly resorbed to be useful. Commercially available India ink is a sterile carbon based dye suspended in stabilizing particles and diluted in normal saline to a 1:100 concentration[46]. India ink is injected through an injection needle and targeted to the submucosal layer of the inter-haustral folds. Common practice is to place a tattoo on more than one side of the lesion in either a two or a four quadrant manner. Injecting at an oblique angle tangential to the colon wall can avoid penetration of the colon wall which can result in inflammation and a diffuse staining of the peritoneum thereby obscuring the surgeon's view during operation[44,47]. To ensure proper ink placement, a double injection technique has been described in which 1 mL of saline is first injected creating a submucosal bleb[48]. Once the

saline bleb is made, the needle is left in place, the saline syringe is changed to an India ink syringe and about 0.1 to 2 mL of tattoo ink is then injected into the bleb space[49,50]. After tattooing the polyp site, the endoscopist should also include in the report the distance of the site from the anal verge in centimeters to aid in future localization.

ENHANCED POLYP DETECTION AND CLASSIFICATION TECHNIQUES

Standard colonoscopy based on white light may have a polyp miss rate of anywhere from 1% to 26%[51]. Also distinguishing truly neoplastic lesions from normal or benign tissue endoscopically can be challenging. Potentially unnecessary biopsies require pathologic evaluation leading to increased costs, so one advantage of enhanced detection techniques includes avoiding this increased cost[52]. Some newer modes of enhanced polyp detection and classification have been developed over the last few years. High definition colonoscopy, chromoendoscopy, and narrow band imaging (NBI) are useful to enhance polyp detection. Confocal Laser Endoscopy, and spectroscopic colonoscopy are more for enhancing polyp classification.

High definition colonoscopy (complete system can cost $215 000 from Olympus America, Center Valley, PA, USA) provides an image containing more pixels and better picture quality than standard definition colonoscopy. One retrospective study by Buchner showed a significantly higher polyp and adenoma detection rate (4%-5% increase in yield) with high definition colonoscopy compared to standard definition colonoscopy for polyps less than 1 cm and in the left colon[53].

Another enhanced detection technique, chromoendoscopy, uses indigo carmine (25 g costs about $40) that is flushed over the colonic mucosa to demarcate polyp architecture, vascular pattern and pit detail. This can highlight subtle differences between normal colonic tissue and polyp tissue making polyp detection easier. NBI, a type of virtual chromoendoscopy, is another enhanced mode that uses special narrow band filters to enhance surface and vascular pattern appearance of potential polyps. NBI may be useful for distinguishing between hyperplastic and adenomatous polyps as well. One study from Japan looked at NBI in the evaluation of 617 colorectal lesions and reported a sensitivity of 90.9% and a specificity of 97.1% for differentiating non-neoplastic from hyperplastic lesions[54]. Round and stellate pit patterns represent benign lesions, and villiform, gyrus-like, and irregular patterns represent neoplastic lesions. Many standard colonoscopies now have NBI capability (which means no additional cost to patients when it is used) which is activated by pushing a button on the head of the scope[55,56].

A new spectroscopic probe (not commercially available yet) has been developed that detects the increased microvascular blood supply in normal tissue at the periphery of a polyp that may be unseen or behind a fold. This alerts the endoscopist "like a metal detector going off" to examine the nearby mucosa more carefully to find the polyp thereby increasing detection[57].

Confocal laser endoscopy (CLE) is an enhanced mode of polyp classification (Cellvizio, Paris, France). Once a potential polyp is endoscopically detected, the lesion is focused on for analysis to determine if it is benign or neoplastic. Thousands of optical fibers bundled together take 12 pictures per second and provide image resolution detailed to the micron level. Pit pattern, crypt architecture, and vascular patters are analyzed; and irregular vessels, presence of mucin and increased tissue density indicate a neoplastic lesion. CLE is either integrated into the scope or used as a separate probe passed through the accessory channel[58]. A study from Mayo Jacksonville found CLE to have a sensitivity of 76% and a specificity of 72% in differentiating non-neoplastic from neoplastic lesions. Interobserver agreement over what the images represented was found to be 78%[59].

HEMORRHAGE

Even though the benefit of polypectomy is significant in terms of reducing the risk of colon cancer development, polypectomy is not without some risk of complications. Most complications are related either to post-polypectomy hemorrhage or perforation. Hemorrhage is the most common and is usually divided into immediate (less than 12 h post-procedure) and delayed (after 12 h post-procedure but up to 30 d). There is a greater risk of immediate hemorrhage associated with cut or blended electrocautery and a greater risk of delayed hemorrhage with the use of coagulation current. These specific risks should be appreciated and weighed when choosing electrocautery type.

Dobrowolski *et al*[60] noted that the risk of post-polypectomy hemorrhage ranges from 0.3% to 6% but can be as high as 24% in large polyps. He found that hemorrhage was more likely in polyps larger than 17 mm, pedunculated polyps with stalks thicker than 5 mm, sessile polyps, and malignant polyps. Watabe found that hypertension also puts patients at risk for a delayed post-polypectomy hemorrhage[61].

Immediate hemorrhages are frequently noticed during colonoscopic examination as bleeding from the polypectomy site is directly visualized. In these cases, either epinephrine injection into the base of the polypectomy site or endoclip placement is often considered as first line hemostatic therapy. Endoloop placement can also be considered and applied either to a stalk or to a larger polypectomy base for hemostasis. If snaring a pedunculated polyp results in a visibly bleeding stalk, sometimes grasping the stalk with the snare and holding pressure for 5 min can stop the hemorrhage[36].

Endoclips can be placed onto a bleeding residual stalk or empirically placed just lateral to the polypectomy site to tamponade any supplying blood vessels[62]. Endoclips can also be placed prophylactically at the polypectomy site after removal of the polyp. A group in Spain looked retrospectively at 34 polypectomies using endo-

clips either before or after resection of polyps 15-40 mm in size with stalks 5-12 mm in thickness. They found that all episodes of bleeding could be controlled with the use of endoclips. They also found that the clips easily catch stalks around 5 mm in thickness but that two clips could be placed on stalks thicker than that[63].

Friedland *et al*[64] described performing polypectomy on polyps less than 1 cm in size in actively anticoagulated patients. He placed endoclips prophylactically at the polypectomy site and had no more incidence of post-polypectomy bleed than in non-anticoagulated patients.

Many forceps polypectomies result in some minor oozing from capillaries at the polypectomy site, and this is usually self-limited and resolves after continued visualization. Delayed hemorrhage can require hospitalization, blood transfusion, and repeat colonoscopy for definitive hemostasis.

PERFORATION

Perforation is a serious complication that can result from polypectomy and can often have major clinical ramifications for the patient after the procedure is over[65]. Factors contributing to perforation include mechanical stress from the scope, barotrauma, electrocautery, and the depth of the polyp resection itself. The risk of perforation with all colonoscopies has been estimated somewhere around 1 perforation per 1000 to 2000 colonoscopies[66-68]. Risk of perforation however increases in polypectomies involving longer electrocautery time, removal of larger polyps, location in the cecum, and large sessile polyps requiring piecemeal removal.

If a perforation is visualized during the procedure itself, the endoscopist can consider an attempt at closure with endoclips. The progress of Natural Orifice Translumenal Endoscopic Surgery research has highlighted the reality of closing a perforation endoscopically with endoclips[69]. However, emergency computed tomography imaging, antibiotic administration, bowel rest and surgical consultation still play an important role. Unfortunately, approximately 5% of perforations result in patient death[11].

Similar to perforation but less serious is post-polypectomy syndrome, another complication where there is a transmural burn not resulting in perforation. Post-polypectomy syndrome presents with leukocytosis, fever and abdominal pain in the absence of free air on imaging. Treatment of post-polypectomy syndrome is usually conservative involving antibiotics, fluids, and bowel rest[8].

CONCLUSION

In summary, colonoscopic polypectomy is a continuously evolving therapy that has been remarkable at reducing the risk of colorectal cancer. Gastroenterologists must be thoughtful and proficient in techniques such as snaring, injection, tattooing, and all other tools related to polypectomy for endoscopic success. Cold forceps seem to be preferred for small polyps and snares for larger. Coagulation current may be the electrocautery mode of choice for polypectomy, although it is associated with higher risk of delayed hemorrhage. Difficult to reach polyps continue to require various endoscopic tricks and an ability to improvise for successful resection. There are several options for prevention of bleeding in large polyps including injection, endoloops, and endoclips. Many complications can actually be managed endoscopically. On the research stage, there is still a shortage of studies about many specific aspects of polypectomy, and there is a significant need for more quality studies in the future.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3638

World J Gastroenterol 2010 August 7; 16(29): 3638-3641
ISSN 1007-9327 (print)

GUIDELINES FOR BASIC SCIENCE

Interleukin-21 triggers effector cell responses in the gut

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Received: April 2, 2010 Revised: April 22, 2010
Accepted: April 29, 2010
Published online: August 7, 2010

Abstract

In the gut of patients with Crohn's disease and patients with ulcerative colitis, the major forms of inflammatory bowel diseases (IBD) in humans, the tissue-damaging immune response is mediated by an active cross-talk between immune and non-immune cells. Accumulating evidence indicates also that cytokines produced by these cells play a major role in initiating and shaping this pathologic process. One such cytokine seems to be interleukin (IL)-21, a member of the common γ-chain-receptor family. IL-21 is produced in excess in the inflamed intestine of patients with IBD mostly by activated CD4+ T helper cells co-expressing interferon-γ and follicular T helper cells. Moreover, both *in vitro* and *in vivo* studies indicate that excessive IL-21 production leads to the activation of multiple signaling pathways that expand and sustain the ongoing mucosal inflammation. In this article, we review the available data supporting the pathogenic role of IL-21 in IBD.

Key words: Interleukin-21; Gut; T cells; Epithelial cells; Fibroblasts

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De Nitto D, Sarra M, Pallone F, Monteleone G. Interleukin-21 triggers effector cell responses in the gut. *World J Gastroenterol* 2010; 16(29): 3638-3641 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3638.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3638

INTRODUCTION

The organized lymphoid tissue of the gastrointestinal tract contains large numbers of immune cells that are deputed both to protect from infectious diseases and to evoke immune tolerance[1,2]. Perturbations in this delicately balanced microenvironment can promote the collapse of tolerance, thus leading to chronic inflammation that alters the integrity and function of the gut[3]. This occurs for example in patients with Crohn's disease (CD) and patients with ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD) in humans[4]. In both these conditions, the tissue damage is mediated by an excessive and poorly controlled immune-inflammatory reaction directed against components of the normal bacterial flora[5,6]. Evidence also indicates that an active and dynamic interplay between immune and non-immune cells plays a major role in initiating and shaping this pathologic process, and that cytokines are essential mediators of this cross-talk[7-9]. One such cytokine seems to be interleukin (IL)-21, a product of activated CD4+ T helper (Th) cells, follicular Th cells (TFH), and Natural killer (NK) T cells, which exerts regulatory effects on multiple cell types[10-13]. In this article, we review the available data supporting the pathogenic role of IL-21 in IBD.

IL-21 IS MADE BY TH1 CELLS AND FOLLICULAR TH CELLS IN THE HUMAN GUT

Initial studies conducted in our laboratory showed that IL-21 protein is over-produced in the inflamed gut of

patients with CD and patients with UC as compared to normal controls[14]. These data were confirmed by a recent study showing that IL-21 mRNA expression is increased in rectal mucosa from patients with active UC compared to UC patients in remission and healthy controls and that, in UC, IL-21 gene expression correlates with histological activity of the disease[15]. A genome-wide association study for IBD has identified risk variants in the chromosomal 4q27 region harbouring the *IL-2* and *IL-21* genes, suggesting that polymorphism(s) in this region might contribute to regulation of IL-21 production/function[16-18]. However, it is noteworthy that expression of IL-21 in the uninflamed mucosa of IBD patients does not differ from that seen in the normal colonic mucosa, and that peripheral blood T cells isolated from IBD patients and healthy controls express similar levels of IL-21[14]. Therefore, it is plausible that up-regulation of IL-21 in IBD is strictly linked to the ongoing mucosal inflammation.

In both CD and UC, IL-21 is made by CD4+ but not CD8+ T cells[14]. By flow-cytometry it was also shown that the majority of IL-21-producing CD4+ T-LPL co-express interferon (IFN)-γ, and to a lesser extent IL-17A, supporting the hypothesis that, in IBD, IL-21 is preferentially made by Th1 rather than Th17 cells[19]. At the present time, it remains unclear how IL-21-positive cells co-expressing IFN-γ differentiate in the human gut. Since Th1 cells are abundant in the human gut, and particularly in CD mucosa[20-22], it is conceivable that Th1 cells can acquire the ability to make IL-21 in response to specific stimuli. Indeed, we have recently shown that *in vitro* stimulation of intestinal lamina propria (LP) CD4+ T cells with IL-12, the major inducer of Th1 cell response, enhances the fraction of cells producing IL-21 or both IL-21 and IFN-γ[19].

IL-21 is also produced by TFH cells in the human gut, and the fraction of IL-21-producing TFH cells is significantly higher in CD than in UC and controls[19]. Interestingly, activation of mucosal T cells with IL-12 leads to enhanced production of IL-21 by TFH cells[19], thus confirming that IL-12-driven signals positively regulate IL-21 production in the gut.

IL-21 ENHANCES INFLAMMATORY PATHWAYS IN THE GUT

A large body of evidence supports the concept that excessive production of IL-21 in the gut has deleterious consequences for the host. IL-21 is highly produced in the gut of wild-type mice with dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid-relapsing (TNBS)-induced colitis[23]. Notably, IL-21-deficient mice are largely protected against disease in both models[23]. Amelioration of both DSS- and TNBS-induced colitis in IL-21-knockout mice is associated with a marked decrease in Th17-related molecules, such as IL-17 and IL-17F. Administration of IL-21R/Fc, a fusion protein that binds to IL-21 and prevents it activating cell-surface receptors, in wild-type mice attenuates DSS-colitis, confirming the pro-inflammatory role of IL-21 in this model[23]. A similar scenario emerges from studies in human IBD[19]. Stimulation of intestinal mucosal T cells with IL-21 results in enhanced activation of transcription factors (i.e. Stat3, Stat4 and T-bet) and marked synthesis of IFN-γ and IL-21 itself[14]. Moreover, treatment of CD mucosal cells with IL-21R/Fc reduces Stat4 and T-bet and inhibits IFN-γ production. Neutralization of IL-21 in CD mucosal cell cultures leads also to a decreased expression of IL-17A[23]. Taken together these data indicate that IL-21 is able to expand Th1 and Th17 cell responses in the gut, even though further experimentation is needed to elucidate the basic mechanism by which IL-21 exerts these regulatory effects.

Initially described as an important regulator of the function of immune cells[24,25], IL-21 has been recently shown to also regulate the activity of non-immune cells. Gut myofibroblasts and epithelial cells express constitutively IL-21R and are able to respond to IL-21[26]. In particular, stimulation of colonic myofibroblasts with IL-21 enhances the synthesis of matrix metalloproteinases (MMPs)[26], a family of proteases that are supposed to participate in the tissue damage and remodelling occurring in IBD[27,28]. The IL-21-driven induction of MMPs can be potentiated by tumor necrosis factor α, and associates with no change in the production of tissue inhibitors of MMPs[26]. Regulation of MMPs by IL-21 does not however seem to occur at the transcriptional level, because stimulation of fibroblasts with IL-21 does not alter the MMP RNA expression[26]. Additionally, the intracellular level of MMP proteins is not increased by IL-21, and the IL-21-induced MMP synthesis is not affected by inhibitors of gene transcription and *de novo* protein synthesis[26]. Therefore, it is plausible that IL-21 preferentially increases the secretion of either pre-constituted or newly synthesized MMPs. The *in vivo* relevance of these findings relates to the demonstration that supernatants of CD mucosal cells induce myofibroblasts to secrete MMP and this is partially inhibitable by IL-21R/Fc[26].

IL-21 induces activation of mitogen activated protein kinases in colonic epithelial cells thereby promoting the secretion of macrophage inflammatory protein (MIP)-3α[29], a chemokine up-regulated on the inflamed gut epithelium of IBD patients and involved in the recruitment of T cells in the gut mucosa[30,31]. In line with these observations, blockade of endogenous IL-21 in cultures of IBD mucosal explants reduces MIP-3α synthesis by epithelial cells[29].

IL-21 INHIBITS REGULATORY T CELL DIFFERENTIATION AND MAKES CD4+ T CELLS RESISTANT TO TREGS-MEDIATED IMMUNE-SUPPRESSION

Regulatory T cells (Tregs) play an important role in maintaining homeostasis and preventing autoimmunity in various organs, including the gut[32,33]. Tregs specifically express the transcription factor forkhead winged helix transcription factor gene (Foxp3), which is also functionally required for their regulatory activity[32]. In addition to naturally occur-

ring Tregs that are produced by the thymus as a functionally distinct and mature population of T cells[33], Tregs can arise in the periphery upon conversion of CD4+CD25- T cells into Foxp3-positive-CD4+CD25+ cells in response to activating stimuli and transforming growth factor (TGF)-β1[34,35]. This phenomenon seems to occur in the normal gut, where TGF-β1 synergizes with other regulatory molecules (e.g. IL-10, retinoic acid) in mounting an effective counter-regulatory response[36,37]. However, if activation of naïve CD4+CD25- T cells occurs in the presence of TGF-β1 and inflammatory stimuli, they tend to differentiate into effector Th17 cells rather than into Tregs[38]. IL-21 seems to accomplish this function, given that it can cross-regulate Tregs induction and direct the development of Th17 cells[39]. Interestingly, colitis induced by the transfer of naïve T cells into severe combined immunodeficient mice is suppressed by TGF-β1-induced Tregs generated *in vitro* in the absence of IL-21 but not by T cells generated in the presence of TGF-β1 and IL-21[40]. By contrast, these latter T cells exacerbate colitis with increased expression of IL-17 and a reduced number of Foxp3-expressing cells in the gut mucosa[40]. Consistent with this is the demonstration that blockade of IL-21 associates with high numbers of Foxp3-positive Tregs and reduced tissue damage in the colon and small intestine of mice with acute graft *vs* host disease[41].

IL-21 is also able to counteract the regulatory effects of Tregs by providing human CD4+ T cells signals that raise their threshold for suppression by Tregs[42]. Collectively these observations delineate another mechanism by which IL-21 contributes to amplify the ongoing inflammation in IBD.

CONCLUSION

There is no doubt that IL-21 modulates the activity of several cell types that orchestrate the tissue damage in IBD, and that blockade of IL-21 signalling attenuates the ongoing mucosal inflammation in experimental models of IBD. Therefore, it is conceivable that the near future will witness the use of novel therapeutic strategies aimed at inhibiting IL-21 activity in IBD. However, some important issues related to the blockade of IL-21 function must be taken into consideration before moving into the clinic. For instance, we should not forget that IL-21 plays a decisive role in the control of B cell and plasma cell function, and that IL-21 signalling may attenuate the course of IgE-mediated diseases[24,25]. Moreover, blockade of IL-21 could potentially enhance the risk of malignancies and exacerbate chronic viral infections given the ability of IL-21 to trigger CD8+ T cell-dependent immune reactions against tumors and viruses[43-48]. At least in some circumstances, IL-21 stimulates IL-10 production, thereby promoting tolerogenic rather than inflammatory responses. If so, anti-IL-21 therapy could paradoxically enhance the risk of autoimmunity.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3642

World J Gastroenterol 2010 August 7; 16(29): 3642-3650
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Non-peptidyl low molecular weight radical scavenger IAC attenuates DSS-induced colitis in rats

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Supported by A Grant from the University of Bologna (Ricerca Fondamentale Orientata)
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Received: January 27, 2010 Revised: May 29, 2010
Accepted: June 5, 2010
Published online: August 7, 2010

Abstract

AIM: To investigate the effects of the free radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate (IAC) in the dextran sodium sulphate (DSS) experimental model of ulcerative colitis.

METHODS: Colitis was induced in Sprague Dawley male rats by administration of 5% DSS in drinking water. IAC (30 mg/kg, lipophilic or hydrophilic form) was administered daily (orally or ip) for 6 d until sacrifice. Colonic damage was assessed by means of indirect (Disease Activity Index score) and direct measures (macroscopic and microscopic scores) and myeloperoxidase (MPO) activity. Neutrophil infiltration within the tissue and glutathione S-transferase activity were also investigated.

RESULTS: DSS-induced colitis impaired body weight gain and markedly increased all inflammatory parameters. Six-day treatment with lipophilic IAC significantly reduced intestinal damage caused by inflammation, induced a down-regulation in MPO activity (0.72 ± 0.12 and 0.45 ± 0.12 with lipophilic IAC *po* and ip, respectively, *vs* 1.10 ± 0.27 in untreated DSS colitis animals) and minimized DSS-induced neutrophil infiltration, while hydrophilic IAC administered orally did not ameliorate DSS-induced damage.

CONCLUSION: These results support the hypothesis that reactive oxygen metabolites contribute to inflammation and that the radical scavenger IAC has therapeutic potential in inflammatory bowel disease.

Key words: Dextran sodium sulphate-induced colitis; Oxidative damage; Inflammatory bowel disease; Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate; Radical scavenger; Animal models

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Vasina V, Broccoli M, Ursino MG, Canistro D, Valgimigli L, Soleti A, Paolini M, De Ponti F. Non-peptidyl low molecular weight radical scavenger IAC attenuates DSS-induced colitis in rats. *World J Gastroenterol* 2010; 16(29): 3642-3650 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3642.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3642

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases of the gut of unclear etiology: environmental and genetic factors regulating mucosal immune response, mucosal barrier function and response to intestinal microflora are all thought to contribute to the pathogenesis of these diseases, characterized by mucosal inflammatory infiltrates, intestinal barrier function alteration and erosive loss of mucosa and submucosa[1,2].

Because of extensive mucosal damage and massive infiltration of polymorphonuclear and mononuclear leukocytes[1,2], reactive oxygen and nitrogen radical species are produced and released, resulting in potential oxidation and peroxidation of a large number of molecules (e.g. proteins, lipids and DNA). Indeed, the intestinal mucosa of patients with inflammatory bowel disease (IBD) is characterized by radical species overproduction and imbalance of the most important antioxidants[3,4] leading to oxidative damage; self-sustaining cycles of oxidant production may amplify inflammation and mucosal injury in UC, where activated neutrophils and macrophages mainly contribute to active lesions[5,6]. The phagocytes present in the mucosa of IBD patients, indeed, can produce reactive oxygen metabolites such as superoxide and hydrogen peroxide, through both respiration burst and prostaglandin and leukotriene metabolism. Radical species released during inflammation increase mucosal permeability and contribute to the recruitment and activation of further neutrophils, thus initiating and/or propagating inflammation and tissue damage[6].

Antioxidant compounds and free radical scavengers improved colitis in several experimental models[7,8]. Recently, in a proof of concept study, we have shown that the radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) protects the rat colon from DNBS-induced colitis, which mimics human CD[9]. DNBS is a contact sensitizing allergen which causes immunological activation; acute pathological features include focal necrosis and acute inflammation, followed by a chronic infiltration of mononuclear cells[10].

In this present study, we investigated the effects of IAC in a different model of colitis, i.e. one that is induced by dextran sodium sulphate (DSS), which differs from the DNBS model in terms of tissue inflammatory and immunological activation, as well as severity of the inflammatory process, and is thought to closely mimic human UC[10]. In the DSS model, as in UC, only the mucosa is affected by inflammation; early damage includes shortening and dropout of crypts, particularly over lymphoid aggregates, progressing to focal ulceration, mononuclear cell and neutrophil infiltration. Similar to UC, DSS provokes acute inflammation and macrophage activation, with consequent epithelial cell injury and activation of innate immune responses by luminal bacterial components and eventual activation of T cells[2,11,12].

We tested two different forms of IAC in DSS colitis, with either hydrophilic or lipophilic character. Due to its peculiar physico-chemical properties, IAC readily diffuses through the cellular membrane and can reach virtually any compartment where the production of free radicals occurs, possessing equal radical scavenging activity[13]. This represents an advantage, because it can directly react with free radicals.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (180-200 g body weight; Harlan Italy, S. Pietro al Natisone, Udine, Italy) were used in this study. Animals were housed in a controlled environment and had free access to food and water throughout the study. Before starting any experimental procedure, in order to minimize stress, animals of all experimental groups were weighed and gently manipulated in the laboratory environment for 30 min every day for at least 1 wk. All experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. The protocol for induction of colitis was reviewed by the Institutional Committee on the care and use of experimental animals of the University of Bologna and was authorized by the Italian Ministry of Health. A persistently hunched posture and labored respiration, a markedly erect coat and a weight loss of more than 20% were considered as humane end-points at which to euthanize the animals.

Induction of colitis

Colitis was induced using a previously described method[14]. DSS (molecular weight, 40 kilodaltons; ICN Biomedicals Inc, Aurora, OH) was added to drinking water at a final concentration of 5% (wt/vol) for 5 d. Controls were all time-matched and consisted of rats receiving normal drinking water only. The DSS solution was replenished daily and mean DSS consumption was noted per cage at the end of 5-d treatment.

Experimental design

We studied groups of rats with and without colitis (n = 6-12 per group), which were treated with the radical scavenger IAC (Figure 1) synthesized in our laboratory[13], starting the day before the induction of colitis for 5 d. IAC (30 mg/kg), hydrophilic or lipophilic form, was administered once daily at the same time (orally or ip) as water solution or suspension. In our previous work, we observed that treatment with hydrophilic IAC *po* in DNBS-induced colitis induced only a minor protective effect, while when administered ip, it was unable to reduce inflammation, therefore we decided to only test the hydrophilic form *po* in this study[9].

The dose of 30 mg/kg was selected on the basis of previous studies, in which IAC showed the best antioxidant activity[15] and protective effect in DNBS-induced colitis[9]. At the end of this 7-d period, the animals were killed and their colons collected for further analysis.

Tissue collection

Before tissue samples were collected, the entire colon was

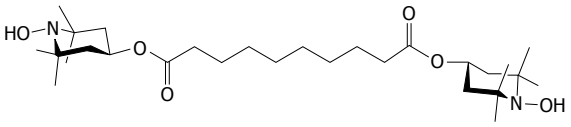

Figure 1 Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate structure.

removed, the length starting from 1 cm above the anus to the top of the cecum and the weight of the colon still containing fecal contents were measured. Then the colon was opened longitudinally and washed with phosphate buffered saline (PBS) to remove luminal contents; at this time, the consistency of any stools found within the colon and the gross macroscopic appearance of the colon were noted. Whole-wall colonic samples were pinned flat on wax, fixed in cold neutral 4% formalin and then placed in 25% sucrose in PBS at 4℃ for cryoprotection and embedded in optimal cutting temperature tissue freezing medium. Seven-micron-thick sections of colon were cut, serially mounted on glass and processed for routine hematoxylin-eosin (HE) staining and naphthol AS-D chloroacetate esterase assay. Specimens of colonic tissue were also removed, snap frozen in liquid nitrogen and stored at -80℃ until subsequent assays.

Assessment of the severity of colitis

Disease activity index: Disease activity index (DAI) scores have historically well correlated with pathological findings in a DSS-induced model of IBD[16]. DAI is the combined score of weight loss, stool consistency and bleeding, as detailed in Table 1. All parameters were scored from day 0 to day 5 during DSS treatment.

Total macroscopic score: When rats were sacrificed, the colon was removed, opened longitudinally and washed with PBS to remove luminal contents and macroscopic damage was immediately assessed. The macroscopic parameters analyzed before tissue samples were collected were: colon length starting 1 cm above the anus to the top of the cecum, weight of the colon still containing fecal contents, stool consistency and gross macroscopic appearance of the colon. Decreases in filled colon weight are indicative of colonic hypermotility[17]. Indeed, colons from animals with severe colitis can be seen to be nearly devoid of fecal contents. Colon shrinkage is also commonly observed in DSS colitis and is indicative of colonic smooth muscle contraction[17,18]. Total macroscopic score was assigned according to a previously described scoring system[16]; details for each parameter are reported in Table 2.

Histology: Seven-micron-thick sections of colon were cut, serially mounted on glass and processed for routine HE staining. Colonic damage was scored based on a published scoring system that considers amount of inflammation (from 0, none, to 3, severe), extent of inflammation (from 0, none, to 3, transmural), crypt damage (from 0, none, to 4, entire crypt and epithelium lost) and tissue regeneration (from 4, no tissue repair, to 0, complete regeneration or normal tissue)[11].

Table 1 Disease activity index score parameters

Stool consistency	Bleeding	Weight loss	Maximum score
0 = formed	0 = normal color stool	0 = no weight loss	10
1 = mild-soft	1 = brown color stool	1 = 5%-10% weight loss	
2 = very soft	2 = reddish color stool	2 = 11%-15% weight loss	
3 = watery stool	3 = bloody stool	3 = 16%-20% weight loss	
		4 = > 20% weight loss	

Myeloperoxidase assay

Specimens of colonic tissue (50 mg) were assayed using a previously described method[19]. Myeloperoxidase (MPO) is a granule-associated enzyme present in neutrophils and other cells of myeloid origin, and widely used as a marker of intestinal inflammation. Colonic tissues were homogenized in ice-cold potassium phosphate buffer (pH 6.0) and centrifuged for 10 min at 6000 *g* at 4℃. The supernatants were then collected, added to a solution of *O*-dianisidine (Sigma-Aldrich, Milan, Italy) and hydrogen peroxide and assayed to assess MPO activity.

MPO was expressed in units per milligram of tissue, where 1 U corresponds to the activity required to degrade 1 μmol of hydrogen peroxide in 1 min at room temperature.

Glutathione S-transferase activity

Colonic samples (25 mg) were obtained using a previously described method[9]. The supernatant was collected and then assayed for glutathione S-transferase (GST) activity[20]. Assessment of protein content in colonic samples was performed using the Quick Start™ Bradford Protein Assay (BIO-RAD, Hercules, CA, USA); the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. GST activity was expressed as μmol/mg of protein per min.

Naphthol AS-D chloroacetate esterase assay

In order to identify neutrophil infiltration within the tissue, we used a commercially available kit (naphthol AS-D chloroacetate esterase; 91C, Sigma-Aldrich, Milan, Italy). This enzyme is considered specific for cells of granulocytic lineage and its sites of activity show bright red granulation. Seven-micron-thick sections of colon were used for this assay. Briefly, tissue sections were fixed in citrate-acetone-formaldehyde solution and assayed according to the manufacturer's protocols. Specimens were mounted with mounting media (glycerol-PBS, 9:1), examined by light microscope (ECLIPSE 90i, Nikon Instruments, Calenzano, Italy) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments, Calenzano, Italy).

Table 2 Total macroscopic score parameters

Stool consistency	Bleeding	Colon damage score	Colon weight score	Colon length score	Maximum score
0 = formed	0 = absent	0 = no inflammation	0 = < 5% weight loss	0 = < 5% shortening	14
1 = loose	1 = present	1 = hyperemia	1 = 5%-14% weight loss	1 = 5%-14% shortening	
2 = liquid		2 = slight erosion	2 = 15%-24% weight loss	2 = 15%-24% shortening	
		3 = extensive erosion/ulceration	3 = 25%-35% weight loss	3 = 25%-35% shortening	
			4 = > 35% weight loss	4 = > 35% shortening	

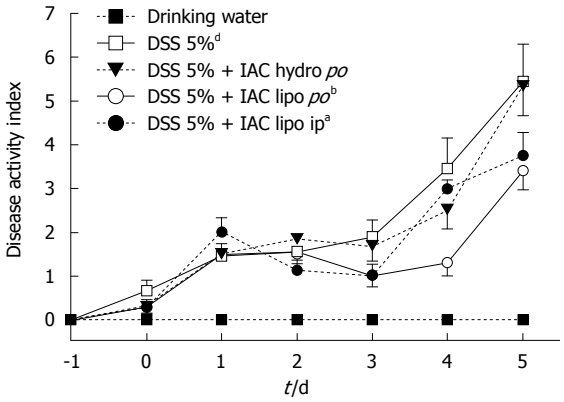

Figure 2 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg on disease activity index in the different experimental groups. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* dextran sodium sulphate (DSS); $^{d}P < 0.001$ *vs* drinking water. Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using analysis of variance (one-way or two-way, as appropriate, with the Bonferroni's correction for multiple comparisons). A P value < 0.05 was considered significant. N refers to the number of animals used for each experiment (n = 8-16). Calculations were performed using GraphPad Prism™ (version 5.01, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Assessment of colitis

All rats with DSS colitis progressively lost weight and manifested bloody diarrhea. DAI was significantly increased together with all inflammatory parameters (Figures 2-6). On day 5 after DSS administration, the colonic mucosa of inflamed rats was edematous and erythematous with occasional areas of mucosal erosion. Compared to non-inflamed rats, the colon weight was decreased, the colon length was significantly shorter and a marked increase in total macroscopic damage score was noted (Figure 3, panel A). Colitis was characterized by mucosal ulceration, crypt dropout and a marked neutrophil infiltration extending throughout the mucosa and submucosal layers (Figure 5B) and by a 20-fold increase in microscopic damage score over the non-inflamed control animals (Figure 3, panel B); crypt abscesses and depletion of goblet cells were observed in some regions of colonic mucosa. A 3-fold increase in MPO activity was found (Figure 3, panel C) compared with healthy rats. Moreover, in DSS-treated animals, we detected neutrophil infiltration (red cells, Figure 6B) with respect to controls (Figure 6A), extending throughout the mucosa and submucosa. GST activity slightly decreased in inflamed animals as compared with healthy controls, although statistical significance was not achieved (1.40 ± 0.14 *vs* 1.27 ± 0.11).

Effect of IAC on DSS-induced colitis

Six-day treatment with lipophilic IAC (30 mg/kg, orally and ip) reduced intestinal inflammation and damage: indeed, treated rats had neither bloody diarrhea nor perianal injury and an improvement was observed in gross findings (clinical signs and symptoms of colitis) such as weight loss (Figure 2). Total macroscopic score was significantly improved by drug treatment (Figure 3, panel A). DSS-induced colon shrinkage and weight loss were significantly improved by IAC treatment (lipophilic) as compared to inflamed rats, and these features were similar to healthy controls (Figure 4). Moreover, lipophilic IAC (orally and ip) significantly decreased DSS-induced microscopic damage (Figure 3, panel B and Figure 5D), down-regulated MPO activity (Figure 3, panel C) and also minimized DSS-induced neutrophil infiltration within the colonic wall (Figure 6D).

In contrast, hydrophilic IAC 30 mg/kg orally was able to decrease only microscopic damage score, but substantially failed to protect the colon from DSS-induced damage (Figures 2-4, 5C and 6C).

GST activity was not significantly affected either by hydrophilic IAC *po* (1.27 ± 0.10 *vs* 1.27 ± 0.11 in DSS 5%) or lipophilic IAC *po* (1.28 ± 0.07 *vs* 1.27 ± 0.11 in DSS 5%) or ip (1.00 ± 0.07 *vs* 1.27 ± 0.11 in DSS 5%, P = 0.07).

DISCUSSION

This study shows that the lipophilic form of IAC at the dose of 30 mg/kg significantly ameliorates damage and inflammation in DSS-induced colitis, an experimental model of UC, and is far better than its hydrophilic form, since it positively affected all the parameters under scrutiny. This different activity on inflammation can be ascribed to the differing ability of the two distinct forms of IAC (hydrophilic *vs* lipophilic) to distribute in cell membranes and intra-/extra-cellular compartments; hydrophilic IAC may have a particular profile of absorption and distribution, leading to a lower concentration within areas of major damage.

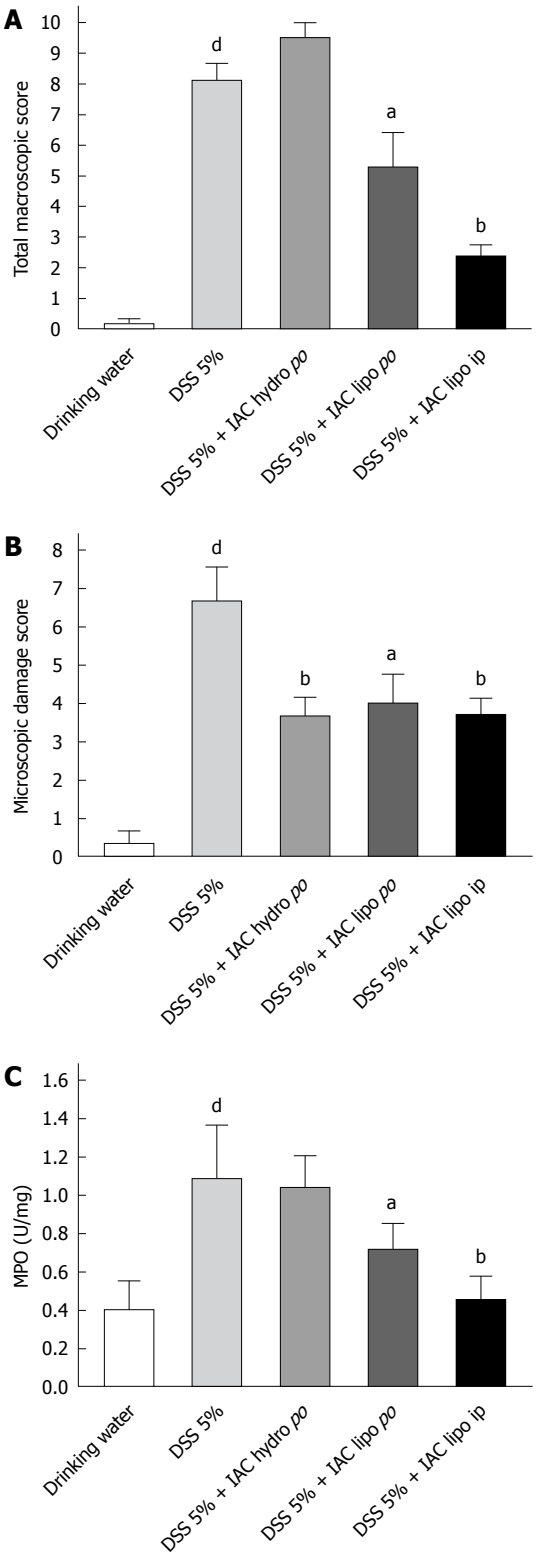

Figure 3 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg (hydrophilic or lipophilic form) on total macroscopic (A) and microscopic damage score (B) and on myeloperoxidase activity (C) in the different experimental groups. Treatment with hydrophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) *po* decreased colonic microscopic damage but had no effect on macroscopic and myeloperoxidase (MPO) activity. Six-day treatment with lipophilic IAC *po* and ip significantly reduced intestinal damage induced by inflammation. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* dextran sodium sulphate (DSS); $^{d}P < 0.001$ *vs* drinking water. ICC hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

Different experimental models can be used for pathophysiological studies and, because of their similarity to human IBD (UC and CD), they represent useful tools to test the therapeutic potential of new drugs. These models allow the study of early events and interactions among different components of IBD and the identification of immunologic processes and genes determining susceptibility to inflammatory disorders. Although there is no unique model of IBD and the various experimental models differ in their pathophysiology, each of them can be useful to gain insight into the multi-factorial nature of IBD[10].

In DSS-induced colitis, as in UC, only the mucosa is affected by inflammation; early damage includes shortening and dropout of crypts in the left colon, particularly over lymphoid aggregates, progressing to focal ulceration, mononuclear cell and neutrophil infiltration.

Reactive oxygen, nitrogen and carbon species are produced and released during the acute phase of inflammation, resulting in epithelial damage with consequent activation of innate immune responses by luminal bacterial components and eventual activation of Th1 and later Th1/Th2 responses during chronic colitis[2,11].

In our study, 5-d treatment with 5% DSS induced weight loss and bloody diarrhea and caused a substantial degree of inflammation and tissue injury in the rat colon, which was edematous, erythematous and characterized by mucosal ulceration. Moreover, inflammation was associated with polymorphonuclear colonic infiltrate (histology, MPO activity and naphthol AS-D chloroacetate esterase assay). Indeed, within the bowel wall of IBD patients and of animals with experimental colitis, a massive infiltration of polymorphonuclear and mononuclear leukocytes, which may produce large amounts of free radicals, is commonly observed[1,9,21,22].

During DSS-induced colitis, we also studied GST activity. GST is a detoxification enzyme catalyzing the conjugation of reactive electrophiles with the thiol glutathione, providing cellular protection from highly reactive electrophiles[23]. A significant decrease in the GST activity was reported in patients with family history of colon cancer and polyps[24]; moreover, GST level changes have been observed both in IBD patients[25] and in some experimental models of colitis[23,26]. The only marginal (not statistically significant) decrease in GST activity observed by us on day 5 after DSS administration is actually in line with data obtained by Clapper *et al*[23], who showed cyclical changes in GST activity during acute DSS colitis, with a significant decrease in enzyme activity on days 2 and 7, while on day 5 they reported only marginally decreased GST activity, exactly as we did.

Treatment with lipophilic IAC (30 mg/kg, orally and ip) significantly ameliorated colonic damage and inflammation induced by DSS, decreased MPO activity and also minimized DSS-induced neutrophil infiltration within the colonic wall. These observations extend and corroborate our previous report that IAC is effective in DNBS-induced colitis[9], a quite different model where DNBS causes transmural immunological activation and inflam-

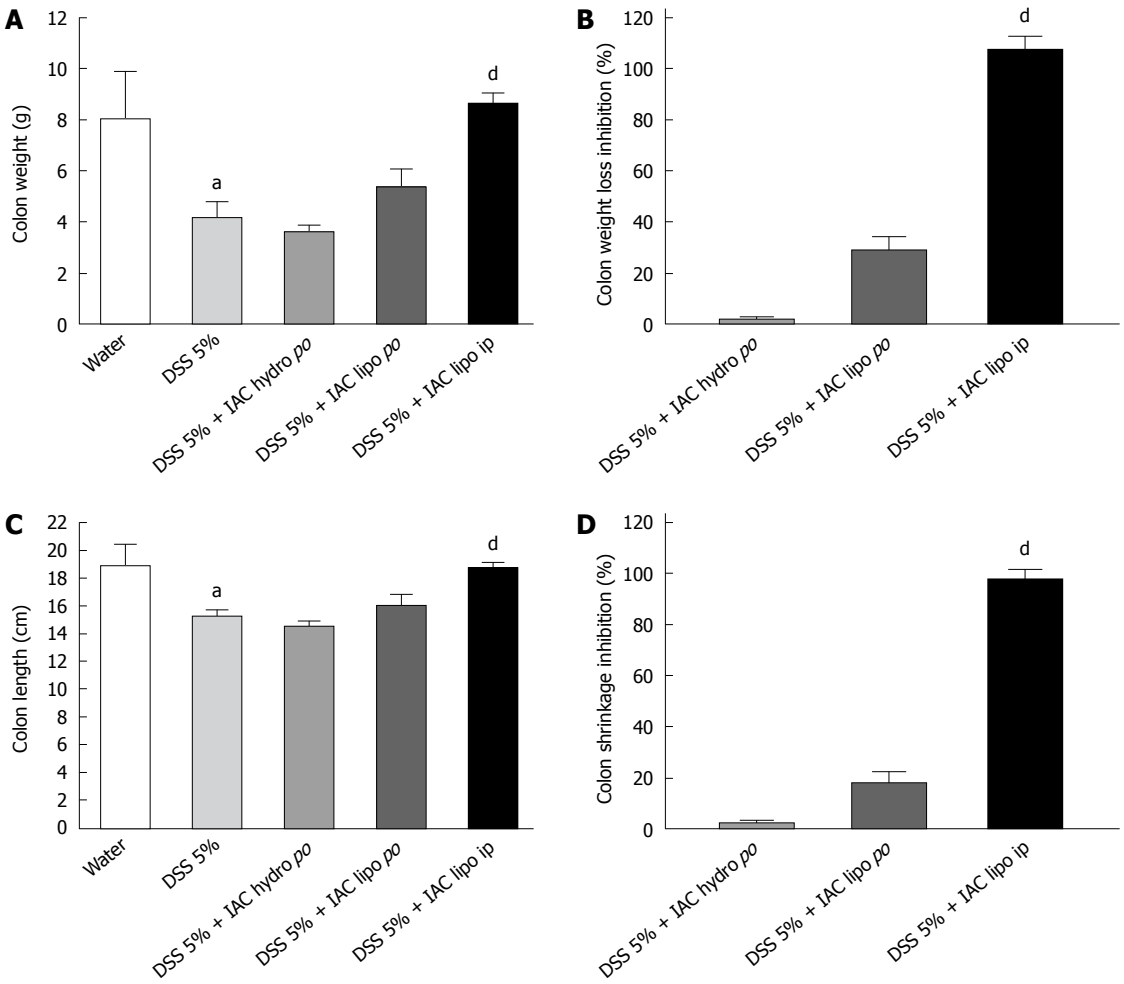

Figure 4 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg (hydrophilic or lipophilic form) on dextran sodium sulphate-induced colon weight loss (absolute value, A, and % inhibition, B) and shrinkage (absolute values, C, and % inhibition, D) in the different experimental groups. Six-day treatment with lipophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) ip significantly inhibited colon weight loss and colon shrinkage induced by inflammation, while the hydrophilic form had no effect. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$ *vs* water; $^{d}P < 0.01$ *vs* dextran sodium sulphate (DSS). IAC hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

mation resembling CD. In our hands, lipophilic IAC was slightly more effective ip than orally, probably because it undergoes protonation at low gastric pH after oral administration and is more quickly excreted from the gut (the protonated form has higher polarity). For clinical use, it may be necessary to protect IAC from low gastric pH using a specific formulation.

Thus, lipophilic IAC is protective both in DNBS and DSS experimental models of inflammation and has a wide spectrum of activity; its lipophilic form (ip) significantly reduced DAI (30%), macroscopic and microscopic damage (70% and 45%, respectively) and decreased MPO activity (58%) in DSS-induced colitis. Likewise, in DNBS-induced colitis, treatment with lipophilic IAC can decrease macroscopic and microscopic damage (55% and 46%, respectively), reduce MPO and tumor necrosis factor-α tissue levels (80% and 30%, respectively) and lipidic peroxidation (40%).

Notably, in both the DSS and DNBS models[9], IAC is able to significantly decrease MPO activity and neutrophil infiltration within the bowel wall. In both experimental models, as in several human inflammatory diseases such as rheumatoid arthritis and IBD, infiltrating neutrophils are the major candidate for the production of reactive oxygen radicals[27]. Oxidative damage may represent a pathogenic factor in IBD because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species and an imbalanced antioxidant response[1,28,29]. Indeed, free radical production is a key mechanism for the appearance and the maintenance of colonic inflammation in experimental models of colitis[7,30,31].

Thus, we can hypothesize that IAC exerts its protective effects by reducing inflammatory neutrophil infiltrate and by scavenging reactive oxygen species produced by infiltrating cells which during inflammation contribute to the recruitment and activation of further neutrophils, and by counteracting this self-sustaining cycle of oxidant production, which propagates inflammation and tissue damage. This hypothesis of reduced oxidative damage by IAC is not contrary to its lack of activity on GST levels because, in our hands, GST levels were only marginally affected by DSS-induced inflammation. It has been shown that treatment with antioxidant compounds and radical scavengers exerts a protective effect in several models of

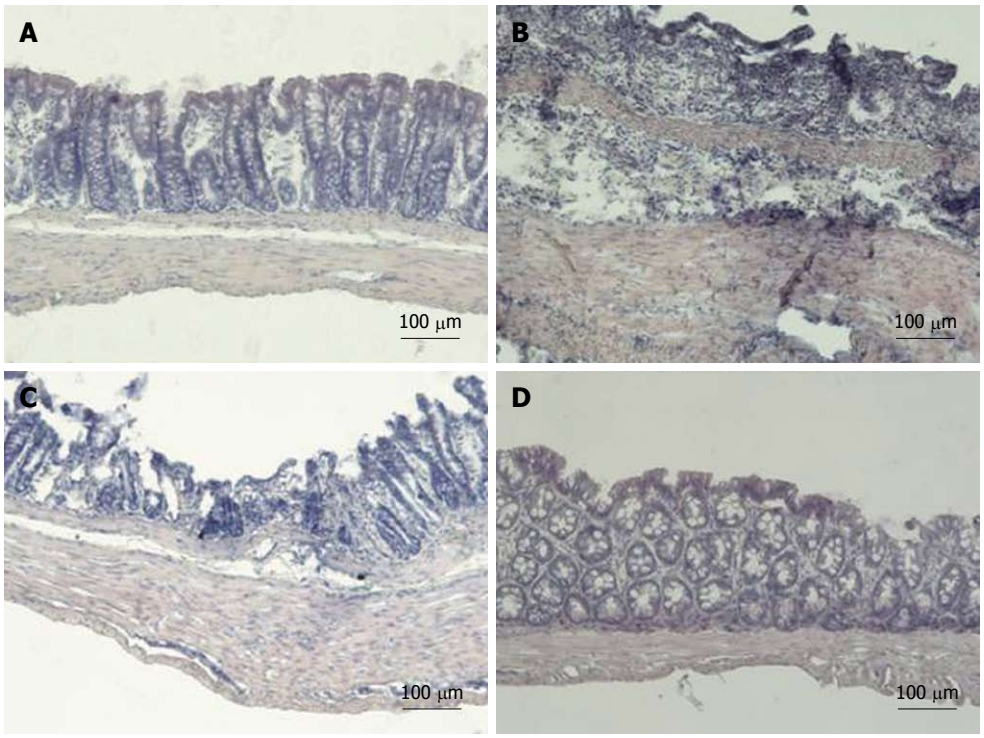

Figure 5 Representative examples of cross sections of distal colon. A: From a non-inflamed rat [drinking water + bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate (IAC) vehicle orally]; B: From an inflamed rat [dextran sodium sulphate (DSS) 5% in drinking water + IAC vehicle orally]. Note the dramatic loss of mucosal architecture with crypt dropout and the granulocyte infiltrate extending throughout the mucosa and submucosa; C, D: Cross sections of distal colon from an inflamed rat treated with hydrophilic (C) and lipophilic (D) IAC 30 mg/kg orally (C) and intraperitoneally (D). Lipophilic (*po*, not shown here, and ip) IAC 30 mg/kg decreased the microscopic damage produced by DSS, facilitating mucosal healing, reducing inflammatory cells infiltration and muscle thickening (panel D). Hydrophilic IAC *po* failed to protect the colon from the damage induced by DSS (panel C).

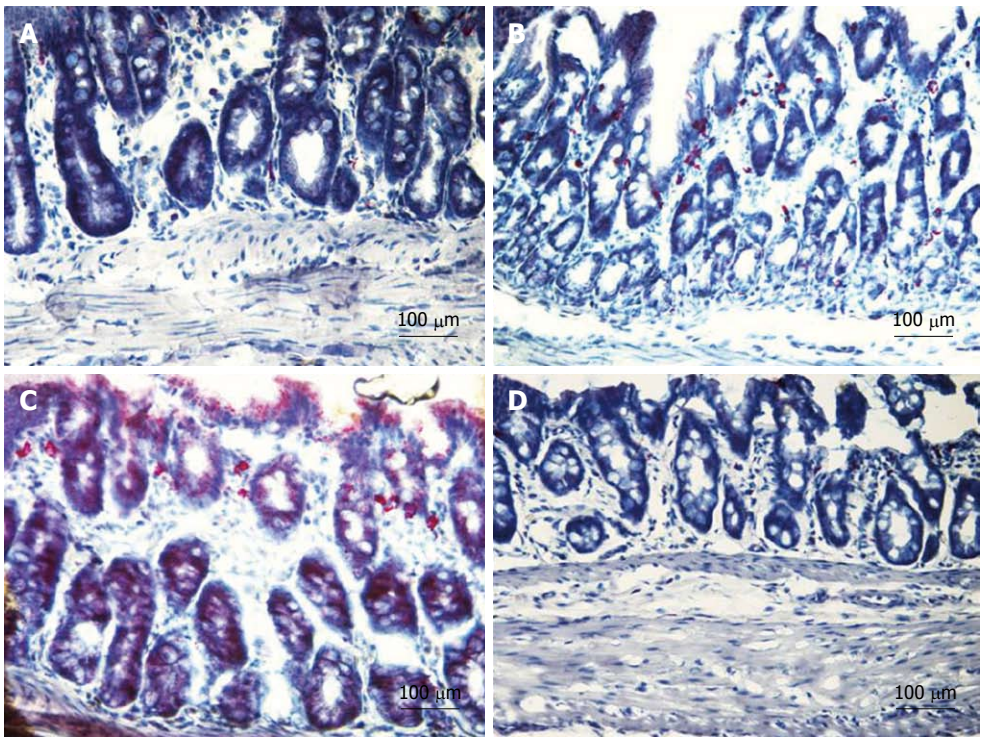

Figure 6 Naphthol AS-D chloroacetate esterase positivity (red) in cross section of distal colon. A: Tissue sections obtained from non-inflamed rats showed occasional red staining indicating a low presence of neutrophils within the bowel wall under physiological conditions; B: From a rat with colitis (5% dextran sodium sulphate in drinking water, panel B). Compared to non-inflamed rats, tissue from rats with colitis showed a massive neutrophil infiltration extending throughout the mucosa (note the scattered degranulation within the crypts and submucosa); C, D: Treatment with bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) 30 mg/kg hydrophilic form *po* (C) was unable to suppress neutrophil infiltration especially in the mucosa. Treatment with lipophilic IAC both *po* (not shown) and ip (D) almost completely suppressed neutrophil infiltration within the colonic wall.

intestinal inflammation[9,32,33]: Cuzzocrea *et al*[7,34] demonstrated that treatment with antioxidants such as tempol and M40403, a superoxide dismutase mimetic, ameliorated TNBS/DNBS experimental colitis, probably by limiting leukocyte recruitment. However, cyclic nitroxides such as tempol are very persistent in water or organic solutions, but when used *in vivo* or in a biological sample are reduced to the parent hydroxylamine by several enzymatic processes mainly involving ascorbate or glutathione. IAC is more stable in physiological solutions and possesses a stronger antioxidant capability than that of the aforementioned cyclic nitroxides; it is easily distributed through cell membranes and intra-/extra-cellular compartments, thus it can directly react with oxidant molecules within the cell, where free radicals are produced[35]. In DNBS-induced colitis, IAC seems to display higher activity than tempol, at least in reducing MPO activity and neutrophil infiltration[7]. Recently, the lipophilic form of IAC was used in different experimental disease models all characterized by oxidative stress (e.g. nonobese mouse diabetes model[15] and a rat model of transient middle cerebral artery occlusion[36]), as well as *in vitro*[37], with positive results.

Notably, IAC is a low molecular weight radical scavenger which can rapidly react with most carbon-, nitrogen- and oxygen-centered radicals of biological interest, including peroxyl, superoxide, and peroxynitrite radicals[13]. Its antioxidant activity is a direct effect of the molecule itself; its activity is due to hydroxylic hydrogen transfer to peroxyl radicals, which generates the corresponding nitroxide, unable to propagate the autoxidation chain. Its peculiar physico-chemical properties affect its partition properties across cell membranes and both intra- and extra-cellular compartments: the free form is highly lipophilic (the calculated logP is 4.01) and easily crosses the cell membrane, allowing distribution to any compartment where the production of free radicals occurs, but it is also in equilibrium with the protonated form, which administered to a biological system is completely water-soluble and distributes in the extra-cellular compartments[13].

In conclusion, our data show that treatment with the lipophilic (but not the hydrophilic) form of the radical scavenger IAC, at the dose of 30 mg/kg, ameliorates DSS-induced colitis in rats. These results, taken together with our previous data showing a protective effect of lipophilic IAC in DNBS-induced colitis, provide further evidence of the involvement of reactive oxygen species in inflammation and support the concept that antioxidant therapy may have an important role in treatment of IBD.

ACKNOWLEDGMENTS

The authors thank Dr. Barbara Bonamassa for her help in performing the GST experiments.

COMMENTS

Background

Ulcerative colitis (UC) and Crohn's disease are chronic inflammatory bowel diseases (IBDs) of unclear etiology: environmental and genetic factors regulating mucosal immune response, mucosal barrier function and response to intestinal microflora are all thought to contribute to the pathogenesis of these diseases, characterized by mucosal inflammatory infiltrates, intestinal barrier function alteration and erosive loss of mucosa and submucosa. Indeed, the intestinal mucosa of patients with IBD is characterized by radical species overproduction and imbalance of the most important antioxidants leading to oxidative damage.

Research frontiers

Over the last two decades, the incidence and the prevalence of IBD seem to have increased; IBDs cause a large number of hospitalizations for the patients affected. Many drugs are used to treat IBD, given for a variety of reasons: to suppress inflammation in patients with active disease, to prevent flare-ups in those with inactive disease, to control symptoms such as pain or diarrhea or to replace or supplement essential nutrients which are poorly absorbed because of extensive disease or surgery. However, the etiology of IBD is still unknown and new therapeutic options are needed, as available drugs are still unsatisfactory to treat and heal IBD.

Innovations and breakthroughs

It is generally hypothesized that oxidative stress is a potential etiological and/or triggering factor for IBD, because the detrimental effects of reactive oxygen molecules have been well established in the inflammation process. Antioxidant compounds and free radical scavengers have been shown to improve colitis in several experimental models suggesting an important role of reactive oxygen species in intestinal inflammation. This study clearly shows the effect of an antioxidant molecule in an experimental model of colitis which resembles human UC, indicating a potential clinical application for this class of compounds in treating IBD.

Applications

The protective effect shown by antioxidant therapy in this experimental model of colitis indicates a potential clinical application for this class of compounds in treating IBD. However, several clinical studies have been largely disappointing, probably due to the inability of the antioxidant to reach sufficient concentrations at the inflammation site. Lipophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) is easily distributed through cell membranes and intra-/extra-cellular compartments and directly reacts with oxidant molecules within the cell, where free radicals are produced. For clinical use, it may be necessary to protect IAC from low gastric pH using a specific formulation in order to improve its bioavailability.

Peer review

The study is well done with adequate supporting documentation, controls and references. The difference in activity between the lipophilic and hydrophilic forms of IAC in suppressing dextran sodium sulphate-induced colitis is a novel and important finding.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3651

World J Gastroenterol 2010 August 7; 16(29): 3651-3663
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Effects of protein deprivation and re-feeding on P2X2 receptors in enteric neurons

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Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo/Proc 04/00746-3
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Received: March 16, 2010 Revised: April 14, 2010
Accepted: April 21, 2010
Published online: August 7, 2010

Abstract

AIM: To investigate the effects of malnutrition and re-feeding on the P2X2 receptor, nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in neurons of the rat ileum.

METHODS: We analyzed the co-localization, numbers and sizes of P2X2-expressing neurons in relation to NOS-immunoreactive (IR), calbindin-IR, ChAT-IR, and calretinin-IR neurons of the myenteric and submucosal plexus. The experimental groups consisted of: (1) rats maintained on normal feed throughout pregnancy until 42 d post-parturition (N); (2) rats deprived of protein throughout pregnancy and 42 d post-parturition (D); and (3) rats undernourished for 21 d post-parturition and then given a protein diet from days 22 to 42 (DR). The myenteric and submucosal plexuses were evaluated by double labeling by immunohistochemical methods for P2X2 receptor, NOS, ChAT, calbindin and calretinin.

RESULTS: We found similar P2X2 receptor immunoreactivity in the cytoplasm and surface membranes of myenteric and submucosal neurons from the N, D and DR groups. Double labeling of the myenteric plexus demonstrated that approximately 100% of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in all groups also expressed the P2X2 receptor. In the submucosal plexus, the calretinin-IR, ChAT-IR and calbindin-IR neurons were nearly all immunoreactive for the P2X2 receptor. In the myenteric plexus, there was a 19% increase in numbers per cm^2 for P2X2 receptor-IR neurons, 64% for NOS-IR, 84% for calretinin-IR and 26% for ChAT-IR neurons in the D group. The spatial density of calbindin-IR neurons, however, did not differ among the three groups. The submucosal neuronal density increased for calbindin-IR, calretinin-IR and ChAT-IR neurons. The average size of neurons in the myenteric plexus neurons in the D group was less than that in the controls and, in the re-fed rats; there was a 34% reduction in size only for the calretinin-IR neurons.

CONCLUSION: This work demonstrates that expression of the P2X2 receptor is present in inhibitory, intrinsic primary afferent, cholinergic secretomotor and vasomotor neurons. Undernutrition affected P2X2 receptor expression in the submucosal plexus, and neuronal and size. These changes were rescued in the re-fed rats.

Key words: Chemical coding; Myenteric neurons; Submucosal neurons; Undernutrition

Peer reviewers: Dr. John R Grider, PhD, Professor, Department of Physiology and Biophysics, Virginia Commonwealth University, PO Box 980551, Richmond, VA 23298, United States; Geoffrey Burnstock, Professor, PhD, DSc, FAA, FRCS (Hons), FRCP

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Misawa R, Girotti PA, Mizuno MS, Liberti EA, Furness JB, Castelucci P. Effects of protein deprivation and re-feeding on P2X2 receptors in enteric neurons. *World J Gastroenterol* 2010; 16(29): 3651-3663 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3651.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3651

INTRODUCTION

ATP is known to be a co-transmitter in the nervous system and a ligand of the P2X receptor family, which is made up of seven known receptor subunits (PX_{1-7})[1]. In the myenteric plexus, electrophysiological studies have found P2X receptors in 80%-90% of neurons[2]. P2X receptors play an important role in synaptic transmission within the neural pathways and mediate intestinal motility[3-5]. Immunohistochemical studies have documented the distribution of P2X receptors in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. There is only one earlier study in which the authors have reported expression of $P2X_2$ receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats[12].

The effects of undernutrition on enteric and other autonomic neurons have been investigated[17-25]. In the enteric nervous system, a 27% decrease in the number of enteric neurons in the jejunum of rats submitted to severe prenatal malnutrition has been reported[18], and a mean neuronal loss of 13% in the myenteric plexus of the proximal colon has been observed after malnutrition[20]. Experiments that have examined the effect of re-feeding on enteric neuronal number suggest that, when neurons are reduced in number by undernutrition, they do not recover[18]. However, other reports have described a 15% decrease in the sizes of myenteric neurons from the large intestine of pre- and postnatally protein-deprived animals, as well as recovery of normal size after re-feeding[21]. A 45% size reduction in enteric neurons of the small intestine has also been observed following undernutrition[22].

The present work analyzed the effects of pre- and postnatal protein undernutrition and postnatal re-feeding on neurons immunoreactive for the $P2X_2$ receptor, by specifically examining the expression of nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in these neurons, as well as neuronal density and somatic size in the myenteric and submucosal plexuses in the rat ileum.

MATERIALS AND METHODS

Experimental animals

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo. Young male and female Wistar rats (200-240 g body weight) were mated. After conception, which was assumed to have occurred when vaginal sperm plugs were found, the females were placed in individual cages. During pregnancy, the nourished mothers received an AIN-93G normal protein diet (protein, 20%; fat, 7%; carbohydrate, 20% and fiber, 5%), and the undernourished mothers received the AIN-93G diet with low protein (protein, 5%; fat, 7%; carbohydrate, 20% and fiber, 5%) (Rhoster Indústria e Comércio Ltda, São Paulo, Brazil). The rats were maintained under standard conditions at 21℃, with a 12-h light/dark cycle, and all groups were supplied with water *ad libitum*. After parturition, the dams and pups received the same diet that the dam had during pregnancy. Only the male animals in the litters were used for experimentation. Females remained in the litters but were not investigated. There were three experimental groups. The first group of rats was maintained on normal feed throughout pregnancy until examined at 42 d (P42) (N, n = 5). The second group was protein-deprived throughout pregnancy and postnatally for 42 d (P42) (D, n = 5). The third group of rats was the deprived plus re-feeding group (DR, n = 5), in which animals were undernourished until P21, and then received the AIN-93G normal protein diet from P22 to P42[21,22]. At P42, animals were weighed and euthanized in a CO_2 chamber and the anterior abdominal wall was opened. The small intestine was removed and washed in PBS. The surface area of the small intestine was measured using a planimeter.

Immunohistochemistry

Fresh segments of ileum were removed from each animal of the N, D and DR groups and placed in PBS (0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) that contained nicardipine (10^{-6} mol/L; Sigma, St Louis, MO, USA) to inhibit tissue contraction. The dissected pieces were opened along the mesenteric border and cleaned of their contents using PBS. They were then pinned out tautly, mucosa-side down, onto a balsa-wood board and fixed overnight at 4℃ in paraformaldehyde in 0.2 mol/L sodium phosphate buffer (pH 7.3). The next day, the tissue was cleared of fixative with three 10-min washes in 100% DMSO, followed by three 10-min washes in PBS. All tissue was stored at 4℃ in PBS that contained sodium azide (0.1%). The fixed tissue was dissected and the mucosa, submucosa and circular layers were removed to obtain longitudinal muscle-myenteric plexus whole mounts. In the second type of preparation, the mucosa and muscularis externa were removed to reveal the intact submucous layer. Whole-mount preparations of the myenteric and submucosa of the ileum were preincubated in 10% normal horse serum in PBS that contained 1.5% Triton X-100 for 30 min at room temperature, to reduce non-specific binding and to permeabilize the tissue (Table 1). To localize $P2X_2$ receptor immunoreactivity, we used a rabbit antiserum raised against amino acid sequence 457-472 of the rat $P2X_2$ receptor, with a single Cys extension at the N-terminal (AB5244; Chemicon, Temecula, CA, USA). Incubation was for 48 h at 4℃ at a dilution of 1:120 in 10% normal horse serum in PBS that contained 1.5% Triton X-100. Double labeling was achieved using combina-

Table 1 Characteristics of primary and secondary antibodies

Tissue antigen	Host	Dilution	Code and reference
NOS	Sheep	1:2000	H205
Calbindin	Mouse	1:500	Swant 300
Calretinin	Goat	1:100	CG1 Swant
ChAT	Goat	1:50	Chemicon
Donkey anti-rabbit IgG Alexa 488		1:500	Molecular probes
Donkey anti-sheep IgG Alexa 594		1:100	Molecular probes
Donkey anti-mouse IgG Alexa 594		1:200	Molecular probes

NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

tions of antisera (Table 1). Following incubation in primary antisera, tissue was given three 10-min washes in PBS and incubated in a mixture of secondary antibodies (Table 1). Further 10-min washes in PBS were made before tissue was mounted in glycerol buffered with 0.5 mol/L sodium carbonate buffer (pH 8.6).

Imaging

Preparations were examined on a Leica microscope equipped with the appropriate filters for Alexa 488 (450-490 nm excitation filter and 515-565 nm emission filter) and Alexa 594 (530-585 nm excitation filter and 615 nm emission filter). Images were recorded using an Image-Pro-Plus-coupled camera and Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). Preparations were also analyzed using confocal microscopy on a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope (Carl Zeiss). The system had a krypton/argon laser for differential visualization of the fluorophores using a 488-nm excitation filter and a 522/535-nm emission filter for 488 and 568 nm excitation filters and a 605/632 nm emission filter for Alexa 594. The images were 512 × 512 pixels in size and the thickness of each optical section was 0.5 μm. Immunoreactive cells were scanned as a series of optical sections with a center spacing of 0.2 μm. Confocal images were collected using LSM 5 Image Zeiss processing software (Carl Zeiss MicroImaging, Germany). Images were further processed using Corel Photo Paint and Corel Draw software programs (Corel Corporation).

Quantitative analyses

The proportions of neurons in which antigen immunoreactivity was co-localized were determined by examining double-labeled neurons. Neurons were first located by the presence of a fluorophore that labeled one antigen, and then the filter was switched to determine whether the neuron was labeled for a second antigen, located with a second fluorophore of a different color. In this way, proportions of neurons labeled for pairs of antigens were determined. The cohort size was 100 neurons and data were collected from preparations obtained from at least four animals. The percentage of neurons immunoreactive to a second neurochemical was calculated and expressed as mean ± SE. The numbers of P2X$_2$-immunoreactive (IR), NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons and nerve cell perikarya were measured by examining the whole-mount preparations under a binocular microscope at a magnification of 100 ×. All neurons present in each 1 cm^2 were counted. The nerve cell perikarya profiles area, major axes, and minor axes of 50 nerve cell perikarya from each animal were obtained on a semiautomatic morphometry device, the Image-Pro Plus Program.

Table 2 Body weight and small-intestinal area of pre- and postnatal protein-deprived rats at 42 d postnatal

	N	D	DR
Body weight (g)	160 ± 10	40 ± 18[a]	100 ± 22
Area of small intestine (cm^2)	20.41 ± 3.7	13.3 ± 0.6[a]	16.5 ± 0.4

[a]$P < 0.05$ *vs* protein deprived, Tukey's test for multiple comparisons.

Statistical analysis

mean ± SE were calculated and compared by analysis of variance and Tukey test for multiple comparisons, as appropriate. The level of significance was set at $P < 0.05$.

RESULTS

The mean body weight of animals of the N group (160 ± 10 g) was approximately 400% greater than that of the D group (40 ± 18 g). The body weight of the DR group (100 ± 22 g) was restored to within 20% of normal at P42 ($P < 0.05$). The small intestine area of the D group was 34% less ($P < 0.05$) than that of the N group, and there was no statistical difference between the N and DR groups (Table 2).

The qualitative results demonstrated that P2X$_2$ receptor immunoreactivity was found in the myenteric and submucosal plexuses of the ileum of all groups. Positive labeling was seen in the cytoplasm and surface membranes of most nerve cells of the nourished, undernourished and refed groups (Figure 1). The labeling intensity of the P2X$_2$ receptor in the myenteric and submucosal ganglia of the N, D and DR groups was similar. Double-labeling studies were conducted to identify neurons that had P2X$_2$ immunoreactivity co-localized with NOS, calbindin, calretinin and ChAT in ileal myenteric neurons (Figure 1), and calbindin, calretinin and ChAT in the ileal submucosal plexus of the N, D and DR groups (Figure 2). In all groups, the cellular morphology of the myenteric plexus showed that NOS-IR neurons had a Dogiel Type I morphology, while calretinin-IR neurons exhibited Dogiel Type II morphology, and calbindin-IR neurons had both small and large Dogiel Type II neurons. In the submucosal plexus, calbindin-IR neurons had Dogiel Type II morphology. The intensity of ChAT immunoreactivity was reduced in some neurons of the myenteric and submucosal ganglia of undernourished rats.

Co-localization

The quantitative results revealed that, in the myenteric plexuses, the majority of NOS-IR, calbindin-IR, calretinin-

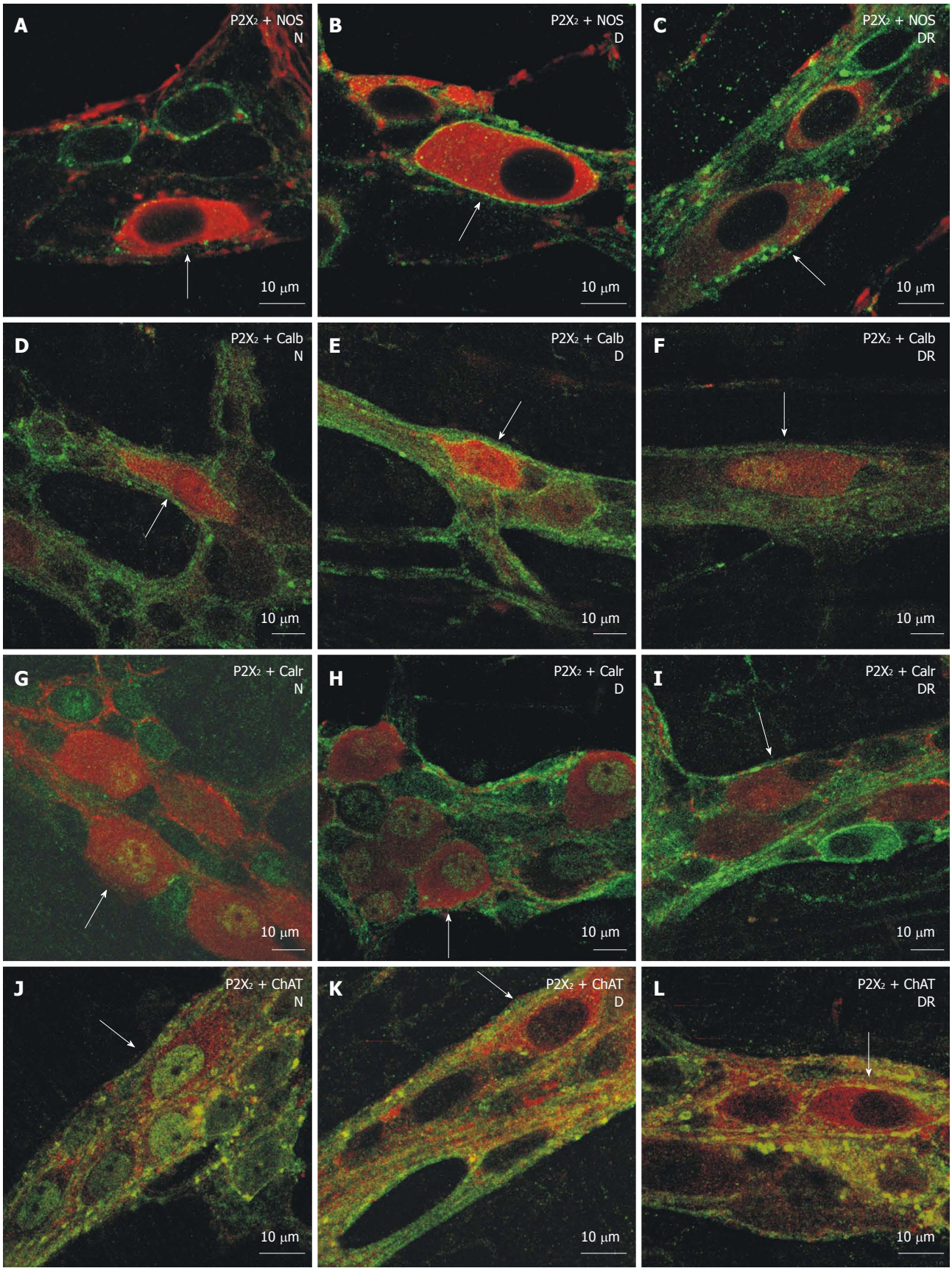

Figure 1 Co-localization of P2X2 receptor immunoreactivity with nitric oxide synthase, calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal myenteric plexus in the N, D and DR groups. A-C: P2X2 receptor-IR (green) co-localized with nitric oxide synthase (NOS)-IR (red); D-F: P2X2 receptor-IR (green) co-localized with calbindin-IR (red); G-I: P2X2 receptors (green) co-localized with calretinin (red); J-L: P2X2 receptors (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.

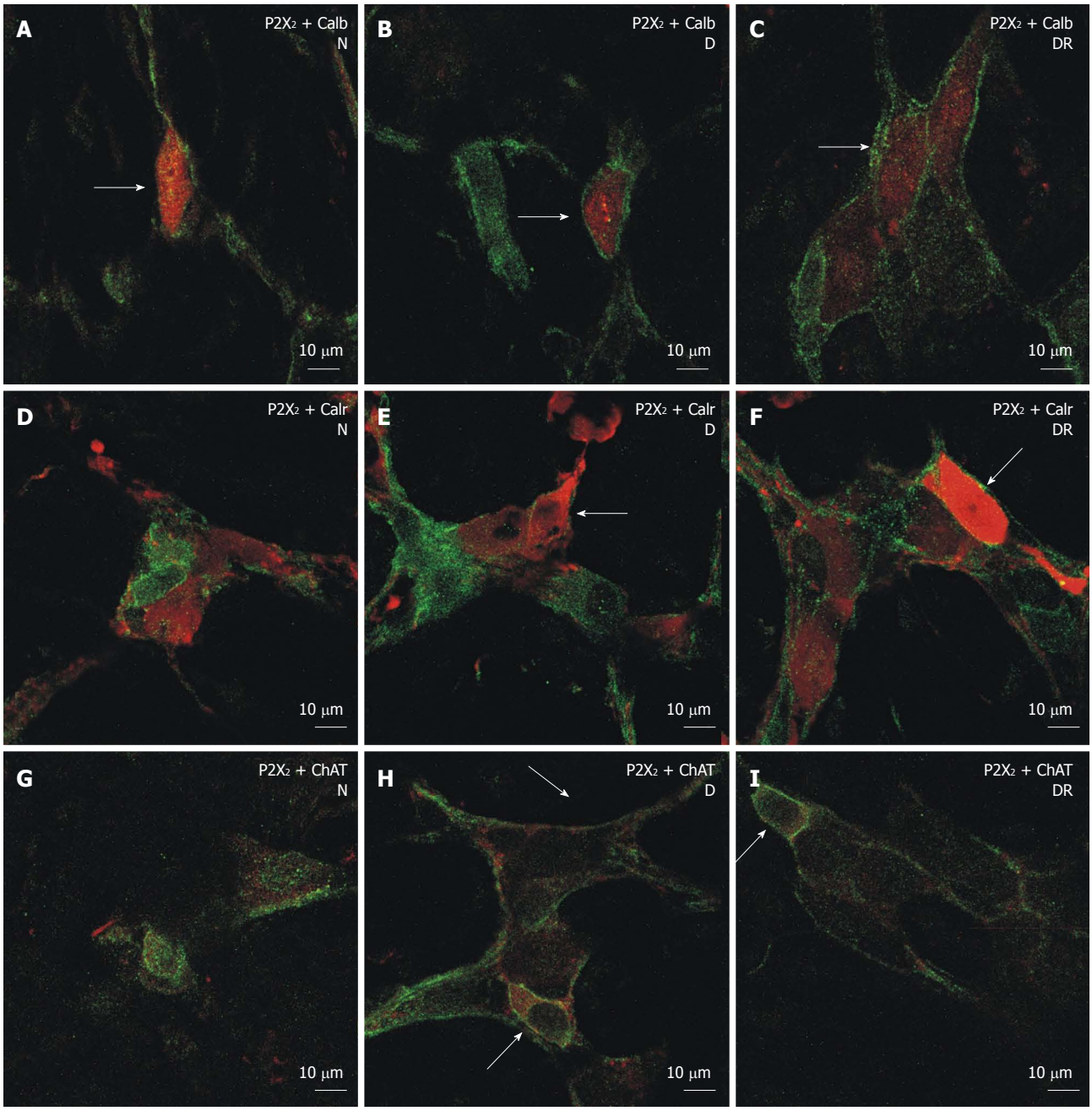

Figure 2 Co-localization of P2X2 receptor immunoreactivity with calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal submucosal plexus in N, D and DR groups. A-C: P2X2 receptor (green) co-localized with calbindin (Calb) (red); D-F: P2X2 receptor (green) co-localized with calretinin (Calr) (red); G-I: P2X2 receptor (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.

IR and ChAT-IR neurons also were immunoreactive for the P2X2 receptor. In the submucosal plexus of the ileum, P2X2-IR neurons were also calbindin-IR, calretinin-IR, and ChAT-IR.

In the myenteric plexus, the majority of NOS-IR neurons were immunoreactive for the P2X2 receptor (N group was 99% ± 0.6% co-localized, D group was 100%, and DR group was 99% ± 0.4%). Also, the majority of calbindin-IR neurons were IR for the P2X2 receptor (N group was 98% ± 0.4%, D group was 100%, and DR group was 99% ± 1%). The majority of calretinin-IR neurons were also IR for the P2X2 receptor (group N was 100%, D group was 98% ± 0.6%, and DR group was 98% ± 1%). Most ChAT-IR neurons were also IR for the P2X2 receptor in the N, D and DR groups (96.2% ± 2%, 96.2% ± 2%, and 97% ± 3%, respectively).

In the submucosal plexus, co-localization between calbindin-IR and P2X2 receptor-IR neurons was complete in the N, D and DR groups. The co-localization between P2X2 receptor-IR and calbindin-IR was 16% ± 0.7% in the N group, 31% ± 2% in the D group, and 24% ± 3% in the DR group ($P < 0.002$). In all three groups, calretinin-IR and ChAT-IR neurons co-localized 100% with P2X2 receptor-IR neurons.

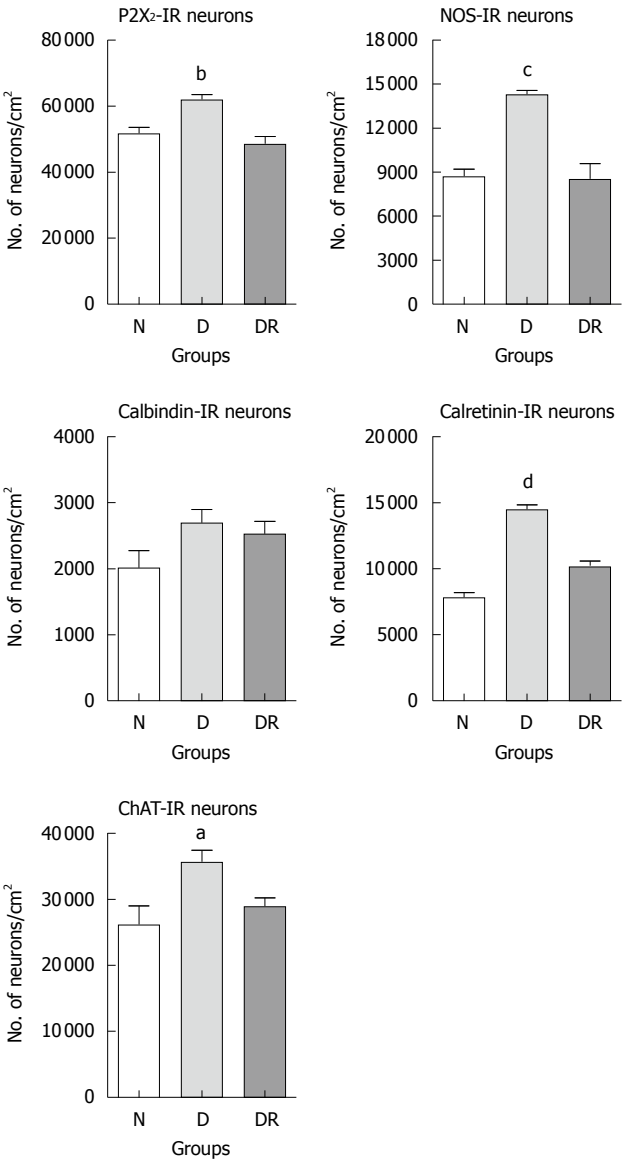

Figure 3 The density (neuron/cm^2) of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin, and choline acetyltransferase in the ileal myenteric plexus in the N, D and DR groups. $^aP < 0.02$, $^bP < 0.01$, $^cP < 0.002$, $^dP < 0.001$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

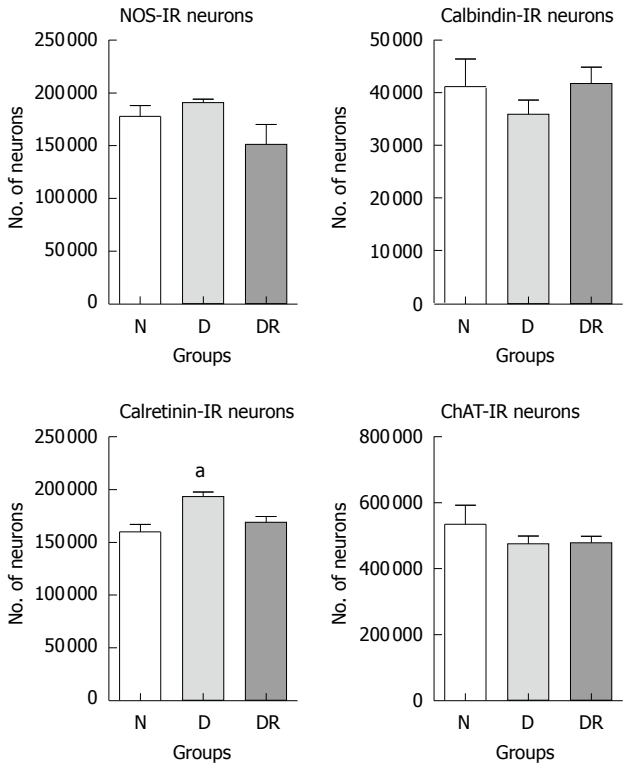

Figure 4 Calculated numbers of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin and choline acetyltransferase in the small intestine of the N, D and DR groups. $^aP < 0.05$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

Neuronal density

In the myenteric plexus, the number of neurons per unit area was increased by 19% for P2X$_2$ receptor-IR neurons ($P < 0.01$), 64% for NOS-IR neurons ($P < 0.002$), 84% for calretinin-IR neurons ($P < 0.001$), and 26% for ChAT-IR neurons in group D ($P < 0.02$); calbindin-IR neuron density, however, did not differ among the three groups ($P > 0.05$, Figure 3). In the myenteric plexus, the total number of NOS-IR neurons, taking into account the change in intestinal surface area (Figure 4), calbindin-IR neurons and ChAT-IR neurons did not differ significantly between the three groups. There was, however, a 20% increase in the numbers of calretinin-IR neurons and decrease in P2X$_2$ receptor cells with undernutrition relative to controls ($P < 0.05$, Figure 4).

In the submucosal plexus, the density of P2X$_2$ receptor-IR, calbindin-IR and ChAT-IR neurons increased significantly in the undernourished group (67%, $P < 0.0003$; 189%, $P < 0.001$ and 42%, $P < 0.01$, Figure 5). Calretinin-IR neuron density did not differ among the three groups ($P > 0.05$, Figure 5). In the submucosal plexus, the total numbers of the calretinin-IR neurons decreased by 23% ($P < 0.05$), and this was accompanied by an 89% increase in the calculated numbers of calbindin-IR neurons. In this region, there was no change in the numbers of ChAT-IR neurons (Figure 6).

Nerve cell perikarya

Neuron size (nerve cell perikarya, the major and minor axes of the myenteric plexus neurons) of the calretinin-IR neurons were approximately 34% smaller in the protein-deprived rats ($P < 0.001$) than the control or re-fed rats. There was an increase of 35% in the nerve cell perikarya of calbindin-IR neurons and a 14% increase in the minor axes of the calbindin-IR neurons in the DR group, as well as a decrease of 15% in the major axes of the NOS-IR neurons (Table 3).

In the submucosal neurons, there were group differences ($P < 0.05$) with respect to the neuron size of calbindin-IR, calretinin-IR and ChAT-IR neurons. There was a 13% decrease in the major axes of calretinin-IR and ChAT-IR neurons ($P < 0.05$) and an 18% increase in the minor axes of calbindin-IR neurons ($P < 0.05$). Neu-

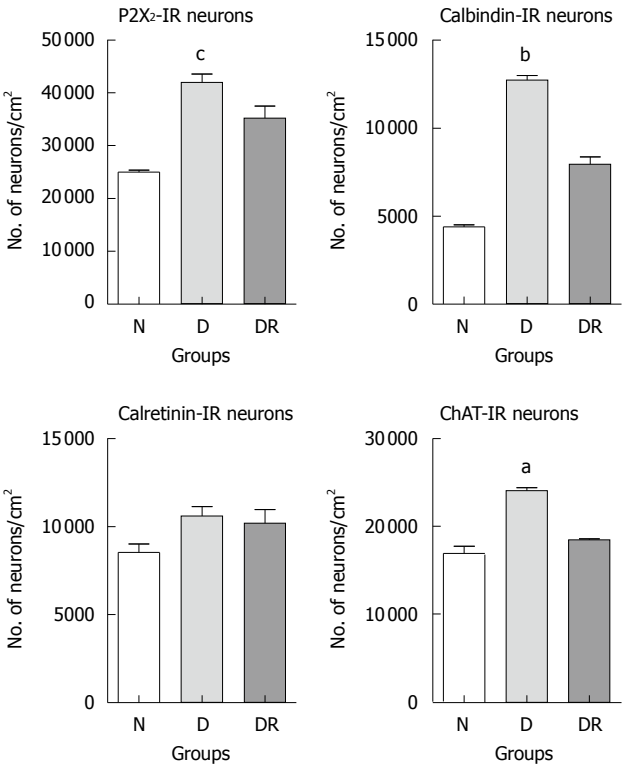

Figure 5 The density of neurons (neuron/cm^2) immunoreactive for P2X$_2$ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus in the N, D and DR groups. $^aP < 0.01$, $^bP < 0.001$, $^cP < 0.0003$ *vs* N and DR groups. ChAT: Choline acetyltransferase.

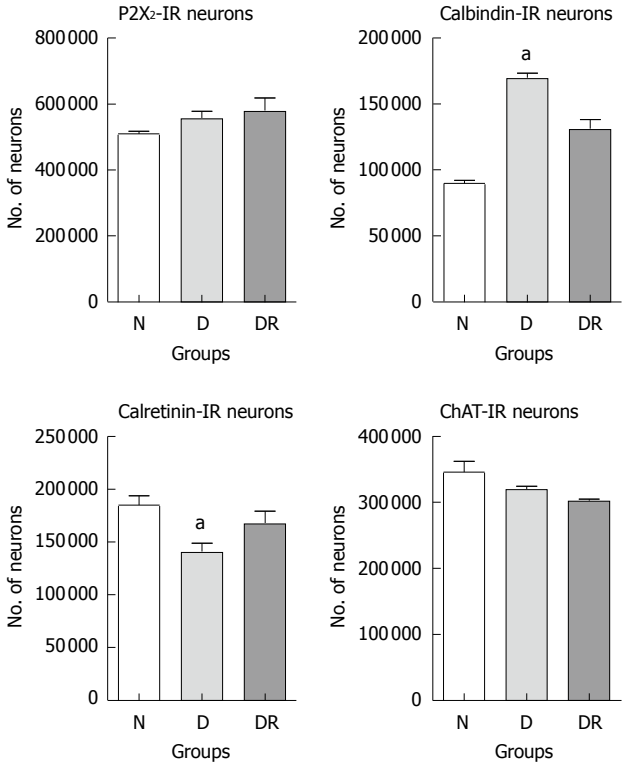

Figure 6 The calculated numbers of neurons immunoreactive for P2X$_2$ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus of the N, D and DR groups. $^aP < 0.05$ *vs* N and DR groups. ChAT: Choline acetyltransferase.

Table 3 Results of the profile area (μm^2), major and minor axes of nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons in the ileal myenteric and submucosal plexuses of the N, D and DR groups

	N	D	DR
Myenteric plexus			
NOS			
Profile	240.4 ± 30.7	197.1 ± 25.8	225.8 ± 26.7
Major axes	25.2 ± 2.1	21.7 ± 1.6^a	25.1 ± 1.9
Minor axes	12.1 ± 0.6	11.3 ± 0.8	11.4 ± 1.1
Calbindin			
Profile	227.7 ± 43.1	223.6 ± 26.3	307.5 ± 58^c
Major axes	23.5 ± 2.8	24.1 ± 2.9	27.9 ± 4.6
Minor axes	12.2 ± 1.1	11.9 ± 0.3	14.0 ± 0.8^c
Calretinin			
Profile	397.7 ± 39.6	259.2 ± 48.8^a	331.5 ± 24.5
Major axes	29.3 ± 2.1	22.3 ± 3.5^a	27.5 ± 2.7
Minor axes	16.7 ± 0.6	14.1 ± 0.5^a	15.1 ± 0.5
ChAT			
Profile	229.4 ± 39.4	183.6 ± 39.3	198.7 ± 37.5
Major axes	21.8 ± 2.4	19.4 ± 2.4	20.1 ± 2.5
Minor axes	12.8 ± 1.0	11.7 ± 0.7	12.1 ± 0.8
Submucosal plexus			
Calbindin			
Profile	244.2 ± 47.6	256.7 ± 34.8	310.7 ± 46.2
Major axes	24.8 ± 1.7	27.4 ± 2.2	27.4 ± 1.7
Minor axes	12.4 ± 1.4	12.2 ± 0.8^b	14.5 ± 1.2
Calretinin			
Profile	233.8 ± 51.5	200 ± 4.6	242.5 ± 41.6
Major axes	24.1 ± 2.1	20.8 ± 0.5^b	25.7 ± 3.1
Minor axes	12.4 ± 1.7	12.1 ± 0.3	12.1 ± 1.4
ChAT			
Profile	185.5 ± 18.4	154.3 ± 22.4	175.3 ± 20.0
Major axes	20.1 ± 1.7	17.5 ± 0.8^b	18.8 ± 0.7
Minor axes	11.4 ± 0.2	11.1 ± 0.9	11.5 ± 1.1

$^aP < 0.05$, $^bP < 0.001$ *vs* N and DR groups; $^cP < 0.05$ *vs* N and D groups. Tukey's test for multiple values, mean ± SE, $n = 5$. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

ron size distributions in the myenteric and submucous plexuses of the N, D and DR groups are shown in the histograms of Figures 7 and 8.

DISCUSSION

Various methods have been used to induce experimental undernutrition[26]. The protocols of undernutrition and re-feeding employed in this study were effective, because malnourished animals lost weight, which was then recovered by re-feeding. These findings agree with those of other studies that have used similar protocols[21,22].

The antigen markers for different functional classes of neurons have been determined for guinea pig and mouse small intestine, and to a lesser extent in other mammals[27-31]. The expression patterns have been partly described in the rat[32,33]. NOS is expressed in inhibitory motor neurons in all species in the small and large intestine, whereas all other neuron types, such as excitatory motor neurons, interneurons, and intrinsic primary afferent neurons (IPANs) are immunoreactive for ChAT in the mouse and rat myenteric plexus[30,33-37]. Dogiel Type II neurons, which are intrinsic primary afferent neurons in all species

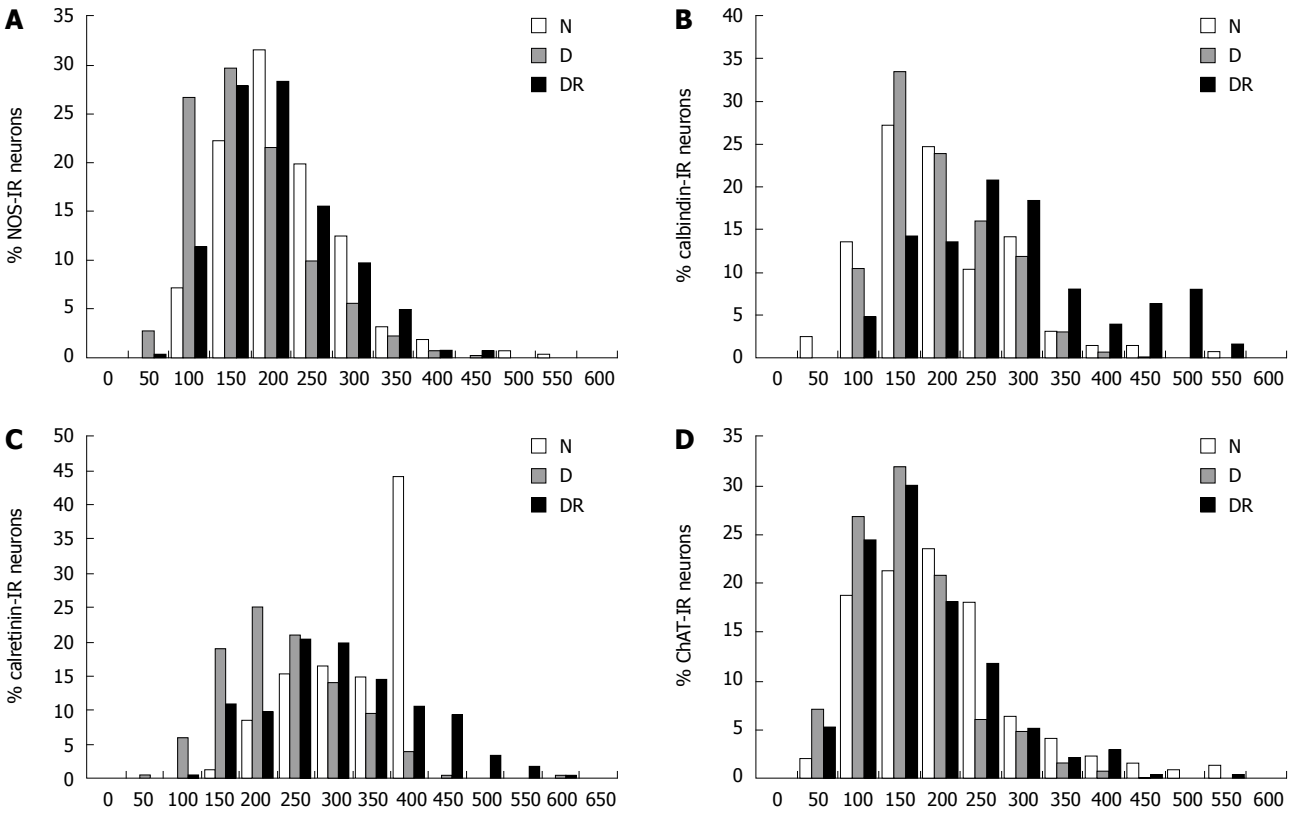

Figure 7 Histograms showing the distribution of areas (μm^2) of neurons immunoreactive for nitric oxide synthase (A), calbindin (B), calretinin (C) and choline acetyltransferase (D) in the ileal myenteric plexus of the N, D and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

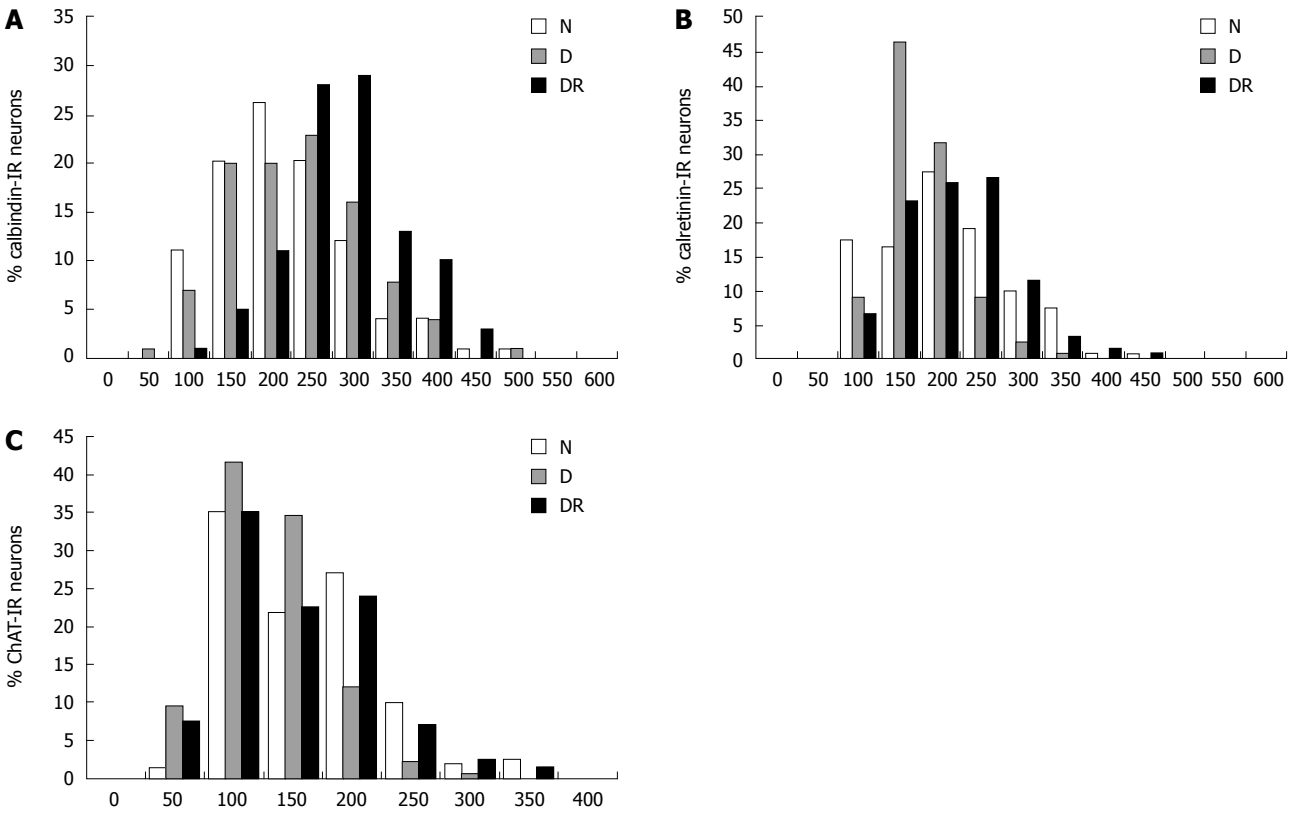

Figure 8 Histogram showing the distribution of areas (μm^2) of neurons immunoreactive for calbindin (A), calretinin (B) and choline acetyltransferase (C) in the ileal submucosal plexus of the N, D and DR groups. ChAT: Choline acetyltransferase.

studied, including rats[38], are immunoreactive for calretinin in the rat small intestine[33]. The subclasses of neurons in the submucosal ganglia of rat ileum have not been extensively studied but, by analogy with other small mammals, they are likely to include cholinergic and non-cholinergic secretomotor neurons and, possibly, IPANs[27,29,37]. In accordance with the data from rats and other small mammals, we chose the enzyme NOS to identify inhibitory motor neurons, ChAT to identify excitatory motor neurons and interneurons, calretinin to identify IPANs, and calbindin, which is a marker of many neurons in the rat small intestine. Within the three groups, the NOS-IR neurons had Dogiel Type I morphology and the calretinin-IR neurons in the myenteric and submucosal plexuses had Dogiel Type Ⅱ morphology while presenting various sizes. Calbindin-IR neurons exhibited four distinct morphologies: the Dogiel type Ⅱ neurons (large and small) and Dogiel type I neurons (small and elongated). These findings are consistent with the literature[37].

By qualitative analyses, there were no differences in neuron morphology between the N, D and DR groups. There was also no observed change in the labeling intensity of neurons immunoreactive for NOS, calretinin, and calbindin among the three groups. However, the intensity of ChAT immunoreactivity was reduced in some neurons of the undernourished group and increased in the re-fed group. These results are consistent with other studies in which a decrease in the intensity of ChAT immunoreactivity in the myenteric neurons of malnourished animals has been reported[22,39]. Other enzymes, such as NADH diaphorase, also show decreased immunoreactivity in malnourished and recovery in re-fed animals[21,22].

Previous studies have revealed the presence of $P2X_2$, $P2X_3$ and $P2X_7$ receptor-containing neurons in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. In the current work with the rat enteric nervous system, we found that $P2X_2$ receptors were present in both the cytoplasm and cytoplasmic membrane in neurons of the myenteric and submucosal plexuses.

The co-localization of different neuronal markers described in this work confirm the presence of $P2X_2$ receptors in NOS-IR, calretinin-IR, and calbindin-IR enteric neurons, as well as in ChAT-IR neurons of the myenteric and submucosal plexuses. ATP has been reported to depolarize 70%-90% of guinea-pig enteric neurons, which indicates that many enteric neurons have ionic P2X receptors[2,3]. In the myenteric plexus of rats, we found that the $P2X_2$ receptors exhibited complete co-localization with NOS-IR, calretinin-IR, calbindin-IR and ChAT-IR neurons in the three groups examined, without significant differences among them. This finding is consistent with the literature, which demonstrates the presence of the receptor in inhibitory neurons, as well as intrinsic excitatory and secretomotor/vasodilator primary afferent neurons in guinea pigs[7] and rats[12].

Our analyses of co-localization in the submucosal plexus showed that all calretinin-IR, ChAT and calbindin-IR neurons co-localized with $P2X_2$ receptor-IR neurons. However, there was a significant increase ($P < 0.05$) in the co-localization of $P2X_2$ neurons with calbindin-IR neurons in the undernourished group, which recovered in the re-fed DR group. This result agrees with Xiang and Burnstock's[12] findings, in which they reported expression of $P2X_2$ receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats. The co-localization that we described in the myenteric and submucosal plexuses suggested that malnutrition did not change neurochemical coding, for the markers that were used, in the enteric nervous system.

Changes in the density of myenteric neurons have been observed in various regions of the gastrointestinal tract in models of undernutrition[20-23,25,39] and recovery is observed in re-fed rats[21,22,25]. The increase in neuronal density in undernourished protocols is likely due to decreases in the surface area of the small or large intestine[20-22,39]. In our work on the myenteric plexus, neuron densities were increased for $P2X_2$ receptor-IR, NOS-IR, ChAT-IR and calretinin-IR neurons in the D group, and went back to control levels in the DR group. This increase in neuron density was due to a reduction of approximately 34% in small-intestinal area in the D group. There was recovery of the intestinal area in the DR group. In contrast, the density of calbindin-IR neurons in the myenteric plexus did not differ among the three groups ($P > 0.05$). Moreover, the increases in neuron density in the myenteric plexus in the D group were dependent upon the neuronal class examined. NOS-IR neuron density increased by 64%, calretinin-IR neurons by 84%, and ChAT-IR neurons by 26%; these data suggest that undernourishment affects the neuronal subtypes differently. There was no change in the calculation of the total number of NOS-IR, calbindin-IR or ChAT-IR neurons in the small intestine of the three groups. However, the calretinin-IR neuron numbers were increased (20%) in the undernourished group and $P2X_2$ receptor-IR neurons were decreased by around 25% in the D and DR groups.

The density of $P2X_2$-receptor-expressing neurons in the myenteric plexus in group N was about 51 000/cm^2 in our study. This value is higher than the combined sum of the two major neuronal subtype populations of the myenteric neurons: NOS (8000/cm^2) + ChAT (26 000/cm^2). This discrepancy could be due to $P2X_2$ receptor staining in another neuronal class, which was not immunoreactive for NOS, calbindin, calretinin or ChAT. Also, $P2X_2$ receptor labeling could have also stained enteric glial cells. The presence of P2X and P2Y receptors has been described in astrocytes and microglia of the central nervous system[40,41] and in enteric glial cells[9,42]. In the mammalian enteric nervous system, the proportion of glial cells to neurons is about three to one[43-45].

The tonic release of ATP into the extracellular space without a particular stimulus is a widespread physiological process. However, the release of ATP into the extracellular environment is also caused by pathophysiological events like inflammation, ischemia, injury as a consequence of cell damage or acute cell death, and metabolic

stress[46]. All physiological effects of ATP including fast purinergic transmission and co-transmission, the secretion of neuropeptides, and mechanosensory transduction might be amplified by overtly increased extracellular concentrations of ATP[46].

Studies from the literature have reported changes in the expression of purinergic receptors in different dietary conditions in the central nervous system. A diet deficient in zinc, for example, increases expression of $P2X_6$ receptors in the hippocampus of rats[47], which suggests that dietary zinc levels also affect protein expression and could act as a modulator of the receptor function. Increased $P2Y_1$ receptor mRNA expression in the hypothalamus after food restriction has been reported in rats[48], and the data indicate that expression of ADP/ATP-sensitive $P2Y_1$ receptors in the hypothalamus is dependent on feeding conditions. The enhanced expression of the $P2Y_1$ receptor during the early and late interval of restricted feeding suggests an increased demand for purinergic signaling to enhance the activity of hypothalamic neurons. Also, there is an indication of $P2Y_1$ and A_{2A} that purinergic receptor mRNA expression is altered during acute and chronic food deprivation[49]. Some authors have suggested that ATP/ADP, acting as extracellular signal molecules in the rat brain, is involved in the regulation of food intake, possibly depending on $P2Y_1$-receptor-mediated nitric oxide production[50].

During metabolic stress, such as hypoglycemia or brain ischemia, activation of different P2 receptors has been demonstrated *in vivo* and *in vitro*. The $P2X_2$ and $P2X_4$ receptors are upregulated after oxygen and glucose deprivation in organotypical slice cultures and in CA1 and CA3 pyramidal cells after *in vivo* ischemia in gerbils[51]. During *in vivo* and *in vitro* ischemia, the $P2X_7$ receptor density is upregulated in microglia and on astrocytes and neurons[52]. Prenatal protein malnutrition might increase circulating concentrations of ATP, and this increases $P2X_2$ expression in cells. Enhancement of $P2X_2$ receptors in the D group suggests an increased demand for purinergic signaling. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

Changes in neuronal expression of $P2X_{1-7}$ purinoceptors are frequently seen not only as a result of maturation and neuronal differentiation, but also after various types of acute insults to the central nervous system such as ischemia, hypoxia, mechanical stress, axotomy, and inflammation. Purinergic mechanisms are involved in the etiology of many neurodegenerative conditions, especially due to the large extracellular release of ATP, adenosine, and other neurotransmitters[46,53] upon neural damage. Prolonged stimulation of ATP receptors results in changes in the location and density of P2 receptors in the cell membrane[46]. Increased $P2X_3$ receptor expression has been observed in inflammatory bowel disease of the large intestine, which suggests that changes in this receptor can cause pain and dysmotility of the bowel[54].

Nitrergic neurons (using nitric oxide synthesized by NOS) and cholinergic neurons (those that use the acetylcholine synthesized by ChAT) represent two major subpopulations of myenteric neurons[55], although these patterns vary between guinea pigs[56], mice[30,57] and rats[33,36].

Our work implies that differences between groups in the total neuronal density in the myenteric plexus are comprised principally of changes in the NOS-IR-and ChAT-IR neuronal populations. The total neuron density in the myenteric plexus was approximately 34 800/cm^2 in the nourished group. This neuronal density is greater in comparison with that in previous studies, which have reported values of 15 000 to 20 000/cm^2[58], 10 000/cm^2[59] and 18 000/cm^2[60]. These differences could be, in part, due to methodology as well as the different ages and strains of rats used.

Marese *et al*[61] have quantified neuronal numbers in the myenteric plexus of the duodenum using the Giemsa histological method and myosin V pan-neuronal immunohistochemical labeling. These studies have demonstrated that the number of neurons/cm^2 decreases with animal age between 21 and 428 d. Our experiments used 42-d-old rats of the same lineage (Wistar), and the estimate of neuron density in our work is within the 21-60 d range reported by Marese *et al*[61] (21 d: Giemsa 89 335 neurons/cm^2; myosin V: 59 364/cm^2; 60 d: Giemsa: 47 814/cm^2; myosin V: 30 291/cm^2).

In the present work, the proportions of NOS-IR and ChAT-IR neurons were 32% and 68%, respectively, in the myenteric plexus of the malnourished group. Consistent with previous studies[35,56], we found that these proportions were maintained in the D and DR groups.

Submucosal plexus

ChAT-IR neurons comprised the majority of submucosal plexus neurons in the three groups. These findings agree with prior studies, which have described most neurons of the submucosal plexus as ChAT-IR[33,56]. We demonstrated, for the first time, an increase in the density of $P2X_2$ receptor-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in the deprived animals, which returned to control levels in the re-fed animals in the submucosal plexus. This increase was due to a 34% reduction in the area of the small intestine in the deprived animals.

The total number of calbindin-IR submucosal neurons increased in the small intestine of undernourished animals, in contrast to a decrease in calretinin-IR neurons and no change in the number of ChAT-IR neurons. These data indicate that the lack of protein nutrition can also have an impact on the chemical coding of the submucosal plexus. Differences in these measures between the myenteric and submucosal plexuses might reflect a differential effect of malnutrition or undernutrition on these two regions, as well as a differential effect of undernourishment on each neuronal subtype. In addition, the increase of calbindin-expressing neurons in the submucosal plexus could be a compensatory mechanism in response to the decrease in these neurons in the myenteric plexus.

Neuronal sizes

Previous immunohistochemical studies have shown that

undernutrition affects the neuron size profile of the gastrointestinal tract[20,21,39]. Analyses using the Giemsa technique[20,62] and histochemistry[22] have found no significant differences in the neuronal sizes in the small intestine in nourished, undernourished and re-fed animals. The present work, using an immunohistochemistry technique, was unable to verify exactly which neuronal class showed changes in size. In the myenteric plexus, there were decreases in size of the calretinin-IR neurons in groups D and DR. There was also an increase in the size of calbindin-IR neurons in the DR group, compared to the N and D groups. There was no change ($P > 0.05$) in the size of ChAT-IR neurons among the three groups. The size of NOS-IR neurons also did not change, consistent with previous reports[58]. In the submucosal plexus, the sizes of calbindin-IR, calretinin-IR and ChAT-IR neurons were not affected by undernutrition. However, the major axes of the calbindin-IR and minor axes of the calretinin-IR and ChAT-IR neurons decreased in group D, with recovery in group DR. The differences between the submucosal and myenteric plexuses suggest again that undernutrition affects the two plexuses differently. The distribution areas of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in our study ranged from 100 to 500 μm^2, in agreement with previous reports[22].

The current study demonstrates that both undernourishment and re-feeding has a different impact on neuronal subtypes. Undernutrition also differently affects the myenteric and submucosal plexuses; changes in calbindin-IR neuronal density in the submucosal plexus were not reflected in the myenteric plexus, where only the profile of the calretinin-IR neurons was affected by dietary restriction. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

ACKNOWLEDGMENTS

We would like to thank Professor Jackson Cioni Bittencourt and Assistant Professor Carol Fuzeti Elias for providing the Leica Microscope for the analyses.

COMMENTS

Background

The myenteric and submucous plexuses are affected in undernourished and re-fed rats.

Research frontiers

The chemical coding and $P2X_2$ receptor expression following malnourishment and re-feeding are unknown.

Innovations and breakthroughs

The present study showed the effects of undernourishment and re-feeding on the morphology of the $P2X_2$-immunoreactive (IR), nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons of the myenteric and submucosal plexuses.

Applications

The present study suggests that re-feeding can restore almost all of the changes to inhibitory intrinsic primary afferent neurons and cholinergic neurons seen in undernourished animals.

Peer review

This is a study of the effects of deprivation and restoration of protein on neuronal development in the submucosal and myenteric plexuses. The results describe changes induced by protein deprivation that are largely reversible by re-feeding.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3664

World J Gastroenterol 2010 August 7; 16(29): 3664-3673
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Proteomic analysis reveals molecular biological details in varioliform gastritis without *Helicobacter pylori* infection

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Received: January 13, 2010 Revised: March 19, 2010
Accepted: March 26, 2010
Published online: August 7, 2010

Abstract

AIM: To investigate and elucidate the molecular mechanism underlying varioliform gastritis for early detection, prevention and intervention of gastric cancer.

METHODS: A combination of two-dimensional gel electrophoresis and mass spectrometry was used to detect the differentially expressed proteins between varioliform gastritis and matched normal mucosa. The selected proteins were confirmed by Western blotting and reverse transcription polymerase chain reaction (RT-PCR) in additional samples and the function of some proteins in varioliform gastritis was analyzed by bio-method preliminarily.

RESULTS: We identified 21 differentially expressed proteins in varioliform gastritis, and compared them with matched normal mucosa. Eleven proteins were upregulated and ten downregulated in varioliform gastritis when compared with the same proteins in individual-matched normal gastric mucosa. These proteins are related to metabolism, oxidation, cytoskeleton, apoptosis, signal transduction and other aspects of cells. Two novel proteins, thioredoxin domain-containing protein 5 (TXNDC5) upregulated in varioliform gastritis, and neuropolypeptide h3 [phosphatidylethanolamine-binding protein 1 (PEBP1)] downregulated in varioliform gastritis, were further investigated. Their expressions were validated by Western blotting and RT-PCR in 12 cases of varioliform gastritis which was matched with normal mucosa. The expression level of PEBP1 in varioliform gastritis was significantly lower ($P < 0.05$) while that of TXNDC5 was significantly higher than that in matched normal gastric mucosa ($P < 0.05$).

CONCLUSION: There are some changes of protein expression in varioliform gastritis. Downregulation of PEBP1 and upregulation of TXNDC5 are involved in the development of varioliform gastritis.

Key words: Differentially expressed proteins; Varioliform gastritis; Proteomic study; *Helicobacter pylori*

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Zhang L, Hou YH, Wu K, Zhai JS, Lin N. Proteomic analysis reveals molecular biological details in varioliform gastritis without *Helicobacter pylori* infection. *World J Gastroenterol* 2010; 16(29): 3664-3673 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3664.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3664

INTRODUCTION

Varioliform gastritis is currently recognized as a special kind of chronic gastritis characterized by nodules, thickened fugal folds and erosions. These features appear to be

unusual and different from those seen in chronic gastritis. The diagnosis can be easily made by endoscopic examination. But the morbidity of varioliform gastritis has increased quickly recently in China. Very little is known about the etiopathogeny, clinical significance and evolution of this disease. The molecular biological researches on varioliform gastritis are very limited and no proteomics research on this disease has been found up to date. So the molecular mechanism of this disease is still unclear. The role of *Helicobacter pylori (H. pylori)* remains unknown. Although a close relationship between this gastritis and the bacteria was suggested to exist over the last few years, But no *H. pylori* infection was found in the gastric mucosa of some patients with varioliform gastritis. What is the reason?

Gastric cancer is the second most common malignancy in the world. Each year, about 798 000 people are diagnosed as having gastric cancer (9.9% of total cancer cases) and 628 000 people die from the disease (12.1% of cancer deaths)[1]. In eastern Asian countries including China, the morbidity and mortality of gastric cancer have ranked the first among all kinds of cancer and grown rapidly in the past two decades. Gastric carcinogenesis is not a well-known process, and the central paradigm for the initiation and development of gastric carcinoma is still not very clear.

In 1960, Munoz Monteavaro *et al*[2] reported varioliform gastritis with "*in situ*" carcinomatous transformation. It was reported a case of ampullary carcinoma accompanied with gastroenteropathy due to diffuse varioliform gastritis. Similarly, Cappell *et al*[3] reported adenomatous transformation in a patient with varioliform gastritis who had serial gastroscopies. This report also suggests a possible association between varioliform gastritis and gastric neoplasia. Several other groups have reported similar findings and performed a more comprehensive analysis of relationship between varioliform gastritis and gastric cancer[4-6].

The elevations could persist and appear as sessile polyps after the erosions heal and symptoms relieved after treatment. Adenomatous transformation was reported in some patients with varioliform gastritis. These reports suggested a possible association between varioliform gastritis and gastric neoplasia. Although this disease was concluded as a kind of precursor disease of gastric cancer at Sydney Conference, the mechanism of carcinogenesis from varioliform gastritis was unknown. Gastric cancer might be effectively controlled if this premalignant lesion-varioliform gastritis-is detected and treated before invasion occurs. Therefore, it is crucial to elucidate the molecular mechanism underlying varioliform gastritis. Some current mechanistic models focus almost on the localized lesion or *H. pylori* infection, with much less attention paid to pathologic changes occurring in the normal-appearing mucosa without *H. pylori* infection from which such lesions emerge.

The pattern of expressed proteins can reflect the information about the functional status and health of the tissue. Recently, the development of new methods for protein analysis has led to the emergence of a new field of clinical proteomics, in which these techniques are harnessed to identify functional molecular or biomarkers of cancer and other diseases[7], but there is hardly any study on the differential expressions of proteins in varioliform gastritis and normal-appearing mucosa.

In the present study, we used proteomic techniques to test the hypothesis that normal gastric mucosa from a patient with varioliform gastritis would exhibit different pattens of protein expression with the disordered mucosa from the same patient. By this approach, comparison of anatomically normal and disordered tissues against the same genetic background could be made.

MATERIALS AND METHODS

Sample collection

Samples were taken from 17 patients with varioliform gastritis in the Second Affiliated Hospital of General Hospital of PLA (Table 1). These patients were examined by ^{13}C urea breath test and the results were all negative. The results of autoantibody detection were also negative in these patients. The case of *H. pylori* infection and autoimmune disease was excluded. Normal gastric mucosa was defined as that 5cm adjacent to the elevations. All samples were obtained by biopsy in endoscopic examinations for these patients. Four pieces of elevatory tissues and normal mucosa were collected from each patient, respectively. One piece of the elevatory tissue underwent pathological diagnosis, and the others were saved for future studies. The patients were well informed in accordance with the disciplines of the Ethics Committee of Biomedicine, General Hospital of PLA, China.

All samples were snap-frozen in liquid nitrogen and stored in a deep freezer (-80℃) until used. Tissues (80-150 mg) were crushed in liquid nitrogen and lysed in 1 mL of 7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 65 mmol/L dithiothreitol (DTT), and 0.2% Bio-Lyte (pH 5-8, Bio-Rad, Hercules, CA) with sonication on ice. The lysates were centrifuged at 20 000 × *g* for 1 h at 4℃. Supernatants were removed and concentrations were determined by the Bio-Rad AC DC protein assay kit (Bio-Rad). The protein samples were stored at -80℃. Before 2-DE was performed, the protein samples were purified using the Readyprep 2-D cleanup kit (Bio-Rad) according to the manufacturer's instructions.

Clinical data of samples

Detailed clinical and pathological data from the health care information center were reviewed. None of the patients had received treatment prior to endoscopic examination. Of the 17 patients, 11 were men, and six were women; the mean age was 51 years (range, 34-72 years, Table 1). No patient suffered from varioliform gastritis with other concurrent gastric diseases. All tissues of varioliform gastritis had definite histologic diagnoses: acute and chronic mucosal inflammation (n = 12), acute and chronic mucosal inflammation with lymphocytic infiltration (n = 5). None of them had *H. pylori* infection or low-to-moderate dysplasia.

Table 1 Characteristics of varioliform gastritis patients in this study

Patient No.	Sex	Age (yr)	Lesion site	Histology
1	F	77	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
2	F	54	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
3	F	59	Gastric body and gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
4	F	43	Gastric body and gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
5	F	62	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
6	F	68	Gastric body and gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
7	M	44	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
8	M	36	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
9	M	76	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
10	M	67	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
11	M	55	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)
12	M	45	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
13	M	72	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
14	M	57	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)
15	M	61	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
16	M	51	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
17	M	78	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)

H. pylori: *Helicobacter pylori*.

Two-dimensional gel electrophoresis

Individual paired samples of normal gastric mucosa and varioliform gastritis were analyzed by 2-DE as described by Xing previously[8]. Briefly, linear gradient 24-cm (pH 5-8) ready strip (Bio-Rad) was rehydrated overnight at 16℃ with 200 μg of protein samples in 500 μL of rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L DTT, and 0.2% Bio-Lyte). Isoelectric focusing (IEF) was performed using PROTEAN IEF Cell (Bio-Rad). After IEF, the immobilized pH gradient strip was immediately equilibrated in equilibration buffer Ⅰ [6 mol/L urea, 2% sodium dodecyl sulfate (SDS)], 0.375 mol/L Tris-HCl pH 8.8, 20% glycerol, and 2% DTT) for 15 min and then in equilibration buffer Ⅱ (6 mol/L urea, 2% SDS, 0.375 mol/L Tris-HCl pH 8.8, 20% glycerol, and 2.5% iodoacetamide) for 15 min. SDS-polyacrylamide gel electrophoresis was carried out on 12% SDS-polyacrylamide gels (25 cm × 20.5 cm × 1.0 mm) using the PROTEAN Plus Dodeca Cell (Bio-Rad) at a constant voltage of 200 V at 20℃. After electrophoresis, the gels were stained using the Silver Stain Plus Kit (Bio-Rad). The above processes were performed in triplicate for each sample.

Gel imaging and analysis

The silver-stained 2-DE gels were scanned on a GS-800 Calibrated Imaging Densitometer (Bio-Rad) at a resolution of 300 dots per inch. Intensities of protein spots were analyzed with Amersham Biosciences-Imagemaster v5.0. The differential protein spots were defined as those having a 5-fold higher or lower level of differential expression in at least 9 cases compared with the normal mucosa.

Spot cutting and in-gel digestion

The 17 samples were used for spot cutting. Equal protein masses of each sample (normal gastric mucosa and varioliform gastritis tissue) were pooled, and 300 μg of the mixture was loaded for 2-DE. The differentially expressed protein spots were identified as described in the preceding text. These spots were excised from gels by Proteomeworks Spot Cutter (Bio-Rad), destained for 20 min in 30 mmol/L potassium ferricyanide/100 mmol/L sodium thiosulfate [1:1 (v/v)], and washed in Milli-Q water until the gels shrank and bleached. The gel pieces were incubated in 0.2 mol/L NH_4HCO_3 for 20 min and dried by lyophilization. Twenty microliters (20 μg/mL in concentration) trypsin (proteomics grade, Sigma, St. Louis, MO) was added into each gel piece, and incubated at 37℃ overnight. The peptides were extracted three times with 50% acetonitrile and 0.1% trifluoroacetic acid and dried in a vacuum centrifuge.

Mass spectrometry

The digests were analyzed using a Bruker Autoflex Ⅱ TOF/TOF mass spectrometer with delayed extraction in which α-cyano-4-hydroxycinnamic acid was exploited as the matrix. The total 2-μL solution was applied onto a target disk and allowed to air-dry. Mass-to-charge ratios were measured in a reflector/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 63%-65%, positive polarity, and a delay time of 200 nanoseconds. Laser shots at 300 per spectrum were used to acquire the spectra from 800 to 4000 Daltons. Trypsin autolysis products were used for internal mass calibration. Database searching was performed using Mascot software (http://www.matrixscience.com). The search parameters were the nrNCBI database, human, 10-150 kDa, trypsin (1 missed enzymatic cleavage), and 100-ppm mass tolerance. The best match was the one with the highest score, and a significant match was typically a score of more than 70 ($P < 0.05$).

Western blotting analysis

After the analysis of selected proteins, two differential proteins were confirmed by Western blotting analysis in additional samples for validating the 2-DE results. West-

ern blotting analysis was performed in 12 cases of varioliform gastritis with individual-matched normal mucosa. Tissue samples were lysed as described above and protein extracts (50 μg) were separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a poly-vinylidene difluoride membrane (Bio-Rad). After blocking, the membranes were incubated with a rabbit monoclonal antibody of phosphatidylethanolamine-binding protein 1 (PEBP1) (dilution of 1:2000; Epitomics, California, MA) and polyclonal goat anti-thioredoxin domain-containing protein 5 (TXNDC5) antibody (dilution of 1:1000; Cell Signaling Technology, Danvers, MA). Subsequently, the membranes were incubated in horseradish peroxidase-anti-rabbit and horseradish peroxidase-anti-goat IgG (Abcam, Cambridge, UK), respectively. The specific proteins were visualized with chemiluminescent reagent (Pierce Biotechnology, Rockford, IL). As a control for equal protein loading, blots were restained with anti-actin antibody (dilution of 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). The band intensity was analyzed by PDQuest software v7.1. The relative expression level was calculated as the intensity ratio of PEBP1 or TXNDC5 to that of actin. The association between categorical data was analyzed using the SPSS11.0 software package.

Reverse transcription polymerase chain reaction of TXNDC5 and PEBP1

The total RNAs of additional samples were extracted by homogenization in Trizol (Invitrogen) for validating the 2-DE results. cDNA synthesis was performed in 20 μL reaction system of reverse transcription including 5 μg RNA. Amplification of TXNDC5, PEBP1 and β2-MG acting as internal control was carried out in DNA thermal cycler (Perkin Elmer) using equal cDNA as template. PCR products were separated by 1.5% agarose gel electrophoresis, scanned and analyzed with VDS ImageMaster system (Pharmacia).

Preliminary functional analysis of TXNDC5 and PEBP1

To understand the function of TXNDC5 and PEBP1 in varioliform gastritis, they were imported into Pathway Studio (demo), and a visualized interaction map was generated with information from Ensembl database, the Pfam protein family database, Prosite database, GNF GeneAtlas database and PDB database. Each node represents either a protein entity or a control mechanism of the interaction. We intended to find the key pathway including TXNDC5, PEBP1 and other proteins in our proteomics research by analyzing the protein interaction networks.

Statistical analysis

SPSS11.0 statistical software was used for the statistical analysis.

The gray values of the protein candidates were analyzed by the nonparametric Wilcoxon test. The intensity ratio of PEBP1 or TXNDC5 to that of internal control in Western blotting or reverse transcription polymerase chain reaction (RT-PCR) analysis was analyzed by one-factor analysis of variance.

RESULTS

Differential protein expression of varioliform gastritis

The 2-DE protein patterns were studied in 17 patients with varioliform gastritis and individual-matched normal mucosa tissues. About 1800 proteins were detected in each gel. The proteins expressed in varioliform gastritis were compared with those in matched normal tissues. The differentially expressed candidates were the protein spots having a 5-fold higher or lower level of differential expression in at least 9 cases (Figure 1A and B). In this study, 21 significantly different candidate protein spots were found. They were also present in the 2-DE gel. Eleven proteins were upregulated and 10 downregulated in varioliform gastritis compared with the same proteins in individual-matched normal gastric mucosa. The quantities of all detected spots were analyzed by the nonparametric Wilcoxon test. These candidate spots were then analyzed by mass spectrometry (MS), and a total of 18 proteins (Figure 1C and Table 2) were identified. We failed to detect three protein spots. There might be several reasons, such as lower abundance, errors in the operation, lower reliability of the MS results, and characteristics of these proteins. More work will be done on the three protein spots in the future studies.

Validation of PEBP1 and TXNDC5 by Western blotting

The two novel candidate proteins, PEBP1 and TXNDC5, were studied further among the differentially expressed proteins. Their expression profiles in varioliform gastritis have not been reported previously. Western blotting analysis showed that TXNDC5 was upregulated significantly in varioliform gastritis but not in normal gastric mucosa (mean ± SD: 0.37 ± 0.05) (Figure 2B). Compared with that in normal mucosa (mean ± SD: 0.76 ± 0.12), PEBP1 was significantly downregulated in varioliform gastritis (mean ± SD: 0.18 ± 0.08) ($P < 0.05$, by Student's test or the Friedman test) (Figure 2A).

Validation of PEBP1 and TXNDC5 by RT-PCR

Using semiquantitative RT-PCR, 476 bp fragment of TXNDC5, 451 bp fragment of PEBP1 and 876 bp control fragment of β2-MG were amplified (Figure 3). The mean ratios of the absorbency of PEBP1 band normalized to the control band were 0.35 ± 0.09 and 1.23 ± 0.27 in 12 cases of varioliform gastritis and normal mucosa. *P* value was lower than 0.05 when Student's *t* test was used to compare the ratios of the two groups (Figure 3B). Those of TXNDC5 were 1.15 ± 0.07 and 0.23 ± 0.06 in 12 cases of varioliform gastritis and normal mucosa, respectively ($P < 0.05$) (Figure 3A). The results suggested that the difference of TXNDC5 and PEBP1 between varioliform gastritis and normal mucosa could be obvious at the mRNA level.

Preliminary functional analysis

TXNDC5 and PEBP1 were imported into Pathway Studio (demo) to build an interaction network. The connectivity of TXNDC5 and PEBP1 was 40 and 145, respectively. The average connectivity of proteins identified was about

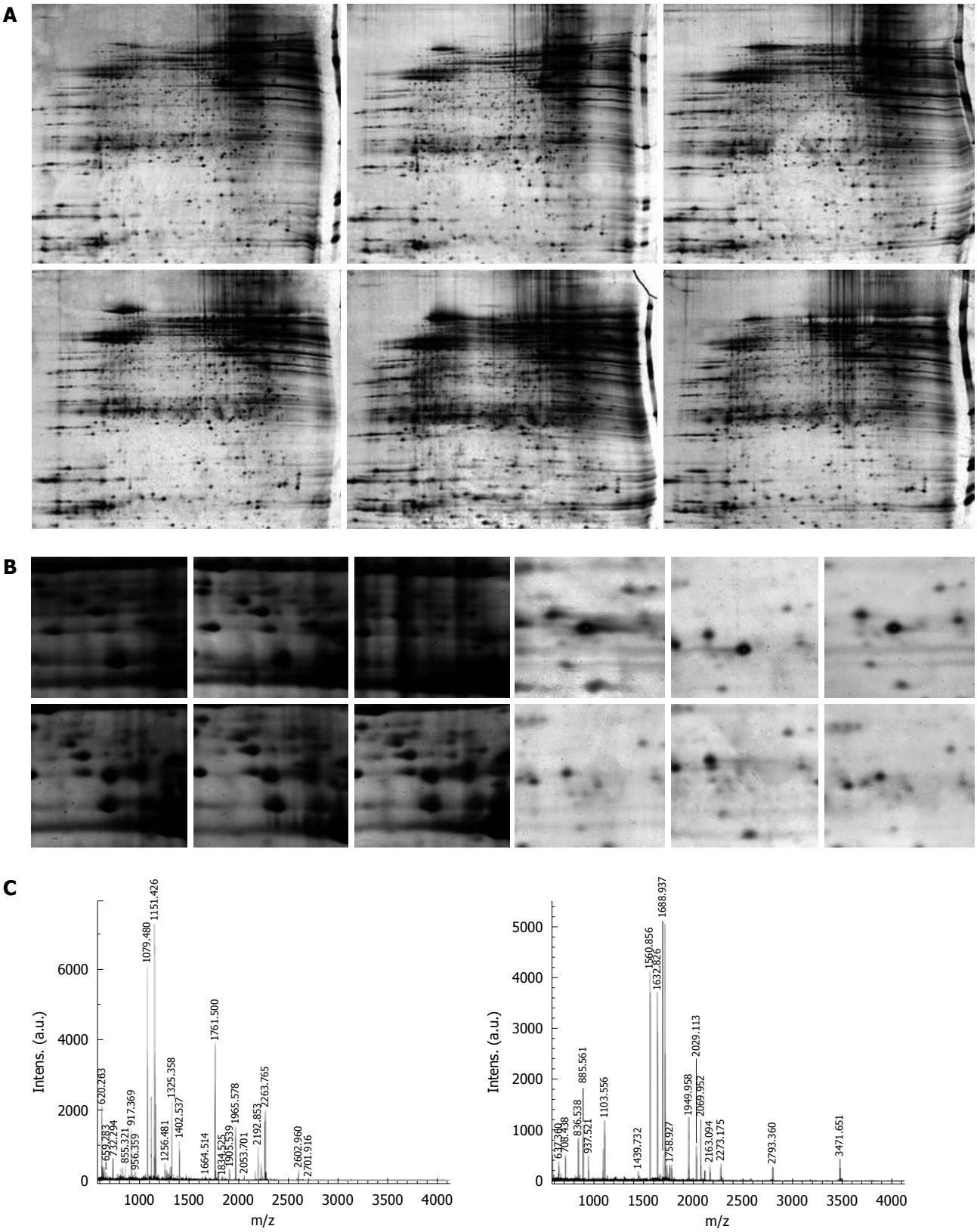

Figure 1 Detection and analysis of differentially expressed proteins in varioliform gastritis. A: Representative 2-DE images of matched varioliform gastritis and normal gastric mucosa tissue. The proteins expressed in varioliform gastritis were compared with those expressed in matched normal tissue. The protein spots that showed more than 5-fold differential expression in at least nine cases were taken as differentially expressed candidates. Of 21 differentially expressed protein spots, 18 were identified by mass spectrometry (MS) (protein nomenclature can be seen in Table 2); B: The magnified regions of the 2-DE gel of upregulated thioredoxin domain-containing protein 5 (TXNDC5) (left) and downregulated phosphatidylethanolamine-binding protein 1 (PEBP1) (right) in varioliform gastritis, compared with normal tissue; C: MS of in-gel trypsin digests of these proteins and analysis of the depicted peptide spectrum resulted in the identification of TXNDC5 (left) and PEBP1 (right).

Table 2 Differentially expressed proteins in varioliform gastritis

ID No.	Protein name	Gene name	Accession No.	Mass (Da)/pI	Cover rate (mean, %)	Mascot scores	Intensity of candidate protein spots[a] (positive rate)	General function/comments
	Up-regulated proteins							
1	Thioredoxin domain containing protein 5 precursor	*TXNDC5*	Q8NBS9	47629.2/5.3	25	228	36.5 ± 3.1, 2.7 ± 0.4 (100%, 44.4%)	Controlling the oxidative protein folding in endoplasmic reticulum
2	Proliferating cell nuclear antigen	*PCNA*	P12004	28762.4/4.7	37	184	18.6 ± 4.2, 3.3 ± 0.9 (94.4%, 70.5%)	Cell growth and maintenance
3	40S ribosomalprotein SA	*RPSA*	P08865	32702.4/4.79	46	104	64.3 ± 11.1, 11.5 ± 2.8 (100%, 88.3%)	Originally known as laminin receptor precursor and p40
4	Heat-shock protein β-1	*HSPB1*	P04792	22768.5/5.9	33		73.8 ± 13.4, 12.7 ± 3.4 (100%, 100%)	A HSP27 isoform (pI5.68)
5	Inorganic pyrophosphatase2	*PPA2*	Q9H2U2	35472/5.9	69	245	24.1 ± 2.7, 3.6 ± 1.2 (94.4%, 58.9%)	Overexpressed in some cancer tissues
6	S100 calcium-binding protein A10	*S100A10*	P60903	11195.5/6.8	62	148	43.0 ± 5.7, 5.9 ± 2.7 (88.9%, 41.2%)	It may function as a regulator of protein phosphorylation in the ANXA2 monomer
7	Nucleoside diphosphate kinase A	*NME1*	P15531	17137.7/5.8	49	193	59.4 ± 12.6, 13.2 ± 5.8 (82.4%, 70.6%)	It plays a major role in the synthesis of nucleoside triphosphates other than ATP
8	Proteasome activator complex subunit 1	*PSME1*	Q06323	38966.2/7.6	53	138	83.8 ± 17.4, 10.5 ± 4.7 (100%, 41.2%)	Implicated in immuno-proteasome assembly and required for efficient antigen processing
9	Ubiquitin thiolesterase L3	*UCHL3*	P15374	26337/4.7	42	174	62.4 ± 11.9, 13.7 ± 7.1 (88.9%, 29.4%)	Ubiquitin-protein hydrolase involved in the processing of both ubiquitin precursors and ubiquitinated proteins
10	S100 calcium-binding protein A6	*S100A6*	P06703	11732.8/5.6	71	234	21.6 ± 5.3, 3.7 ± 1.1 (82.4%, 23.5%)	Preferentially expressed when quiescent fibroblasts are stimulated to proliferate
	Down-regulated proteins							
11	Cell division cycle 2-like protein kinase 5	*CDC2L5*	Q14004	48212.2/8.3	37	168	1.7 ± 0.5, 14.2 ± 2.6 (17.6%, 52.9%)	May be a controller of the mitotic cell cycle involved
12	BTG3 protein	*BTG3*	Q14201	29117.3/9.1	64	265	5.8 ± 2.3, 47.4 ± 6.1 (29.4%, 82.4%)	Overexpression impairs serum-induced cell cycle progression from the G0/G1 to S phase
13	Neuropolypeptide h3	*PEBP1*	P30086	31270.6/5.7	58	220	2.9 ± 1.4, 58.6 ± 11.8 (17.6%, 100%)	Binds ATP, opioids and phosphatidylethanolamine
14	Heat-shock protein 17 kDa	*HSPB3*	Q12988	16966/5.7	39	105	16.8 ± 4.4, 89.5 ± 14.7 (100%, 100%)	Inhibitor of actin polymerization
15	Caspase-5 precursor	*CASP5*	P51878	47815/9.2	48	201	7.6 ± 2.6, 45.9 ± 11.7 (41.2%, 77.8%)	Mediator of apoptosis
16	Cytokeratin 20	*KRT20*	P35900	48487/4.9	52	131	2.9 ± 0.4, 17.5 ± 3.6 (29.4%, 52.9%)	It plays a significant role in maintaining keratin filament organization in intestinal epithelia. When phosphorylated, it plays a role in the secretion of mucin in the small intestine
17	Eukaryotic translation initiation factor 3 subunit 2	*EIF3I*	Q13347	23354/4.9	44	173	8.9 ± 2.3, 46.3 ± 17.2 (35.3%, 100%)	Binds to the 40S ribosome and promotes the binding of methonyl
18	Ribosomal protein S12	*RPS12*	P25398	14526.0/5.6	56	141	5.7 ± 1.4, 52.6 ± 10.3 (29.4%, 70.6%)	Belongs to the ribosomal protein S12e family

[a]$P < 0.05$ *vs* the normal gastric mucosa. *t* test was used for analyzing the difference of the intensity of candidate protein spots. PEBP1: Phosphatidylethanolamine-binding protein 1; TXNDC5: Thioredoxin domain-containing protein 5.

57. Our results showed that some members of mitogen-activated protein kinase (MAPK) family and some molecules involved in nuclear factor (NF)-κB and tumor necrosis factor were hot points with higher connectivity. From the mimical molecular network, we concluded that NF-κB, MAPK and interferon γ (IFN-γ) pathways were the cores of the whole network. The downstream-related cancer and other phenotypes were linked to the three pathways. The reaction of cell to IFN-γ could be the initiating agent of this molecular interaction network (Figure 4).

DISCUSSION

Because of limited knowledge on varioliform gastritis, the molecular events underlying this disease were still unknown, and the patients could be faced with more risk of gastric cancer. It was confirmed that the mucosal lesion of varioliform gastritis could develop into malignant tumor. Proteomic studies can help understand the early stages in the genesis of varioliform gastritis and has the potential to aid in the prevention and intervention for gastric can-

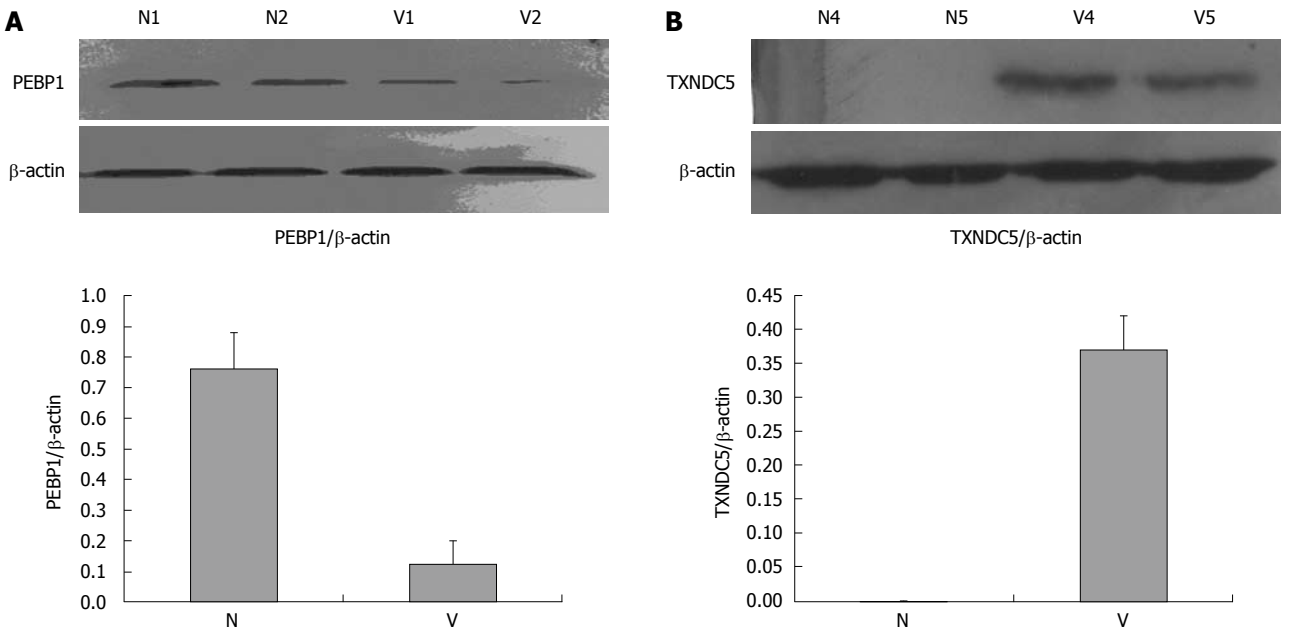

Figure 2 Western blotting analysis of phosphatidylethanolamine-binding protein 1 and thioredoxin domain-containing protein 5. A: Marked downregulation of phosphatidylethanolamine-binding protein 1 (PEBP1) in varioliform gastritis (V) tissue. Protein extracts (50 μg) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to a poly-vinylidene difluoride membrane. After blocking, the membranes were incubated with rabbit monoclonal antibody of PEBP1 (dilution of 1:2000) and subsequently incubated with HRP-anti-rabbit IgG. The specific proteins were visualized with chemiluminescent reagent. As a control for equal protein loading, blots were restained with anti-actin antibody. Immunosignals were quantified by densitometry scanning. The relative quantification was calculated as the ratio of PEBP1 expression to actin expression as shown in the followed chart; B: Upregulation of thioredoxin domain-containing protein 5 (TXNDC5) in varioliform gastritis in comparison with that in normal (N) mucosa. The same experimental process was performed, except that the membranes were incubated with polyclonal goat anti-TXNDC5 antibody (dilution of 1:1000).

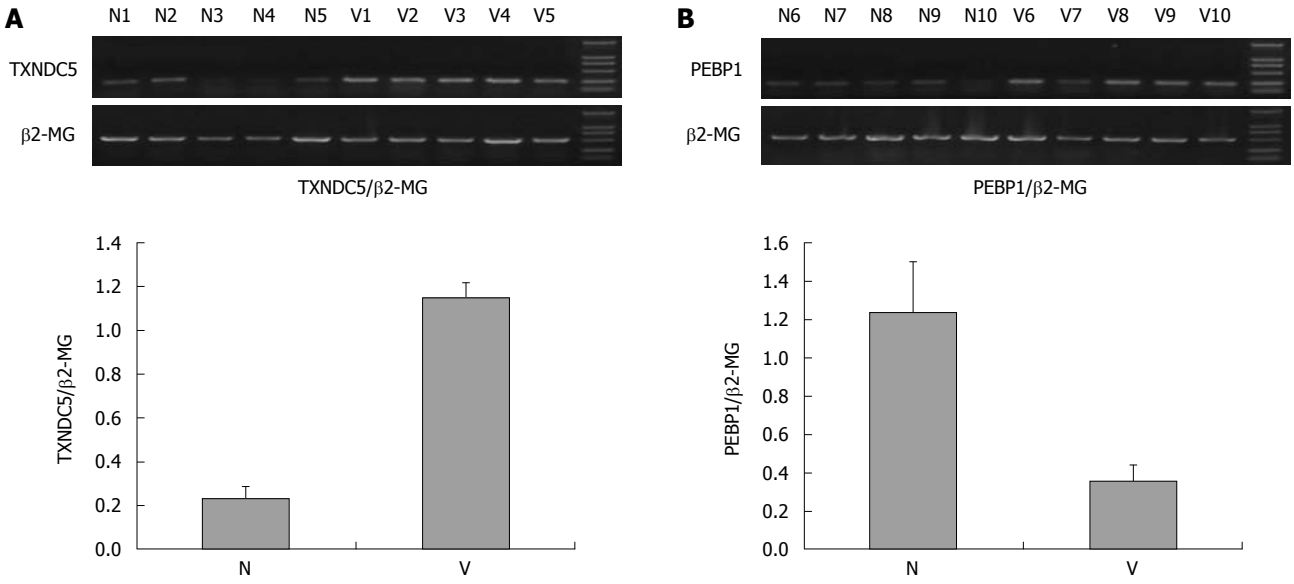

Figure 3 Reverse transcription polymerase chain reaction analysis of thioredoxin domain-containing protein 5 and phosphatidylethanolamine-binding protein 1. A: Marked upregulation of thioredoxin domain-containing protein 5 (TXNDC5) in varioliform gastritis (V). The total RNAs of additional samples tissues were extracted by homogenization in Trizol (Invitrogen), cDNA synthesis was performed in 20 μL reaction system of reverse transcription including 5 μg RNA. Amplification of TXNDC5, with β2-MG acting as internal control, was carried out in DNA thermal cycler. PCR products were separated by 1.5% agarose gel electrophoresis. The bands were quantified by densitometry scanning. The relative quantification was calculated as the ratio of TXNDC5 expression to β2-MG expression as shown in the followed chart; B: Downregulation of phosphatidylethanolamine-binding protein 1 (PEBP1) in varioliform gastritis in comparison with that in normal (N) mucosa. The same experimental process was performed. The relative quantification was calculated as the ratio of PEBP1 expression to β2-MG expression as shown in the followed chart.

cer. In this study, we used the common approach of 2-DE coupled with MS to study the differentially expressed proteins in individual-matched cases of normal mucosa and lesion of varioliform gastritis and confirmed the differential expression of PEBP1 and TXNDC5 by Western blotting or RT-PCR.

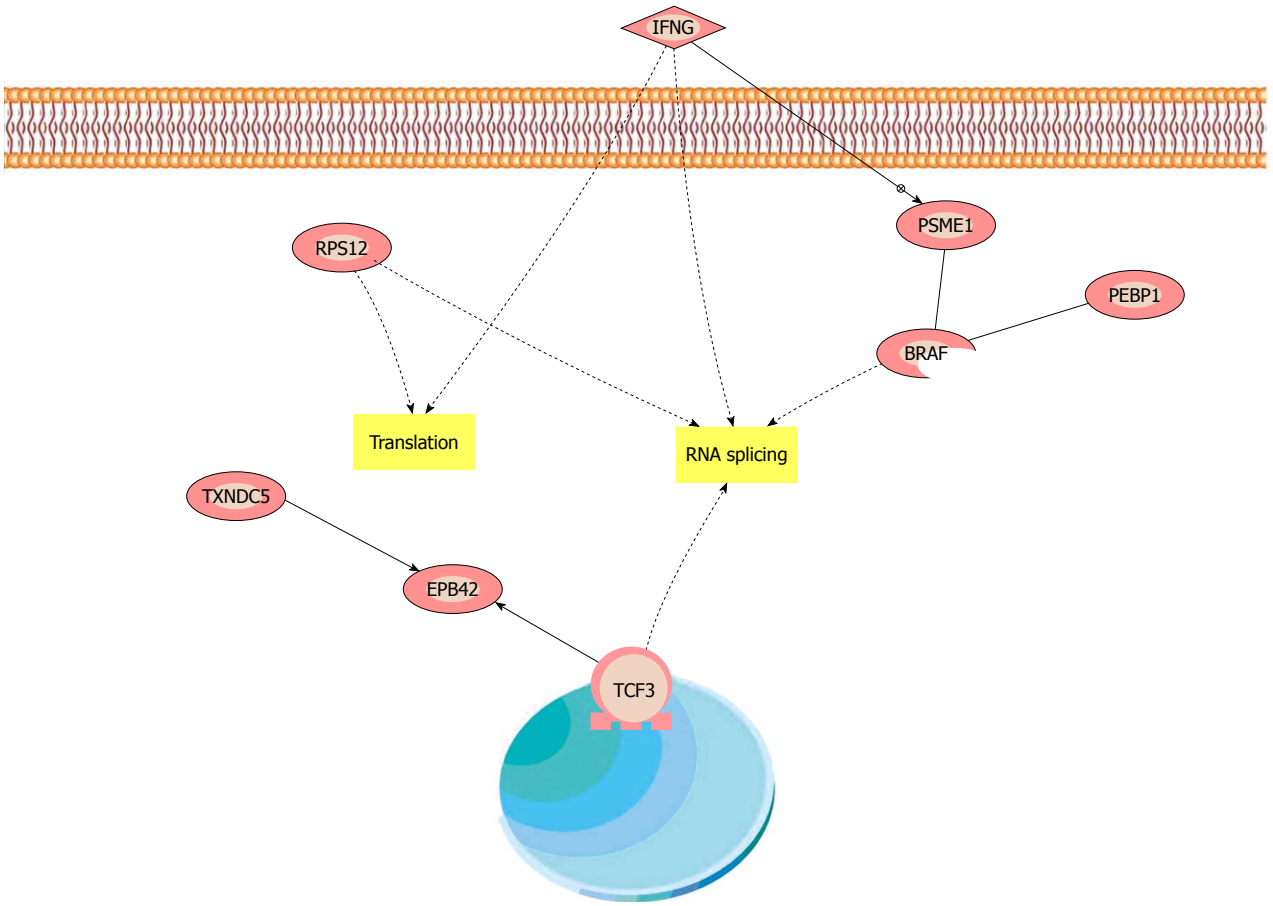

Figure 4 Compendious biological interaction networking of phosphatidylethanolamine-binding protein 1 and thioredoxin domain-containing protein 5 in varioliform gastritis. Phosphatidylethanolamine-binding protein 1 (PEBP1) and thioredoxin domain-containing protein 5 (TXNDC5) were imported into Pathway Studio (demo), and an interaction map was generated. Compendious molecular interaction pathway which linked PEBP1, TXNDC5 and interaction pathways and interferon γ (INFG). INFG could induce some complex molecular interaction in cells and impact on cell proliferation and apoptosis, and then could promote the formation of varioliform lesion.

A concept in cancer biology is that tumors arise and grow from some precancerous lesions as a result of the multiple changes of the genes or proteins which could influence the functions of cells *via* different molecular biological pathways. So it could be very important to find out these molecular changes and their functional pathways. The changes can be detected and analyzed in genomics and proteomics. A differential protein expression profile is a snapshot of the proteomics composition of a specific tissue at a specific time, which can be a key clue for further studies on the underlying mechanisms.

In this study, we identified 21 differentially expressed proteins in varioliform gastritis. However, none of these proteins (Table 2) had been reported in previous studies on this disease. We used a 5-fold cut-off according to the previous studies[9,10], and only found 21 differentially expressed proteins between varioliform gastritis and normal mucosa in the 2-DIGE study. We believe that some major molecular mechanisms underlying the disease should be implicated. There are also some methodological discrepancies in the process of our proteomic study, including the sample collection, the separation and identification of proteins and the analysis of results. Some low-abundance protein spots could not be displayed clearly, which should be further analyzed by a more advanced method.

PEBP1 expression was strong in normal mucosa, but significantly downregulated in varioliform gastritis. An alternative name of PEBP1 was Raf kinase inhibitory protein (RKIP) that belongs to the PEBP family. It is an inhibitor of the Raf/MEK/MAP kinase signaling cascade and is a suppressor of cancer metastasis[11]. Some researches[12,13] have confirmed that PEBP1 regulates activation of MAPK, NF-κB and G protein coupled receptors. As a modulator of key signaling pathways, PEBP1 affects various cellular processes, including cell differentiation, the cell cycle, apoptosis and cell migration. To date, emerging evidence[14-21] suggests that PEBP1 plays a crucial suppressing role in tumorigenesis and metastasis of prostate cancer, ovarian cancer, cervical cancer, colorectal cancer, liver cancer and breast cancer. It represents a novel effector of signal transduction pathways leading to apoptosis and a prognostic marker of the pathogenesis of human cancer cells and tumors. Chatterjee *et al*[22] have examined the expression patterns of PEBP1 and STAT3 in samples from 143 patients with gastric adenocarcinoma using tissue microarrays. Their results indicate the predictive and protective role of PEBP1 expression in gastric adenocarcinoma of the intestinal subtype. Downregulated expressions of PEBP1 could decrease patients' survival. Collectively, these studies suggest that the PEBP1 or RKIP gene, as a poten-

tial tumor suppressor gene, is involved in gastric cancer initiation and progression, and expression of PEBP1 could be downregulated at initiation of tumorigenesis. Our results confirmed that PEBP1 expression was downregulated in varioliform gastritis compared with that of normal mucosa. We, therefore, postulate that this downregulation of PEBP1 might denote a step of the potential canceration.

TXNDC5 was significantly upregulated in varioliform gastritis although its expression remained in normal mucosa. TXNDC5 was first detected by 2-DE analysis of the luminal environment of the endoplasmic reticula of hepatic tissues in 2003[23]. As a novel PDI-like protein, TXNDC5 was highly expressed in endothelial cells. This tissue-specific expression is unusual among members of the PDI family. Sullivan *et al*[24] have confirmed that TXNDC5 could protect endothelial cells from stress-induced apoptosis. In contrast to PDI, which is essential for the survival of endothelial cells in the resting as well as the stressed state, TXNDC5 protects endothelial cells only under conditions of stress. They have found that loss of TXNDC5 results in reduced secretion of adrenomedullin and endothelin-1 together with a reduction in membrane-bound CD105, while TXNDC5 is essential for folding of CD105. The results of Edman's and Freedman's researches[25,26] suggested that TXNDC5 could play important roles in antioxidative injury, antianoxia-induced apoptosis, and promotion of proliferation in cells. Some recent studies showed that upregulation of TXNDC5 was found in tumors of the cervix, uterus, stomach and lung[24]. Nissom *et al*[27] found that a variant of the TXNDC5 gene was upregulated in poorly differentiated hepatocellular carcinoma (HCC) but unchanged in well-differentiated HCC. According to these reports, we think that TXNDC5 gene could be a tumor-enhancing gene, but the detailed biological roles of TXNDC5 in varioliform gastritis and gastric cancer remain to be elucidated. The upregulation of TXNDC5 in varioliform gastritis suggests that this disease could be related to gastric cancer, with higher risk than what was thought before.

The NF-κB and MAPK signaling pathways regulate growth in many tumors or inflammation, suggesting the cooperative role of these two pathways in the regulation of cell proliferation and apoptosis. *H. pylori* is known to be the cause of most gastric diseases, including both peptic ulcer disease and gastric cancer. Fox *et al*[28] think that the induction by *H. pylori* of cytokines and chemokines and growth-related genes is mediated by the MAPK and NF-κB signaling pathways, and Shibata *et al*[29] and Lee *et al*[30] have confirmed this conclusion. Kacar *et al*[31] and Chen *et al*[32] found that MAPK signaling pathway could be a causative factor in the alterations of the gastric mucosa infected by *H. pylori* and MAPK activation seems to be a significant and persistent event in the *H. pylori*-induced neoplastic transformation. IFN-γ acts through distinct cell surface receptors and induces transcription of an overlapping sets of genes. MHC class I genes are inducible by this interferon. IFN-γ is the gastric mucosal immunological reaction produced by T helper cells when gastric mucosa is infected by *H. pylori*[33-35]. It could induce the changes of TXNDC5 or PEBP1 and impact on the NF-κB and MAPK signaling pathways in our molecular interaction network of varioliform gastritis. We supposed that varioliform gastritis should be the results of a series of molecular interactions induced by IFN-γ or other molecules as an immunological reaction against microorganism infection according to previous reports and our research. But most of the previous studies focused on *H. pylori*, and the researches on other pathogens were very limited. So there could be some other bacteria or viruses which could induce an analogous immunological reaction against *H. pylori* in the gastric mucosa of varioliform gastritis patients without *H. pylori* infection.

In summary, our study showed a differential protein expression profile of varioliform gastritis compared with that of matched normal mucosa. The candidate proteins may confirm the previous conclusion that varioliform gastritis is one of the major precursor diseases of gastric cancer. The risk of potential canceration could be higher than what was thought previously, so effective treatment strategies should be studied and adopted for this disease in the future.

ACKNOWLEDGMENTS

We thank the Beijing Proteomic Research Center, the Institute of Biochemistry and Cell Biology, the Fourth Military Medical University.

COMMENTS

Background

Varioliform gastritis is a chronic gastritis with a potential of developing into gastric cancer. To date, the etiopathogeny of the disease is unclear. So it is crucial to elucidate the molecular mechanism underlying the disease for preventing gastric cancer.

Research frontiers

At present, the researches on varioliform gastritis focused mostly on endoscopic diagnosis and treatment or clinical feature. The reports on the molecular mechanism of the disease are very limited. As a new field of clinical proteomics is emerging, many new techniques have been developed to identify functional molecules or biomarkers of cancer and other diseases.

Innovations and breakthroughs

In this research, a differential protein expression profile of varioliform gastritis was indicated compared with that of matched normal mucosa. The important differential proteins and potential signal pathways have been provided for the future studies.

Applications

The results of this study provided some valuable clues for elucidating the molecular mechanism of varioliform gastritis and the relationship between the disease and gastric cancer. Some potential biomarkers were indicated for the early diagnosis of gastric cancer and therapeutic targets for this tumor.

Terminology

Varioliform gastritis: a special kind of chronic gastritis characterized by nodules, thickened fugal folds and erosions. Although it is a very common gastritis, its features appear to be unusual and different from those seen in chronic gastritis.

Peer review

The authors used proteomic techniques to identify differences in protein expression patterns in normal gastric mucosa *vs* mucosa characterized by varioliform gastritis. The study is well designed, represents a large amount of work, and will potentially be very helpful to further studies in the field of cancer research and treatment.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3674

World J Gastroenterol 2010 August 7; 16(29): 3674-3679
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Magnetically labeled mesenchymal stem cells after autologous transplantation into acutely injured liver

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Supported by (partly) the Natural Science Foundation of Jiangsu Province, No. BK2007537; key program of Nanjing Municipal Bureau of Public Health, No. ZKX06015
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Received: February 5, 2010 Revised: April 1, 2010
Accepted: April 8, 2010
Published online: August 7, 2010

Abstract

AIM: To evaluate tracking of magnetically labeled mesenchymal stem cells (MSCs) after intraportal transplantation.

METHODS: Mononuclear cells were isolated from bone marrow aspirates of pigs by density gradient centrifugation, cultured and expanded, after which, they were incubated with super paramagnetic iron oxide (SPIO). Prussian blue staining was performed to highlight intracellular iron. To establish swine models of acute liver injury, 0.5 g/kg D-galactosamine was administrated to 10 pigs, six of which were injected *via* their portal veins with SPIO-labeled MSCs, while the remaining four were injected with unlabeled cells. Magnetic resonance imaging (MRI) was performed with a clinical 1.5T MR scanner immediately before transplantation and 6 h, 3 d, 7 d and 14 d after transplantation. Prussian blue staining was again performed with the tissue slices at the endpoint.

RESULTS: Prussian blue staining of SPIO-labeled MSCs had a labeling efficiency of almost 100%. Signal intensity loss in the liver by SPIO labeling on the FFE (T2*WI) sequence persisted until 14 d after transplantation. Histological analysis by Prussian blue staining confirmed homing of labeled MSCs in the liver after 14 d; primarily distributed in hepatic sinusoids and liver parenchyma.

CONCLUSION: MSCs were successfully labeled with SPIO *in vitro*. MRI can monitor magnetically labeled MSCs transplanted into the liver.

Key words: Magnetic resonance imaging; Mesenchymal stem cells; Super paramagnetic iron oxide; Stem cell transplantation

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Shi XL, Gu JY, Han B, Xu HY, Fang L, Ding YT. Magnetically labeled mesenchymal stem cells after autologous transplantation into acutely injured liver. *World J Gastroenterol* 2010; 16(29): 3674-3679 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3674.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3674

INTRODUCTION

In recent years, cell transplantation has had the advantages of lower cost, lower risk, and simpler manipulation of the procedure compared with orthotopic liver transplantation. A large body of evidence has suggested that mesenchymal stem cells (MSCs) could differentiate into liver-like cells with partial hepatic functions under appropriate environmental conditions *in vivo* and *in vitro*[1,2]. Given that autolo-

gous cell transplantation helps prevent immunological rejection, which is always a problem for orthotopic liver transplantation, MSCs can be regarded as seeding cells for transplantation in relation to liver diseases.

The major issue in liver cell transplantation is monitoring migration, distribution, and differentiation of the transplanted cells. Conventional tissue slicing is unable to distinguish between transplanted donor cells and the recipient cells, therefore, the tissue or organ has to be removed at certain time points and processed with special biochemical procedures to visualize the tagged cells. However, these tagging methods require *in vitro* preparation and examination of histological materials, which are unsuitable for noninvasive and repeated monitoring of *in vivo* transplanted cells under clinical conditions. Therefore, more recent research has focused on *in vivo* real-time tracking and detecting the fate of transplanted cells by using appropriate imaging technologies[3].

The present study had two purposes. First, we incubated swine autologous MSCs with super paramagnetic iron oxide (SPIO) *in vitro*, followed by stem cell transplantation performed *via* the portal vein in acutely injured liver models. Second, we investigated the characteristics of magnetically labeled swine MSCs by magnetic resonance imaging (MRI), as well as intrahepatic dynamic distribution.

MATERIALS AND METHODS

Animal care

Ten outbred white swine of either sex weighing 15-20 kg each were maintained under conventional conditions in the Laboratory Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All animal procedures were approved by the Animal Care Ethics Committee of Nanjing Drum Tower Hospital.

MSC isolation, culture and characterization

Porcine MSCs were isolated by bone marrow aspirates from the iliac crests of the animals as previously described, with slight modification[4]. Mononuclear cells were collected by gradient centrifugation over a Ficoll histopaque layer (20 min, 400 *g*, density 1.077 g/mL) (TBD, China) and seeded at a density of 1×10^6 cells/cm^2 in growth medium that contained low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL). The non-adherent cells were removed after the first 24 h and changed every 3-4 d thereafter. When the cells reached 80% confluence, they were detached using 0.25% Trypsin-EDTA (GIBCO, USA) and re-plated at a density of 1×10^4 cells/cm^2 for expansion. Surface marker identification of the cultured MSCs was performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) by fluorescein isothiocyanate (FITC)-labeled monoclonal antibody staining to CD45 (Antigenix America, Huntington Station, NY, USA) and phycoerythrin (PE)-conjugated antibodies against CD29 (VMRD, Pullman, WA, USA), CD44 and CD90 (Becton Dickinson). Isotypic antibodies served as the control.

MSCs were labeled with Feridex (Advanced Magnetics, Cambridge, MA, USA), as previously described[5]. Briefly, the polyamine poly-l-lysine (PLL) hydrobromide (Sigma, St Louis, MO, USA) was used as the transfection agent. A stock solution of PLL (1.5 mg/mL) was added to DMEM at a dilution of 1:1000 and mixed with Feridex (50 μg/mL) for 60 min at room temperature on a rotating shaker. MSCs of passage 5 were added to the culture medium that contained the Feridex-PLL complex, so that the final concentrations of Feridex and PLL were 25 μg/mL and 0.75 μg/mL, respectively. The cells were placed into six-well plates (Corning, NY, USA) overnight at 37℃ in a 95% air/5% CO_2 atmosphere.

Prussian blue staining

After being incubated overnight with the Feridex-PLL complex, the MSCs were washed three times to remove excessive contrast agent. For Prussian blue staining, which indicates the presence of iron, the coverslip samples were fixed with 4% paraformaldehyde for 30 min, washed, incubated for another 30 min with 2% potassium ferrocyanide in 6% hydrochloric acid, washed again, and counterstained with nuclear fast red.

Cell viability assay

Firstly, MSCs were inoculated in 96-well plates at 1×10^4 cells per well at 37℃ in a 95% air/5% CO_2 atmosphere. Twenty-four hours later, final concentrations of Feridex in the Feridex-PLL complex (25, 50, 100 and 200 μg/mL) were added to each well with 11 other duplicates and incubated overnight. The remaining cells, which were not labeled with the complex and served as control cells, were kept under identical conditions. The magnetically labeled and non-labeled cells were then maintained in fresh culture medium for 2 d and washed twice. Ten microliters of cholecystokinin octapeptide (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added per well for 4 h. The absorbance was then measured at a wavelength of 450 nm.

Swine model of acute liver injury

Under general anesthesia with mechanical ventilation *via* an endotracheal tube, animals were administered a dose of 0.5 g/kg of D-galactosamine (D-Gal; Sigma) dissolved in 5% glucose solution, *via* the external jugular vein. Venous blood samples were drawn 6, 12 and 24 h after the operation for biochemical analysis.

Intraportal transplantation of MSCs

Animals were randomly assigned to either control ($n = 4$) or experimental (liver injured) groups ($n = 6$). The abdomens of the liver-injured animals were opened to expose the portal vein, and approximately 1×10^7 labeled MSCs suspended in 2 mL DMEM were slowly injected into the portal vein. A 30-gauge needle was used for the procedure. The pinhole at the injection site was pressed for hemostasis. Thereafter, the laparotomy incision was enclosed

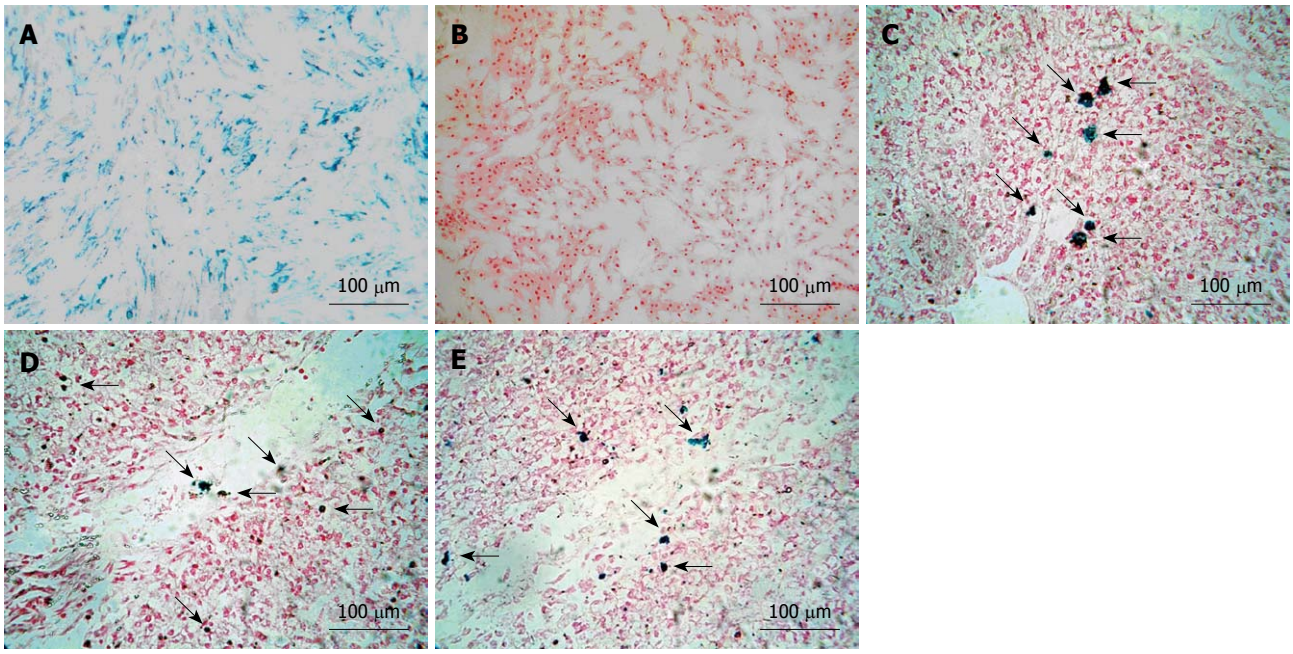

Figure 1 Characterization of labeled mesenchymal stem cells. A: Almost 100% of labeled mesenchymal stem cells (MSCs) were positive for Prussian blue staining (magnification 100 ×); B: No blue particles were observed in unlabeled group (magnification 100 ×); C-E: Prussian blue staining for liver tissue slicing displayed several blue-positive cells scattering in and around sinusoids on day 3 and 7, and the experimental group at the endpoint of the experiment (magnification 100 ×). Arrows indicate Prussian blue positive MSCs.

in layers. The control group underwent the identical procedure except that the injected cells were unlabeled.

MRI and data acquisition

Animals underwent MRI of the liver immediately before, and 3, 7 and 14 d after injection of cells. MRI was performed with a 1.5-T imaging device (Philips Medical Systems, Eindhoven, the Netherlands). The pig was anesthetized and placed supine on a plastic flat plate. The scanning sequence was as follows: (1) SE: T1WI, TR 120 s, TE 14 ms; (2) FSE: T2WI, TR 3000 ms, TE 96 ms; and (3) FFE: T2*WI, TR 485 ms, TE 14.0 ms, flip angle, 18°.

Histological assessment

Two weeks after cell transplantation, animals were sacrificed for histological examination. Liver tissues taken from both the control and experimental groups were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-mm sections and stained with hematoxylin and eosin (HE) as well as Prussian blue for examination under a light microscope.

Statistical analysis

Data were shown as mean ± SE. The two-tailed unpaired Student's *t* test was used to evaluate the statistical significance of differences which was set with a *P* value less than 0.05.

RESULTS

MSC phenotype

Twenty-four hours after first seeding, MSCs could be seen in newly formed colonies. Observed under the microscope, the MSCs rapidly grew fibroblast-like cells with a single nucleus. After the first passage, they looked like spindles or asters with a slim body. At passage 5, however, most of the miscellaneous cells were eliminated, and the remaining uniform fibroblast-like cells were MSCs. The expression of different cell surface markers, including CD29, CD44, CD45 and CD90, of MSCs from passage 5 was determined by flow cytometry; the results of which showed that > 90% of MSCs of passage 5 were positive for CD29, CD44 and CD90, but negative for CD45 (data not shown).

Characterization of labeled MSCs

MSCs stained with Prussian blue showed results (blue particles) in all labeled cells. The labeling rate was approximately 100%, which was calculated under a light microscope *via* counting the numbers of positive cells in five random fields (Figure 1A). In contrast, no blue particles were observed in the unlabeled group (Figure 1B).

Proliferation of labeled MSCs

The growth curve of CCK-8 with Feridex-PLL labeled MSCs showed that the cellular proliferation of the 25 and 50 μg/mL subgroups were not significantly influenced by different concentration ($P > 0.05$). Figure 2 shows that MSCs labeled with higher concentrations of complex were somewhat inhibited in proliferation, which indicated that < 50 μg/mL Feridex-PLL would be suitable for MSC labeling in future transplantation.

Establishment of acute liver injury model

Acute liver injury was effectively induced in all animals. Serum alanine aminotransferase (ALT), aspartate amino-

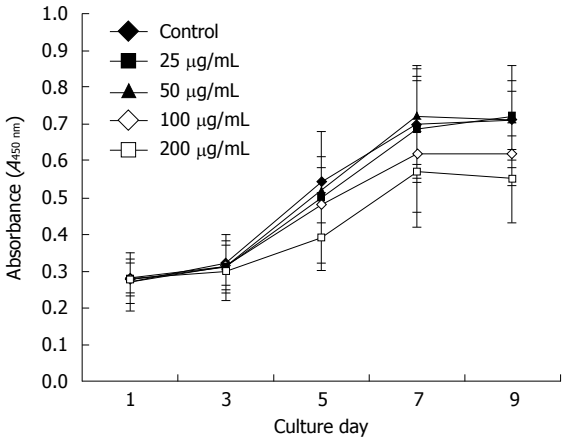

Figure 2 Growth curve of CCK-8 with Feridex-poly-l-lysine-labeled mesenchymal stem cells. Cellular proliferation of 25 and 50 μg/mL subgroups were not significantly influenced ($P > 0.05$). Mesenchymal stem cells (MSCs) labeled with higher concentrations of complex were somewhat inhibited, which indicated that < 50 μg/mL Feridex-poly-l-lysine would be suitable for MSC labeling.

transferase (AST) and bilirubin levels were all significantly and progressively elevated 6 h after D-Gal induction (Table 1, $P < 0.05$). The porcine model clinically presented listlessness, loss of appetite and xanthochromia. Liver tissue samples from the injured model group after 24 h injection of D-Gal demonstrated severe hepatic necrosis in 60%-70% of the lobule, sinusoidal congestion, vacuolization, trabecular fragmentation, and granulocytic infiltration (Figure 3). Twenty-four hours after administration of D-Gal, the animals were ready for transplantation (Table 1).

MR tracking of magnetically labeled MSCs

Signal intensity decreased 6 h after intrahepatic transplantation of labeled MSCs on the FFE sequence, but gradually approached close to normal on day 14 (Figure 4). For the control group, there was no visible difference at each time point after transplantation compared with before.

Histological demonstrations

The Prussian blue staining demonstrated several blue-positive cells scattered in and around the sinusoids in the experimental group on day 3 and 7 and the endpoint of experiment (Figure 1C-E), which indicated the presence of the magnetically labeled MSCs transplanted into the liver.

DISCUSSION

Bone marrow-derived MSCs are multipotent adult stem cells of mesodermal origin with the potential for self renewal. Because MSCs have the ability to differentiate into cells of multiple organs/systems such as hepatocytes, osteoblasts, chondrocytes, adipocytes and myocytes under appropriate stimuli[1,2,6-9], they have generated considerable interest for their potential use in regenerative medicine and tissue engineering. Given the ease of their isolation, extensive expansion rate and differentiation potential, as well as their immunosuppressive properties, MSCs may be suitable candidates for seed cells for hepatocyte transplantation, in addition to being potential carrier cells for gene therapy[10], which holds a promising future in the treatment of acute or chronic liver failure and metabolic liver diseases. Despite their important potential, however, the detailed processes involved in MSC implantation, migration, and differentiation in the liver remain to be elucidated.

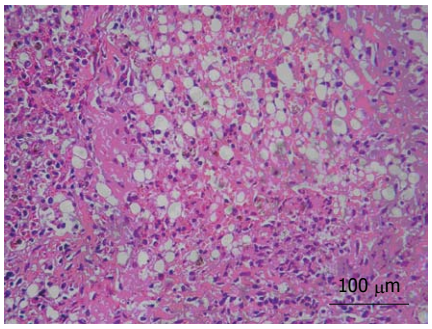

Figure 3 Histology of acutely injured liver tissue. Liver tissue samples from the injured model group after 24 h injection of D-galactosamine demonstrated severe hepatic necrosis of 60%-70% of the lobule, sinusoidal congestion, vacuolization, trabecular fragmentation, and granulocytic infiltration of the portal space and septa (magnification 100 ×).

Table 1 Biochemical parameters before and after the injection of D-galactosamine

	Pre-injection	6 h after injection	24 h after injection
ALT (U/L)	31.2 ± 2.6	87.5 ± 13.2	181.9 ± 12.8
AST (U/L)	28.9 ± 3.8	134.0 ± 7.8	564.8 ± 89.7
TBIL (μmol/L)	3.2 ± 0.45	26.7 ± 3.2	43.0 ± 2.9

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin.

Previous tissue slicing experiments have been unable to monitor the dynamic changes of transplanted cells in the living body. Accordingly, a noninvasive and living labeling technique is needed with respect to MSC intrahepatic transplantation. With the advent of molecular imaging technologies, *in vivo* real-time tracking and detecting the fate of transplanted stem cells may become a reality[3,11,12]. Cells for transplantation have been labeled with MR contrast agents since the beginning of the 1990s. In this regard, MRI appears most promising for dynamically monitoring *in vivo* cell migration after transplantation, due to its well known properties of relative long-term imaging, high spatial resolution, and sharp contrast[13]. Currently, SPIO MRI contrast agents have been most widely used for tracking transplanted cells in various organs because of their strong signal attenuation properties[14-18]. In particular, dextran-coated SPIO nanoparticles have been approved by the US Food and Drug Administration for use in hepatic reticuloendothelial cell imaging, and ultrasmall SPIOs are in phase Ⅲ clinical trials for use as blood pool agents or for use with lymphography[19-21]. However, such contrast agents cannot be used to label efficiently stem cells *in vitro* in their native unmodified form[22]. By conjugating antigen-specific internalizing monoclonal antibodies to the surface dextran coating, cells can be magnetically labeled

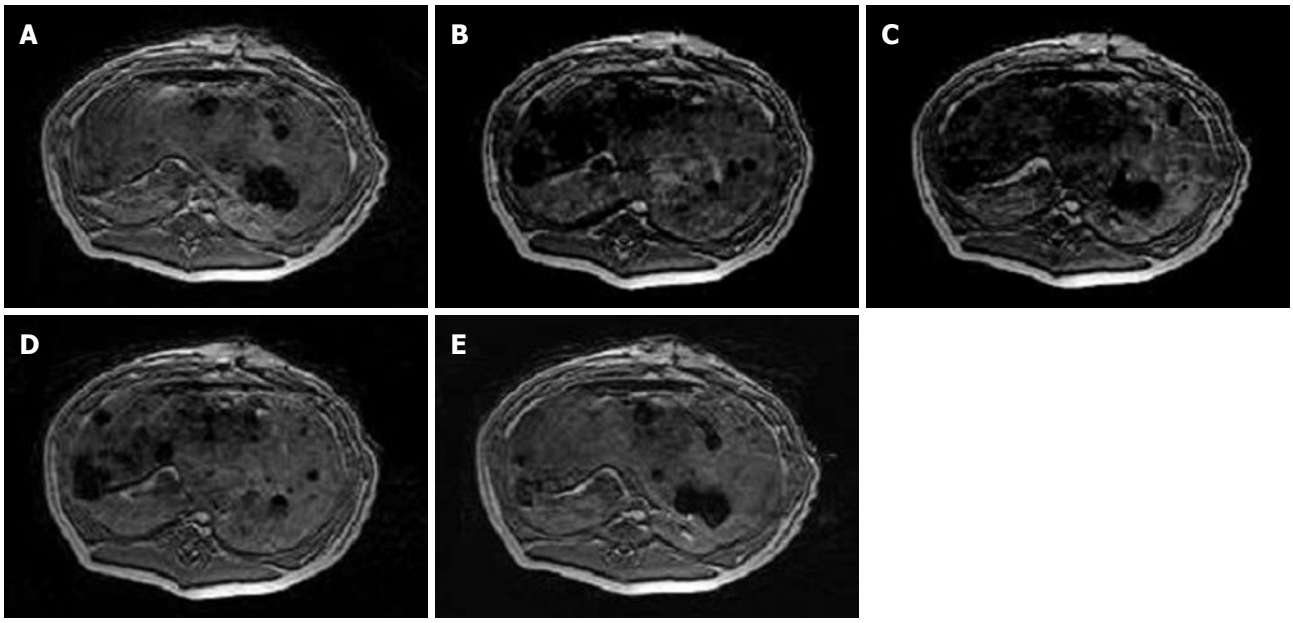

Figure 4 Magnetic resonance imaging of Feridex-poly-l-lysine labeled mesenchymal stem cells in the liver. A: No loss of signal in the liver before transplantation; B: Significant signal intensity loss was observed in labeled mesenchymal stem cells on FFE sequences 6 h after transplantation; C, D: Attenuation of signal loss appeared over time; E: T2*-weighted images gradually approached to the normal level at the endpoint of the study.

during their normal expansion in culture medium. In the present study, we used PLL as a transfection agent to magnetically label MSCs *in vitro* by the establishment of a Feridex-PLL complex through electrostatic interactions. This encourages cellular membrane endocytosis and transportation of Feridex into endosomes without requiring novel synthesis or covalent binding of proteins or antibodies to the dextran coating. Our results indicated that the labeling efficiency in our study approximated to 100% as expected. Subsequent analysis of the inhibitory effects of different concentrations of Feridex-PLL complex on MSCs revealed < 50 μg/mL Feridex was a relatively safe and effective dose for MSC labeling, which was suitable for transplantation study.

So far, most studies concerning MRI of grafted stem cells have been applied to animal brains, spines and hearts[17,23,24]. MRI techniques offer the possibility of tracking labeled cells *in vivo* noninvasively and repeatedly during extended study periods. The potential of MRI for future clinical interventions within the realm of regenerative cell therapy has been elegantly demonstrated in previous studies, and MRI fluoroscopy has been used to guide the delivery of MR-labeled adult stem cells into damaged organs. In our study, migration and retention of porcine MSCs after intraportal transplantation were demonstrated by using *in vivo* MRI. Significant signal intensity loss was observed in labeled MSCs on FFE sequences 6 h after transplantation. Thereafter, it gradually approached normal levels on day 14. The loss of signal could be attributed to either biodegradation of the contrast agent, the process of cellular division, or cellular migration to neighboring organs. To confirm the long-term results, Prussian blue staining was performed to demonstrate positive cells in liver tissue slices at the endpoint of the experiment. Furthermore, the dispersed distribution of labeled MSCs confirmed that the acute injured liver model may offer an ideal microenvironment for cell recruitment and implantation.

In summary, the present study incubated porcine MSCs with Feridex-PLL complex *in vitro*, and *in vivo* real-time tracking and detecting of magnetically labeled MSCs were manipulated by MRI in models of acute liver injury. Future research will focus on the optimized numbers of transplanted MSCs, distribution beyond injured liver, as well as safety and therapeutic effects concerning liver regeneration after MSC intraportal transplantation. In addition, our newly established co-culture system for hepatocytes and MSCs for cell transplantation and bioartificial liver devices should also be monitored[25].

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance from Department of Radiology, Nanjing Drum-Tower Hospital.

COMMENTS

Background

In recent years, cell transplantation has had the advantages of lower cost, lower risk, and simpler manipulation of the procedure compared with orthotopic liver transplantation. Autologous cell transplantation helps prevent immunological rejection, which is always a problem for orthotopic liver transplantation. Moreover, a large body of evidence has suggested that mesenchymal stem cells (MSCs) differentiate into liver-like cells with partial hepatic functions under appropriate environmental conditions *in vivo* and *in vitro*. Therefore, MSCs could be regarded as seeding cells for transplantation in relation to liver diseases.

Research frontiers

One of the major issues in liver cell transplantation is monitoring migration, distribution, and differentiation of the transplanted cells. Present tagging methods require *in vitro* preparation and examination of histological materials, which are unsuitable for noninvasive and repeated monitoring of *in vivo* transplanted cells under clinical conditions. Therefore, more recent research

has focused on *in vivo* real-time tracking and detecting the fate of transplanted cells by using appropriate imaging technologies.

Innovations and breakthroughs

The authors sought to label MSCs *in vitro* with super paramagnetic iron oxide (SPIO) and to monitor the labeled cells with magnetic resonance imaging (MRI). Through this research, they found a new invasive and repeatable monitoring method.

Applications

This method of labeling and monitoring cells could be applied to research in cell transplantation. In future, it could be considered as an invasive and repeatable monitoring method for clinical cell transplantation.

Terminology

SPIO is an MRI contrast agent that shows a high signal during imaging.

Peer review

In this study, authors successfully showed the use of swine mesenchymal stem cells in an acute liver injury model. The study should be considered as a brief report as it is a nice piece of information.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3680

World J Gastroenterol 2010 August 7; 16(29): 3680-3686
ISSN 1007-9327 (print)

BRIEF ARTICLE

ICC density predicts bacterial overgrowth in a rat model of post-infectious IBS

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Author contributions: Jee SR, Morales W, Chang C and Pimentel M designed the study, conducted the experiments and wrote the manuscript; Low K, Zhu A, Pokkunuri V and Chatterjee S conducted the experiments; Soffer E and Conklin JL designed the experiments and wrote the manuscript.

Supported by A grant from Beatrice and Samuel A Seaver Foundation as well as the Shoolman Foundation

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Received: November 12, 2009 Revised: January 7, 2010
Accepted: January 14, 2010
Published online: August 7, 2010

Abstract

AIM: To investigate the interstitial cells of Cajal (ICC) number using a new rat model.

METHODS: Sprague-Dawley rats were assigned to two groups. The first group received gavage with *Campylobacter jejuni* (*C. jejuni*) 81-176. The second group was gavaged with placebo. Three months after clearance of *Campylobacter* from the stool, precise segments of duodenum, jejunum, and ileum were ligated in self-contained loops of bowel that were preserved in anaerobic bags. Deep muscular plexus ICC (DMP-ICC) were quantified by two blinded readers assessing the tissue in a random, coded order. The number of ICC per villus was compared among controls, *Campylobacter* recovered rats without small intestinal bacterial overgrowth (SIBO), and *Campylobacter* recovered rats with SIBO.

RESULTS: Three months after recovery, 27% of rats gavaged with *C. jejuni* had SIBO. The rats with SIBO had a lower number of DMP-ICC than controls in the jejunum and ileum. Additionally there appeared to be a density threshold of 0.12 DMP-ICC/villus that was associated with SIBO. If ileal density of DMP-ICC was < 0.12 ICC/villus, 54% of rats had SIBO compared to 9% among ileal sections with > 0.12 ($P < 0.05$). If the density of ICC was < 0.12 DMP-ICC/villus in more than one location of the bowel, 88% of these had SIBO compared to 6% in those who did not ($P < 0.001$).

CONCLUSION: In this post-infectious rat model, the development of SIBO appears to be associated with a reduction in DMP-ICC. Further study of this rat model might help understand the pathophysiology of post-infectious irritable bowel syndrome.

Key words: Post-infectious irritable bowel syndrome; Bacterial overgrowth; Interstitial cells of Cajal; Campylobacter

Peer reviewer: Kristin Verbeke, PhD, Professor, Laboratory Digestion and Absorption, University Hospital Leuven, E462, Herestraat 49, B-3000 Leuven, Belgium

Jee SR, Morales W, Low K, Chang C, Zhu A, Pokkunuri V, Chatterjee S, Soffer E, Conklin JL, Pimentel M. ICC density predicts bacterial overgrowth in a rat model of post-infectious IBS. *World J Gastroenterol* 2010; 16(29): 3680-3686 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3680.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3680

INTRODUCTION

A recent series of studies suggested a link between small

intestinal bacterial overgrowth (SIBO) and irritable bowel syndrome (IBS)[1,2]. The latest of these is a study that compared jejunal cultures between IBS patients and controls[3]. In that study, the number of coliform bacteria was higher in the small bowel of IBS compared to healthy controls.

Another potential bacterial pathogenesis in IBS is related to the development of IBS symptoms after acute gastroenteritis [post-infectious IBS (PI-IBS)]. In a recent meta-analysis, almost 10% of normal subjects developed IBS after an incident of bacterial gastroenteritis[4]. Initial research in this area focused on possible inflammatory events, as suggested by increased lymphocyte counts in the rectal mucosa[5-7]. Recent evidence has linked post-infectious events to the development of SIBO in a rat model of PI-IBS[8]. In this model, rats exposed to *Campylobacter jejuni* (*C. jejuni*) have persistent altered stool form that is linked to the development of SIBO in 27% of rats, based on quantitative polymerase chain reaction (PCR). This finding in rats suggests that, at least in the case of *C. jejuni*, a possible mechanism for PI-IBS could be the development of SIBO. How SIBO develops in this model is not known. In humans, it has been speculated that SIBO in IBS patients is related to a reduced number of fasting migrating motor complexes[9]. This has been a recognized cause of SIBO since 1977[10].

The important role of interstitial cells of Cajal (ICC) in gastrointestinal physiology has been elucidated over the past 10-20 years. ICC are required for normal intestinal motility. Their role as intestinal pacemakers has been established in a number of model systems[11-13]. Labeling ICC with anti-Kit antibodies provides an opportunity to evaluate ICC of gastrointestinal muscles in patients with various motility disorders. Reduced numbers or loss of ICC is associated with several motor dysfunctions, including achalasia, intestinal pseudoobstruction, infantile pyloric stenosis, diabetic gastroparesis, ulcerative colitis, and slow-transit constipation[14-19]. Loss of ICC also interferes with electrical pacemaker activity, slow-wave propagation, and gastrointestinal motor neurotransmission[12,13,20,21].

Given the significant role of ICC in modulating the neuromuscular activity of the gut, we sought to investigate whether the development of SIBO in the rat model infected with *C. jejuni* is associated with reduction in intestinal ICC.

MATERIALS AND METHODS

Development of post-infectious rats

After confirming no pre-existing presence of *C. jejuni* in their stools, 66 outbred Sprague-Dawley rats were randomized (in a 1:1 ratio) into two groups. One group was gavaged with a 1 mL solution containing *C. jejuni* 81-176 at 10^8 CFU/mL (C+ rats) in Brucella broth. The control rats were gavaged with an identical solution without *C. jejuni* (C- rats).

Following gavage, all the rats were housed at two per cage. However, the rats receiving *Campylobacter* (C+) were housed separately from the control (C-) rats to avoid the possibility of cross contamination of the *C. jejuni* infection between the two groups.

In the first 3 d after gavage, stool was collected from both groups to verify that intestinal colonization in C+ rats had occurred. It was also used to confirm the absence of infection in C- rats. Stool was later obtained in the C+ group on days seven and 14 after infection and then every 2 wk until two consecutive negative cultures for *C. jejuni* were confirmed.

After 90% of the C+ rats no longer had detectable *C. jejuni* in the stool, they were considered to be in the post-infectious time period. At this point, they were housed for three additional months. During this time, both groups were treated equally with respect to food, water, and environment. In the 3 d just prior to euthanasia (at 3 mo into the post-infectious period) stool was collected from each rat and graded blindly according to an *a priori* stool form grading score. This score was based on whether or not there was loose or normal stool in a blinded evaluation. Any loose stool was considered abnormal. The stool was also cultured to determine if there was any lingering case of *Campylobacter* (C+).

Campylobacter gavage

The *C. jejuni* 81-176 strain used in the gavage of the rats was obtained from freezer stocks, plated on selective media, and incubated for 36-48 h under microaerophilic conditions at 42℃ to create a bacterial lawn. This lawn was then harvested from these plates and suspended in Brucella broth. The concentration of bacteria was estimated spectrophotometrically and confirmed *via* serial dilution and plating on selective media. In the 30 min prior to *Campylobacter* gavage, rats were gavaged with a 1 mL solution of 5% sodium bicarbonate using a ball-tipped inoculating needle. This was done to neutralize gastric acid to increase the likelihood of intestinal colonization of the pathogen. Subsequently, a 1 mL suspension of *C. jejuni* in Brucella broth (5×10^8 CFU/mL) was administered by gavage.

Bowel sampling

Three months after clearance of *Campylobacter* from the stool, fresh stool was obtained from all rats. This was used to determine the presence or absence of prolonged *C. jejuni* colonization. Stool was also qualitatively evaluated for stool form as described above.

Rats were then euthanized. Immediately following euthanasia, a laparotomy was performed whereby precisely determined segments of the ileum, jejunum, and duodenum were obtained, as previously described[8]. The first 2 cm segment at each location was a ligated self-contained loop of bowel. Sutures were placed on either side to prevent exposure to air. Samples were kept at 4℃ in an anaerobic bag for transport. These self-contained loops were then used to extract bacterial contents for the determination of bacterial number using quantitative

PCR, as previously described[8]. The quantity of bacteria in these segments was compared between control rats and rats 3 mo after recovery from *Campylobacter*. SIBO was considered to be present when bacterial count in the *Campylobacter* treated rats exceeded two standard deviations above the mean count found in the control group.

In each rat, a 2 cm segment of small bowel immediately adjacent to the closed loop of small bowel was resected and sent for bacterial quantitation. This second piece of bowel was opened longitudinally along the mesenteric border and placed in a solution of 10% formalin (VWR, West Chester, PA). After paraffin and mounting, the tissue was stained.

Immunohistochemistry

Rats were then divided into three groups based on the presence or absence of *Campylobacter* and SIBO. The three groups were a random selection of eight control rats (C-), eight *Campylobacter*-infected rats that were found to have bacterial overgrowth (C+/SIBO+), and eight randomly selected rats that received *Campylobacter* gavage but did not develop bacterial overgrowth (C+/SIBO-). Sections from each paraffin block were stained immunohistochemically using Polyclonal Rabbit Anti-Human CD117, c-kit (Dako-Cytomation, Carpinteria, CA). The positive control used to test the quality of the stain was a c-kit positive gastrointestinal stromal tumor. ICC were quantified by two blinded readers assessing the tissue in a random, coded order. The number of ICC was evaluated in the region of the deep muscle plexus ICC (DMP-ICC) according to the number of villi. The number of DMP-ICC per villus was compared among controls, C+/SIBO-, and C+/SIBO+.

Statistical analysis

Statistical comparisons for the number of DMP-ICC per villus were made by one-way analysis of variance among three groups and differences from controls was further analyzed using the Wilcoxon Rank-Sum Test. To compare the density threshold of ICC per villus, a Fisher's exact test was used. A P value of <0.05 was considered significant.

RESULTS

Campylobacter infection

Out of 66 rats used in the study, none had stool culture demonstrating *C. jejuni* before gavage. Of these rats, 33 were gavaged with vehicle (C-) and 33 rats received approximately 5×10^8 CFU *C. jejuni* 81-176 (C+). Six rats died of trauma secondary to gavage (three in each group). Among the remaining 30 rats that received *C. jejuni*, all had stool cultures that confirmed intestinal colonization by *C. jejuni*, and all but one rat cleared this colonization by 14 d.

As previously reported[8], C- rats were then used to determine the normal range of flora in the duodenum, jejunum, and ileum. Using these control data, eight C+ rats (27%) were found to have SIBO and were designated C+/SIBO+.

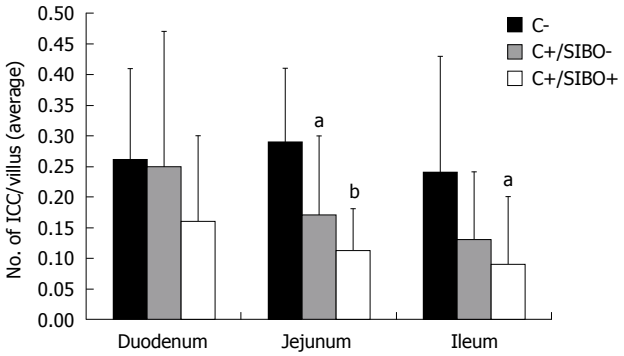

Figure 1 The number of interstitial cells of Cajal per villus in the duodenum, jejunum, and ileum. C-: Rats that received vehicle gavage; C+/SIBO-: Rats gavaged with *Campylobacter* but which did not develop small intestinal bacterial overgrowth (SIBO); C+/SIBO+: Rats gavaged with *Campylobacter* that later developed SIBO. $^aP < 0.05$, $^bP < 0.001$ *vs* control.

The number of DMP-ICC per villus

The number of DMP-ICC per villus was determined in C-, C+/SIBO-, and C+/SIBO+ rats. As shown in Figure 1, the rats with SIBO had the lowest number of DMP-ICC. This was more obvious in the jejunum and ileum than in the duodenum. Although there was a reduction of ICC in the C+/SIBO- group, this was not as great as was seen in the C+/SIBO+ group. Figure 2 shows representative examples of ileal biopsies in the C-, C+/SIBO-, and C+/SIBO+ groups, respectively. There was a reduced number of CD117 stained cells in the deep muscular plexus in the C+/SIBO+ group.

Density threshold of DMP-ICC

The number of DMP-ICC was then used to determine the level of ICC compared to SIBO. The data suggested that rats with < 0.12 ICC/villus were most likely to have SIBO. In fact, 54% of rats with a density < 0.12 ICC/villus in the ileum had SIBO compared to 9% in which DMP-ICC density was ⩾ 0.12/villus ($P < 0.05$). Using all levels of bowel, the density threshold was even more relevant. If the density of DMP-ICC/villus was < 0.12/villus in more than one of the three bowel segments, 88% had SIBO compared to 6% if the figure was ⩾ 0.12/villus ($P < 0.001$).

Differential effects of campylobacter on ICC

When the number of DMP-ICC was noted by segment of bowel according to whether rats received vehicle or *Campylobacter*, a significant difference was seen (Figure 3). There was a graded affect on DMP-ICC population, such that the greatest effect on reduction in DMP-ICC was in the distal small bowel. Reduction was also seen in the jejunum and ileum. This contrasted to the control group, whereby DMP-ICC density was uniform from proximal to distal small bowel.

DISCUSSION

In a previous study, we demonstrated that in a post-infectious rat model, altered bowel function persisted in

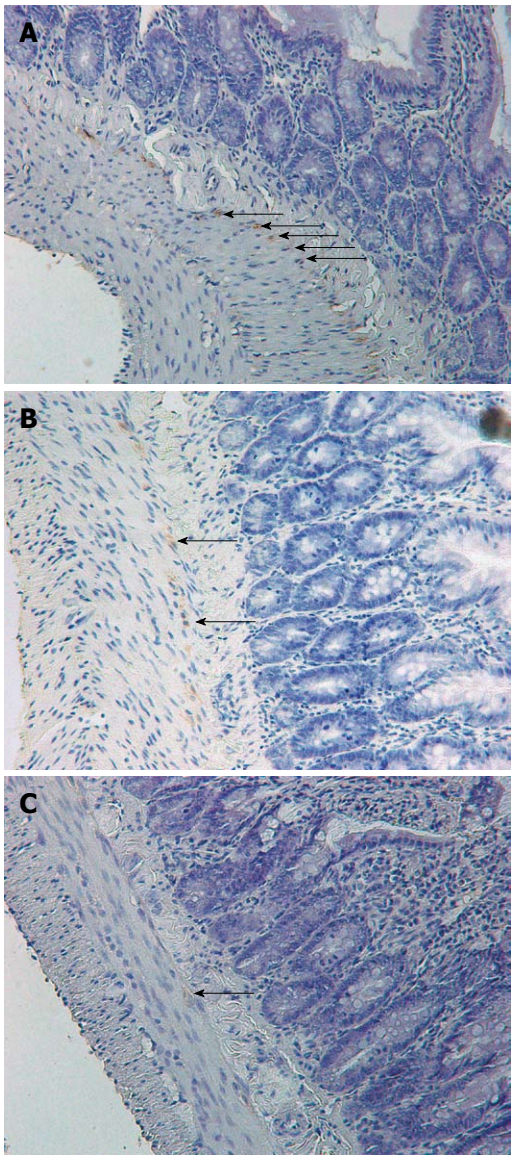

Figure 2 Interstitial cells of Cajal (arrows) in the ileum stained with CD117 (magnification 40 ×). A: Control rat; B: Rat exposed to *Campylobacter* that did not develop small intestinal bacterial overgrowth (SIBO), demonstrating persistent CD117 staining of interstitial cells of Cajal (ICC) cells; C: Rat exposed to *Campylobacter* that developed SIBO, demonstrating a reduction in CD117 staining of ICC cells. In this case, the staining is slight and the arrow indicates a "possible" stained cell.

a large number of rats even 3 mo beyond the clearance of the infection[8]. 27% of rats were found to have SIBO by quantitative PCR. SIBO correlated with those animals that had the most altered stool form. In this study, we attempted to further understand how SIBO could develop in this post-infectious rat model by studying the ICC. We found that long after *Campylobacter* infection had cleared, there was an apparent reduction in the number of DMP-ICC. This reduction appeared most evident in rats that developed SIBO after clearing *Campylobacter*. Furthermore, when the DMP-ICC density is less than 0.12/villus, SIBO is predicted to occur.

Six to seventeen percent of IBS patients believe their symptoms began after acute gastroenteritis[22], and the

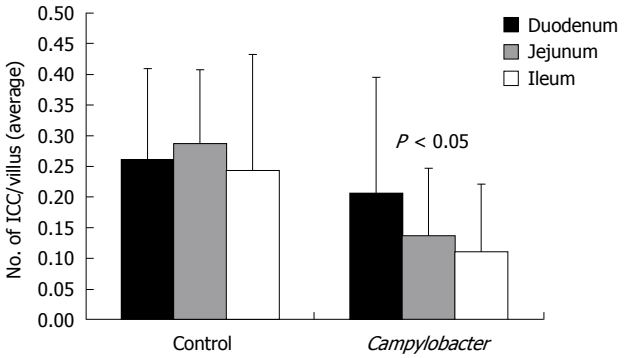

Figure 3 The effect of *Campylobacter* on interstitial cells of Cajal is greater in the distal small bowel (based on one-way analysis of variance).

incidence of PI-IBS following bacterial gastroenteritis is reported to be between 4%-31%[7,23-26]. In a 6-year follow up study, 57% of subjects thought to have developed PI-IBS still had altered bowel function consistent with IBS[27]. This growing body of literature on PI-IBS has led to two recent meta-analyses[4,28]. Both studies estimated that IBS has an incidence rate of about 10% after a case of acute gastroenteritis.

Two issues make PI-IBS very important in research on IBS pathophysiology. First, acute gastroenteritis is very common, and with a 10% rate of IBS development, this phenomenon might be very important in the development of IBS as a whole. Secondly, this is the first demonstration of a direct cause and effect relationship in the precipitation of IBS. How an acute gastrointestinal infectious process produces IBS in humans remains poorly understood, but some investigators have found residual inflammatory changes in the gut among patients with PI-IBS. These include increased numbers of lymphocytes, enteroendocrine cells, and mast cells in PI-IBS[5-7,26,29,30]. It is difficult to guarantee uniformity of population and pathogen in human studies of PI-IBS as they rely on studying a group of humans who emerge from an outbreak of infectious diarrhea. As a result, the study of inflammation and other factors in PI-IBS have been moving into animal models of PI-IBS.

One model that has been studied for some time is a *Trichinella spiralis* (*T. spiralis*)-infected mouse model. Beyond inflammatory changes, research in this model has looked at the long term effect on gastrointestinal motility. A major finding in this model was that, after resolution of the initial pathogen-related inflammatory response and elimination of the nematode from the gut, there was persistent intestinal neuromuscular dysfunction[31]. *T. spiralis*-induced mucosal inflammation led to prolonged effects on intestinal smooth muscle, and to colonic visceral hyperalgesia[32,33]. Although models such as this are vital to our understanding of PI-IBS, no model had been developed to investigate the more common human pathogens believed to cause the bulk of PI-IBS. These include *Campylobacter*, *Salmonella*, and *Shigella*, among others[34].

Two existing theories of bacterial events (post-infectious

and SIBO) now appear to be linked, based on a recent study suggesting that *C. jejuni* infection precipitates altered stool form and SIBO in a rat model[8]. How this occurs is currently unknown, although one study has demonstrated that acute *C. jejuni* infection alters intestinal myoelectric activity. In ileal segments of rabbits, alteration of action potential activity was seen in the small intestine infused intraluminally with *C. jejuni* and its cell-free filtrate[35]. In that study, the rabbits were monitored for only 24 h after exposure to *C. jejuni*. Unfortunately, no long term studies were done, and the cause of these effects was unknown.

An important cell in the control of gut motor function is the ICC. A growing list of animal models appears to support the notion that ICC are impaired in the presence of inflammation-induced changes in motor control. With acetic acid-induced inflammation, a reduction in resting membrane potential and the amplitude and duration of slow waves was related to the damage of the ICC in circular muscle cells in dogs[36]. In another study using the earlier described *T. spiralis*-model, damage to the structure of ICC networks within the region of the myenteric plexus was seen[32]. When jejunal inflammation was induced by *Nippostrongylus brasiliensis* in rats, changes in myenteric neurons, circular muscle cells, and ICC were observed[37].

In this study, we investigated whether the development of SIBO in the postinfectious rat model is related to the alteration of DMP-ICC populations, and whether this could in some way be linked to the development of IBS. In this study, *Campylobacter* gavaged rats were found to have a reduction of DMP-ICC density 3 mo after clearance of *Campylobacter*. More interesting was that the DMP-ICC density also corresponded to the development of SIBO. Rats with SIBO had the lowest DMP-ICC density. We could further evaluate a density threshold of DMP-ICC per villus which predicts SIBO. If the density of DMP-ICC/villus was not greater than 0.12 in more than one location among duodenum, jejunum, and ileum, 88% of the rats had SIBO. Although this finding does not examine the numerous contributions to gut motor function in addition to ICC, the possibility is raised that through some effects on ICC or neuromuscular mechanisms, acute gastroenteritis produces a long term effect that allows for the development of SIBO.

How *Campylobacter* affects DMP-ICC is unknown, but two possibilities include a toxin or a result of the initial acute inflammatory reaction. Although inflammation seems to be an obvious possibility, ICC's have a significant amount of "plasticity". Loss of ICC in pathological conditions does not always mean permanent injury. This "plasticity" of ICC was found in a murine model of partial bowel obstruction[38]. After the onset of the obstruction, hypertrophy of the smooth muscle layers and progressive loss of ICC orad to the site of obstruction were observed. Recovery of ICC and restoration of slow wave activity after removal of the obstruction were achieved within 30 d. In addition, injury to ICC due to inflammation is repaired in the course of time. Structural damage was observed in the network of ICC for 2 wk after *T. spiralis* infection. The structural changes were accompanied by aberrant pacemaker activity and abnormal slow waves. Sixty days after infection, motility and ICC recovered to normal values[32]. Unlike these two studies, we found a persistent decrease in the number of DMP-ICC 3 mo after clearance of infection. This was not due to any persistent *Campylobacter* colonization, as the pathogen was not detectable by culture in any location of the gastrointestinal tract. However, it is presumed that some event related to *Campylobacter* is responsible for this persistent reduction of DMP-ICC. This particular layer of ICC might be important. For example, in the W/W^{v} mouse, the DMP is intact and, despite loss of all other layers of ICC and loss of electrical slow wave, the migrating motor complex also remains intact[39]. Thus, DMP destruction might have an impact on mechanisms that protect against bacterial overgrowth.

An alternative explanation would be that SIBO is contributing to the reduction of ICC. Although not considered in this study, one means of determining this would be to provide antibiotic treatment to rats and count ICC after eradication of SIBO. The challenge with examining this concept is that it is difficult to identify SIBO in a live rat. This would make it difficult to know which animal should receive antibiotics.

It can be considered that ICC is just a marker of more global damage. In our study, smooth muscle was not evaluated. In a mouse model after acute nematode infection, altered neuromuscular function and long-lasting muscle contractility were noted[31,33]. The findings were considered as a model of PI-IBS. Further studies are needed to evaluate smooth muscle, toxin, and the effect of inflammation with *Campylobacter* infection.

In conclusion, rats with SIBO that developed 3 mo after *Campylobacter* gavage had a decreased number of ICC in the jejunum and ileum compared to control rats. Furthermore, the density threshold of ICC per villus appears to predict SIBO. These data suggest that a decrease in the number of ICC in the small intestine is implicated in the pathogenesis of PI-IBS. Elucidating which *Campylobacter*-related factor produces this decrease in the number of ICC may contribute greatly to our understanding of PI-IBS and lead to potential treatments for IBS.

COMMENTS

Background

There is a growing interest in understanding newly discovered bacterial mechanisms in the pathophysiology of functional bowel disease. These mechanisms might result innovative treatments for diseases such as irritable bowel syndrome (IBS).

Research frontiers

Two bacterial hypotheses have emerged in IBS. The first hypothesis is that IBS appears to develop in humans after an episode of acute gastroenteritis. The other bacterial hypothesis is that a proportion of patients who already have IBS appear to have small intestinal bacterial overgrowth (SIBO) and improve with antibiotic therapy.

Innovations and breakthroughs

Recently, a new animal model of post-infectious IBS has been developed on the basis of a common human pathogen, *Campylobacter jejuni* (*C. jejuni*). In

this model, IBS-like symptoms appear to develop in rats 3 mo after clearance of the initial infection. At this time, a proportion of these rats have bacterial overgrowth, based on quantitative polymerase chain reaction. These data now link acute gastroenteritis to the development of bacterial overgrowth and symptoms in an animal model.

Applications

This new animal model will facilitate the discovery of the cascade of events that lead to IBS and SIBO. One candidate in the cascade is likely to be an effect on the neuromuscular apparatus of the gut.

Terminology

Post-infectious IBS is the development of IBS after a self-limited infection of the intestine, such as acute gastroenteritis. Interstitial cells of Cajal (ICC) are nerve cells in the intestinal lining that are important for maintaining the function of the gut.

Peer review

In this manuscript, the authors investigate the potential role of a decreased number of ICC in causing SIBO after a *C. jejuni* infection in rats. The paper is well written and is pleasant to read. The experiments have been carefully designed and executed.

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S- Editor Wang YR **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3687

World J Gastroenterol 2010 August 7; 16(29): 3687-3691
ISSN 1007-9327 (print)

BRIEF ARTICLE

Is chronic hepatitis C virus infection a risk factor for breast cancer?

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Received: September 17, 2009 Revised: December 22, 2009
Accepted: December 29, 2009
Published online: August 7, 2010

Abstract

AIM: To evaluate the prevalence of breast tumors in adult females with chronic hepatitis C virus (HCV) infection.

METHODS: Prospective, single-center study, based on female outpatients consulting in a liver unit, for 1 year. The study group included females with present and/or past history of chronic infection by HCV. Patients with spontaneous recovery were excluded. Chronic hepatitis had been proved by liver biopsy in the majority of cases and/or biological markers of inflammation and fibrosis. The control group included female patients with other well documented chronic liver diseases: chronic hepatitis B, alcoholic liver disease, autoimmune hepatitis, hemochromatosis, non alcoholic liver disease, chronic cholangitis. Participating patients were prospectively questioned during consultation about past breast history and follow-up by mammography.

RESULTS: Breast carcinoma was recorded in 17/294 patients with HCV infection (5.8%, 95% CI: 3.1-8.4) *vs* 5/107 control patients (4.7%, 95% CI: 0.67-8.67). Benign tumors of the breast (mastosis, nodules, cysts) were recorded in 75/294 patients with HCV infection (25.5%, 95% CI: 20.5-30.5) *vs* 21/107 (19.6%, 95% CI: 12.1-27.1) in the control group. No lesion was noted in 202 patients with HCV (68.7%, 95% CI: 63.4-74) *vs* 81 control patients (75.7%, 95% CI: 67.6-83.8). Despite a trend to an increased prevalence in the group with HCV infection, the difference was not significant compared to the control group (P = NS). In patients over 40 years, the results were, respectively, as follows: breast cancer associated with HCV: 17/266 patients (6.3%, 95% CI: 3.4-9.3) *vs* 5/95 patients (5.2%, 95% CI: 0.7-9.7) in the control group; benign breast tumors: 72/266 patients with HCV infection (27%, 95% CI: 21.7-32.4) *vs* 18/95 patients (18.9%, 95% CI: 11-26.8) in the control group; no breast lesion 177/266 (66.5%, 95% CI: 60.9-72.2) in patients with HCV infection *vs* 72/95 (75.7%, 95% CI: 67.1-84.4) in the control group. The differences were not significant (P = NS).

CONCLUSION: These results suggest that chronic HCV infection is not a strong promoter of breast carcinoma in adult females of any age.

Key words: Breast tumors; Breast cancer; Hepatitis C virus infection; Risk factor

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Larrey D, Bozonnat MC, Kain I, Pageaux GP, Assenat E. Is chronic hepatitis C virus infection a risk factor for breast cancer? *World J Gastroenterol* 2010; 16(29): 3687-3691 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3687.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3687

INTRODUCTION

Several viruses have been involved in the occurrence of cancers[1]. For instance, human papilloma virus has been directly implicated in uterus cancer; poliomavirus in mesothelioma and brain tumors; Epstein-Barr virus in B-cell lymphoproliferative disease and nasopharyngeal carcinoma; herpes virus in Kaposi sarcoma[1]. Hepatitis C virus (HCV) is well-known to cause chronic hepatitis, cirrhosis and hepatocarcinoma[2-5]. The prevalence of HCV in France, as in other Western European countries, is around 1% and is estimated to be 1.6% in the United States[6-8]. The potential link between HCV infection and the risk of developing malignancy other than hepatocarcinoma has been recently raised in several studies[9-13]. There are several lines of evidence showing a role in the occurrence in non-Hodgkin lymphoma and lymphoproliferative diseases[9,10]. Recent studies argue for an increasing risk of intra-hepatic cholangiocarcinoma[11] and thyroid cancer[12]. The prevalence of HCV has been evaluated in elderly patients with tumors different from hepatocarcinoma and non-Hodgkin lymphoma (colorectal, prostate, breast, bladder, kidney)[13]. Among 236 patients, 87 (36%) were positive for HCV, a higher prevalence than in the patients of the control group (10%)[13]. A statistically significant difference was observed with kidney cancer, prostate cancer, and bladder cancer[13]. Finally, the link between hepatocarcinoma and another tumor has been assessed in a retrospective study including 37 patients[14]. Five patients (13.5%) had developed another primary cancer before or after hepatocarcinoma: kidney cancer, breast cancer, colorectal cancer, prostate cancer, or lymphoma. A common point between these 5 patients was HCV chronic infection. This suggested that HCV chronic infection may not only promote hepatocarcinoma, but also other solid tumors[14].

Therefore the aim of this study was to assess the frequency of breast tumors in adult females with chronic infection by HCV and whether this disease may be a promoting factor for the onset of benign or malignant breast tumors.

MATERIALS AND METHODS

This was a prospective, single-center study performed over 1 year in female patient aged ⩾ 20 years, consulting in the Liver Unit of Montpellier School of Medicine, France, for chronic liver diseases. The study group included patients with present or past chronic infection by HCV.

Patients

Inclusions criteria: Age ⩾ 20 years, the evidence of chronic infection by HCV based on the presence of serum anti-HCV antibodies, detection of serum HCV RNA by PCR tested on several occasions for a period longer than 1 year; chronic hepatitis C proved by liver biopsy (75% of patients) or non-invasive methods (25% of patients) including biological markers of inflammation and fibrosis of the liver such as Fibrotest-Actitest® and/or elastographic examination (Fibroscan®) as recently published[15-17]; agreement of the patient for participation in the study.

Exclusion criteria: A spontaneous recovery from HCV; co-infection by hepatitis B virus or human immunodeficiency virus; absence of capacity to understand or to answer the questions in the inquiry.

The control group included females seen sequentially and prospectively during the same period and affected by chronic liver disease over 1 year, with well defined characteristics based on clinical, radiological and histological features [chronic hepatitis B, chronic alcoholic liver disease, auto-immune hepatitis, hemochromatosis, non alcoholic fatty liver disease (NAFLD), chronic cholangitis, *etc.*].

Methods

The following information was collected during the consultation by using a questionnaire: past history of breast cancer or benign breast tumor; which type if any (adenoma, mastosis, cyst); performed examinations or treatment (mammography, biopsy, surgery); potential participation in a tumor detection program by mammography. Indeed, in our geographic area, there is a detection program for breast lesions with systematic mammography every 2 years for females over 40 years.

Statistical analysis

The data processing was performed using SAS software packages version 8.1. A general descriptive analysis was done for every parameter of the study. The distribution of qualitative variables (such as breast tumors) between groups was compared using χ^2 test. When the calculated frequency of the categorical data of the contingency table did not allow the use of the χ^2 test, Fisher's exact test was performed. A P level < 0.05 was considered as significant. Stratification was performed according to age brackets: 20 to 40 years, 41 to 60 years and more than 60 years. Unilateral and bilateral power was calculated *a posteriori*.

RESULTS

Four hundred and one patients fulfilled inclusion criteria and all agreed to participate in the study. The study group with HCV infection included 294 patients; the control group included 107 patients with the following chronic liver diseases: NAFLD, 32 cases; chronic hepatitis B, 10 cases; primary biliary cirrhosis, 17 cases; auto-immune chronic hepatitis, 13 cases; chronic alcoholic liver disease, 4 cases; chronic cholangitis, 9 cases; hemochromatosis, 4 cases; and other chronic liver diseases, 18 cases.

Patients' ages ranged from 21 to 84 years (median 58 years) in the HCV group and from 23 to 84 years (median 56 years) in the control group. The distribution by age was comparable in the two groups with a predominance of patients between 40 and 70 years: 20-40 years, n = 36 (8.9%) *vs* n =

Table 1 Characteristics of the 294 patients with chronic hepatitis C

	All patients
Median age (yr)	58 (21-84)
HCV Genotype	
HCV 1	63%
HCV 2	11%
HCV 3	19%
HCV 4-5	7%
Severity of liver disease[1]	
F0-F2	70%
F3	16%
F4	14%
HCV treatment	
Never treated	30%
Past or ongoing treatment	70%

[1]The extent of fibrosis has been expressed according to the METAVIR scale as previously described[15]. HCV: Hepatitis C virus.

15 (9.7%); 41-60 years, n = 132 (33%) *vs* n = 52 (45.9%); > 60 years, n = 126 (42.8%) *vs* n = 40 (37.4%). Other main characteristics of patients with chronic HCV infection, including genotype, severity of fibrosis and anti-viral treatment history, are shown in Table 1. They are similar to the features collected in the data bank of patients with HCV infection in our regional HCV network (3280 patients)[18]. In the control group, the percentage of chronic liver diseases reaching the stage of cirrhosis (stage F4 of METAVIR scale) was 13% (14/107 cases), which was similar to the HCV group (14%) (Table 1).

The programme of mammography for patient aged over 40 years was followed in 212/266 patients (79.7%) of the HCV group and in 74/99 patients (77.8%) of the control group. In younger patients, mammography had only been performed because of symptoms, in less than 15% of patients of both groups.

The prevalence of breast tumors is shown in Table 2. Among all patients, breast cancer was recorded in 5.8% (95% CI: 3.1-8.4) of HCV group patients *vs* 4.7% (95% CI: 0.67-8.67), a benign breast tumor in 25.5% (95% CI: 20.5-30.5) in the HCV group *vs* 19.6% (95% CI: 12.1-27.1) in the control group, no evidence of breast lesion in 68.7% (95% CI: 63.4-74) of patients in the HCV group *vs* 75.7% (95% CI: 67.6-83.8) in the control group. Despite a trend for a higher prevalence of malignant or benign tumors in the HCV group, there was no significant statistical difference with the control group (Table 2). Familial history of breast cancer was recorded only in 1 of the 17 patients in the HCV group and none in the 5 cases of the control group.

The same analysis was performed according age brackets as presented in Table 3. No breast cancer was recorded in females younger than 40 years in the two groups. The frequency was low for females between 41 and 60 years, with a mild predominance but no significant difference in the HCV group compared to the control group: 3.4% (95% CI: 0.5-6.4) *vs* 1.8% (95% CI: 0-5.3). Females older than 60 years exhibited the highest prevalence with 10.0% without any difference between the two groups. In all patients over 40 years, breast cancer in the HCV group was 17/266 patients (6.3%, 95% CI: 3.4-9.3) *vs* 5/95 (5.2%, 95% CI: 0.7-9.7) in the control group.

For benign breast tumors, frequency also varied according to age brackets: it was slightly lower in the HCV group *vs* the control group for females between 20 and 40 years. In contrast, it was slightly higher in the other two age brackets but the difference was not statistically significant.

The absence of breast tumors was slightly higher in females aged between 41 and 60 years, and older than 60 years in the control group *vs* the HCV group but the difference was not statistically significant.

DISCUSSION

In many parts of the world, breast cancer is the most frequent form of cancer in females[19-22]. Similarly in France, there are 49 000 new cases/year and 11 000 deaths for a population of 60 million inhabitants[23-25]. The incidence is 101 cases/100 000 females[25]. Overall, cancer occurs in one female out of 11. As with many other cancers, the risk increases with age (less than 10% of breast cancers are detected in patients younger than 40 years[21-24]. Then the incidence increases with age[21-24]. These observations justify a systematic detection in females from 50 years. The causes of breast cancer are poorly known. Nevertheless, some risk factors have been identified[25-28]: benign breast diseases, fertility (females without pregnancy or with first pregnancy later than 30 years old exhibit a higher risk), obesity particularly after menopause[25]. Familial and genetic factors may also increase the risk, in particular through a gene mutation (BRCA1-BRCA2)[28]. The role of oral contraceptives has been discussed[21-27]. The increase in risk has been mainly observed in oral contraceptive users with a family history of breast cancer[28].

The role of HCV in breast cancer has been recently raised[14] for the following reasons. Chronic HCV infection is clearly involved in the occurrence of hepatocarcinoma and lymphoma[3,4-10] and in several other solid tumors[11-13]. Some cases of breast cancer were observed in a recent study of patients with HCV[14] and several cases have been recorded during the regular follow-up of the large cohort of patients with chronic HCV infection seen in the Liver Unit of Montpellier School of Medicine (personal observation). This led to the present prospective study knowing that a program of systematic detection of breast tumors by mammography every 2 years in all females older than 40 years has been in place in our geographic area for nearly 20 years.

Global results of this study show a breast cancer frequency of 5.8% in adult females with chronic HCV infection. Intentionally, we included a group of young females, aged 20-40 years, to detect a potential signal in an age population known to not exhibit a particular risk. No malignant lesion was recorded. However, only a small proportion of these patients had undergone mammography. Therefore, the detection of a tumor was mainly based on

Table 2 Prevalence of breast tumors

	Patients with HCV infection (n = 294)		Patients with other chronic liver diseases (n = 107)		P value
	n (%)	95% CI	n (%)	95% CI	
Breast cancer - all patients	17 (5.8)	3.1-8.4	5 (4.7)	0.67-8.67	NS
Benign breast tumors	75 (25.5)	20.5-30.5	21 (19.6)	12.1-27.1	NS
No breast lesion	202 (68.7)	63.4-74	81 (75.7)	67.6-83.8	NS

HCV: Hepatitis C virus; NS: Not significant.

Table 3 Prevalence of breast tumors according to age

Age of patients (yr)	Patients with chronic HCV infection (n = 294)		Patients with other chronic liver diseases (n = 107)		P value
	n (%)	95% CI	n (%)	95% CI	
Breast cancer					
20-40	0/28 (0)		0/12 (0)		NS
41-60	5/146 (3.4)	0.5-6.4	1/55 (1.8)	0-5.3	NS
> 60	12/120 (10.0)	4.6-15.4	4/40 (10.0)	0.7-19.3	NS
Benign breast tumors					
20-40	3/28 (10.7)	0-22.2	3/12 (25.0)	0.5-49.5	NS
41-60	41/146 (28.1)	20.8-35.3	12/55 (21.8)	10.9-32.7	NS
> 60	31/120 (25.8)	18-33.7	6/40 (15.0)	3.9-26.1	NS
No breast lesion					
20-40	25/28 (89.3)	77.8-100	9/12 (75.0)	50.5-99.5	NS
40-60	100/146 (68.5)	60.9-76	42/55 (76.4)	65.1-87.6	NS
> 60	77/120 (64.2)	55.6-72.7	30/40 (75.0)	61.6-88.4	NS

HCV: Hepatitis C virus; NS: Not significant.

its clinical expression. This sub-group representing about 10% of the overall group has slightly lowered the global prevalence. Indeed, the prevalence of all patients aged more than 40 years is 6.3% and reaches its highest rate, 10%, in females aged more than 60 years. The prevalence may have been underestimated since the mammography program was not followed in 20% of patients. Results observed in the HCV group were similar to those found in the control group, including females with other types of chronic liver diseases and having the same breast tumor detection program. We observed a similar low frequency in younger patients and the same proportion of patients who did not follow the mammography program. Therefore, this did not influence the comparison between groups. Finally, a familial history of breast cancer was recorded in a single patient with breast cancer (in the HCV group). This factor does not appear to have influenced the result of our study. Overall, these data do not support the idea that HCV chronic infection is a factor which contributes markedly to breast cancer. This view is also reinforced by the fact that prevalence of breast cancer found in this study is within the range of those found in general French and occidental populations[19-24]. Nevertheless, the interpretation of the results needs to be balanced by some limitations, in particular the relatively low number of patients in the control group and the absence of a priori calculation of the number of patients required to show a significant difference between groups with high power. This is largely caused by the fact that the prevalence of breast cancer in patients with chronic liver diseases in general and in HCV infection in particular, was completely unknown when the study was started. Therefore, our study does not allow us to draw definite conclusions. Nevertheless, it may serve as basis to set a more powerful study with matched control groups.

In summary, results of this study allowed the evaluation of the prevalence of breast cancer in patients with HCV chronic infection and suggest that HCV is not a strong promoter of breast carcinoma in adult females of any age.

COMMENTS

Background

Chronic infection by hepatitis C virus (HCV) exhibits a high frequency worldwide and represents a major cause of chronic liver disease leading to cirrhosis and hepatocarcinoma. Its influence on the onset of malignant tumors is under investigation.

Research frontiers

Several recent studies suggest that HCV chronic infection can not only cause hepatocarcinoma and lymphoma but may also promote the onset of several other solid tumors involving biliary ducts, thyroid, prostate, kidney and bladder.

Innovations and breakthroughs

The prevalence of breast cancer in patients with chronic liver diseases in general and HCV chronic infection in particular, is unknown. The relationship between HCV infection and breast cancer has been recently suggested by anecdotal cases. This is the first study designed to evaluate the prevalence of breast malignant and benign tumors in female patients and to assess whether HCV chronic infection is a risk factor. The study has been performed prospectively, using other chronic liver diseases as the control group. The results show the same prevalence of breast tumors in both groups which suggests that HCV does not appear as a strong promoting factor.

Applications

This study has allowed us to estimate the prevalence of breast cancer in females with chronic HCV infection. The interpretation of the results is balanced

by the number of patients included in the study and statistical power. Nevertheless, this constitutes a step to design new studies with matched control groups including a much larger number of patients to evaluate a potential low impact of HCV in breast malignancy.

Peer review

The authors evaluated the association between HCV infection and breast cancer. The study included 294 patients with HCV infection. Control subjects were 107 women seen in the same liver clinic over a 1-year period. Overall, there was no difference in the frequency of breast cancer or benign breast lesions between HCV-infected patients and control subjects. The hypothesis is interesting, but the study has limitations as discussed.

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S- Editor Wang JL **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3692

World J Gastroenterol 2010 August 7; 16(29): 3692-3696
ISSN 1007-9327 (print)

BRIEF ARTICLE

Nasogastric or nasointestinal feeding in severe acute pancreatitis

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Received: February 23, 2010 Revised: May 14, 2010
Accepted: May 21, 2010
Published online: August 7, 2010

Abstract

AIM: To assess the rate of spontaneous tube migration and to compare the effects of naso-gastric and naso-intestinal (NI) (beyond the ligament of Treitz) feeding in severe acute pancreatitis (SAP).

METHODS: After bedside intragastric insertion, tube position was assessed, and enteral nutrition (EN) started at day 4, irrespective of tube localization. Patients were monitored daily and clinical and laboratory parameters evaluated to compare the outcome of patients with nasogastric (NG) or NI tube.

RESULTS: Spontaneous tube migration to a NI site occurred in 10/25 (40%) prospectively enrolled SAP patients, while in 15 (60%) nutrition was started with a NG tube. Groups were similar for demographics and pancreatitis aetiology but computed tomography (CT) severity index was higher in NG tube patients than in NI (mean 6.2 *vs* 4.7, P = 0.04). The CT index seemed a risk factor for failed obtainment of spontaneous distal migration. EN trough NG or NI tube were similar in terms of tolerability, safety, clinical goals, complications and hospital stay.

CONCLUSION: Spontaneous distal tube migration is successful in 40% of SAP patients, with higher CT severity index predicting intragastric retention; in such cases EN by NG tubes seems to provide a pragmatic alternative opportunity with similar outcomes.

Key words: Acute pancreatitis; Enteral feeding; Tube migration; Nasogastric; Safety

Peer reviewers: Pascal Bucher, MD, Chef de Clinique (FMH), Service de Chirurgie Viscérale et Transplantation, University Hospital Geneva, 4, Rue Gabrielle Perret Gentile, Geneva, 1211, Switzerland; Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Piciucchi M, Merola E, Marignani M, Signoretti M, Valente R, Cocomello L, Baccini F, Panzuto F, Capurso G, Delle Fave G. Nasogastric or nasointestinal feeding in severe acute pancreatitis. *World J Gastroenterol* 2010; 16(29): 3692-3696 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3692.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3692

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process which has different grades of severity and is characterized by high mortality rates in the case of infected pancreatic necrosis[1].

Severe AP (SAP) with pancreatic necrosis is therefore a hard challenge for clinicians, and its management is still debated. The aim of treatment is to prevent necrosis

infection and to manage the hypercatabolism secondary to extended pancreatic and extrapancreatic inflammation with an adequate nutritional, volumetric and hydroelectrolytic support. Nutritional support is presently considered a key issue in patient management. Enteral nutrition (EN) should be preferred to total parenteral nutrition (TPN) in patients with SAP[2,3], as also suggested by current guidelines[1,4-6]. EN is indeed associated with reduced mortality, lower septic complications, reduced surgical procedures and hospital stay[2,3], possibly owing to a trophic action on the intestinal wall and prevention/reduction of bacterial translocation.

However, in clinical practice EN is employed far less frequently than it should. A recent survey conducted by the Italian Society of Pancreatology has reported that only about 20% of SAP patients received EN, either as single nutritional support, or in combination with TPN[7]. This figure is slightly more encouraging in Holland where some 50% of patients with SAP received EN in an observational multicentre study[8].

The main obstacle to EN diffusion is that it is considered complicated, and to require specific skills. Indeed, to ensure full pancreatic rest, nutrition tubes should be placed in the jejunum[9,10]. Although spontaneous transpyloric migration of tubes after gastric positioning, and subsequent localization in the distal duodenum or jejunum, is possible, few studies have specifically addressed this issue in patients with AP. Endoscopic placement of a nasojejunal tube is a possible alternative, but it is troublesome, potentially risky, and variable success rates have been reported[11]. Other techniques and devices have been proposed to improve tube positioning beyond the ligament of Treitz[12-14], but results, although appealing, are preliminary and sometimes out of reach in daily clinical practice.

In the past few years, it has been proposed that EN through nasogastric (NG) tubes may be a simple, safe and equally valid alternative to nasojejunal tubes, with the potential advantage of earlier administration of nutrients[15-17]. However, NG feeding cannot be recommended at this time, and it is not clear if a subgroup of SAP patients may benefit more from this approach.

We speculated that a pragmatic possibility in real-world clinical practice would be to employ NG feeding whenever tube migration does not occur spontaneously.

The aims of this study were therefore to assess the rate of spontaneous distal migration of EN tubes in patients with predicted SAP, to identify possible factors associated with it, and to compare the safety and tolerability of EN with an elemental formula in patients who started nutrition with a "proximal", NG or a "distal", naso-intestinal (NI) tube, depending on the success of spontaneous tube migration.

MATERIALS AND METHODS

Patients

This is a pragmatic ("real world") study, prospectively evaluating patients with predicted SAP admitted to our Institution from January 2006 to November 2009. AP was defined by the presence of typical abdominal pain associated with serum amylase levels > 3 times normal value. Patients with predicted SAP, as defined by a Ranson's score of 3 or higher and/or by a CT severity index of 4 or higher (as reviewed in 1), were included.

Treatment protocol

SAP patients received appropriate fluid support, antibiotic prophylaxis with iv imipenem (500 mg every 8 h), antisecretory therapy with iv pantoprazole (40 mg once daily), and were offered EN. The EN protocol included positioning of a feeding tube (10 F Flexiflo tungsten-weighted polyurethane feeding tube**,** Abbott, Baltimore, USA). After lubrication with 20 mL of water, intubation was performed at the bedside in all cases and, once completed and verified by X-ray, the guide-wire was withdrawn and the tube fixed to the nose. Prokinetics (metoclopramide 10 mg) were administered twice a day for 3 d after intubation. Tip position was radiologically assessed after 24 (day 1) and 72 h (day 3). Position was considered "proximal" (NG) if the tube tip was in the stomach or in the duodenum, proximal to the ligament of Treitz, and "distal" (NI) if in the duodenum beyond the ligament of Treitz or in the jejunum. At day 4 EN was started irrespectively of tube position (either "NG" or "NI"). An elemental formula (Survimed®) was employed at increasing volumes, from 20 mL/h up to an energetic target of 2000 kcal per day (100 mL/h).

Measured outcomes

Patients were monitored daily by measurement of clinical and laboratory parameters, and pain through a quantitative score, based on the subjective evaluation and the need for analgesic drugs (0 = no pain, 1 = pain with no need of analgesics, 2 = pain responding to low dose analgesics, 3 = pain responding to high dose analgesics, 4 = pain not responding to high dose analgesics).

Pain recurrence, biochemical changes (amylase, lipase and C-reactive protein), side effects (such as nausea, vomiting or diarrhoea), success of EN in terms of caloric target and days necessary to reach it, as well as possible need to TPN switching, were recorded. Patients received further appropriate clinical and radiologic investigations when needed. Occurrence of pancreatic (infected necrosis) and/or extrapancreatic complications (renal and/or respiratory failure, bleeding) were also recorded, as well as the patients' clinical outcome (mortality, need for surgery) and length of hospital stay.

Statistical analysis

Categorical data (percentages) were compared by means of Fisher's exact test, and continuous data by means of *t*-test for independent samples. Possible associated risks were evaluated by logistic regression analysis.

RESULTS

Patients

During the study protocol, 116 patients with AP were admitted to our unit. Their demographics and clinical features are detailed in Table 1. Among them, there were 28 pa-

Table 1 Characteristics of the 116 patients with acute pancreatitis hospitalized in the study period *n* (%)

Gender (female/male)	53/63
Median age (range, yr)	55.5 (17-92)
Mild/severe pancreatitis	88/28
Etiology	
Biliary	59 (50.9)
Alcoholic	28 (24.1)
Drug-induced	4 (3.4)
Idiopathic	8 (6.9)
Hypertrigliceridemia	3 (2.6)
Iatrogenic	6 (5.2)
Autoimmune	1 (0.9)
Traumatic	1 (0.9)
Pancreas divisum	4 (3.4)
Intrapapillary mucinous tumour	2 (1.7)

Table 2 Demographics and clinical features of the 25 patients with predicted severe acute pancreatitis who received enteral nutrition, according to tube position at nutrition start

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Sex (female, %)	6 (40)	4 (40)	1
Median age (yr)	56 (31-83)	63 (36-89)	0.3
BMI (kg/m^2)	24.8 (21.4-28.2)	25.4 (21.8-29.1)	0.7
Amylase at entry (U/L; nv < 110)	1045.5 (592.2-1498.8)	1141.6 (127.7-2155.4)	0.8
Lipase at entry (U/L; nv < 300)	8559.8 (3676.4-13443.2)	14037 (2026.1-30100.1)	0.4
CT severity index	6.2 (5.1-7.2)	4.7 (3.5-5.8)	0.04
Ranson's score	3.8 (3.1-4.6)	3 (1.6-4.3)	0.2
CRP at 72 h (mg/L)	149.1 (82.5-215.6)	138 (27-249)	0.8
White blood dells count at entry	13620 (10476-16760)	9940 (5200-14675)	0.1
LDH at entry (mU/mL)	841.8 (608.8-1074.7)	862.6 (244.8-1480.4)	0.9
Hematocrit at entry (%)	38.6 (33.9-43.2)	39.6 (35.9-43.2)	0.7
Etiology: biliary/ alcoholic or other	6/9	5/5	0.6

Values expressed as total number (percentage) or as mean (95% CI), but for age. BMI: Body mass index; CT: Computerized tomography; CRP: C reactive protein; LDH: Lactate dehydrogenase.

tients with predicted SAP (24.1%) who were offered EN as part of their treatment. Two patients refused tube positioning and received TPN, another patient spontaneously withdrew the tube on day 1 and refused further invasive treatments. Data concerning the remaining 25 patients were analysed.

Rate of spontaneous nutrition tube migration

Plain abdominal X-ray evaluation at day 3 demonstrated successful transpyloric tube migration and "NI" positioning in 10 (40%) patients. The tube did not migrate and remained "NG" in the other 15 patients (60%). As shown in Table 2, the two groups were similar in terms of sex, age and pancreatitis etiology. The predicted severity was not different according to Ranson's score, C-reactive protein or other biochemical values, but the CT severity index in the NG tube group was significantly higher than the NI group (mean 6.2 *vs* 4.7, $P = 0.04$). At a logistic regression analysis, we could not identify factors associated with the NG tube position, although CT severity index was the variable closest to significance (OR = 1.6 per unit, 95% CI: 0.95-2.9). Moreover, in all 4 patients (100%) with a CT severity index > 6, the tube did not migrate beyond the pylorus, compared to 11 of the 21 patients (52.3%) with an index ⩽ 6; however this difference was not statistically significant, probably due to the small number of patients.

Table 3 Tolerability and success of nutrition according to tube position *n* (%)

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Tube malpositioning	0	0	-
Epistaxis or Sinusitis	1 (6.6)	1 (10)	1
Accidental tube removal	0	1 (10)	0.4
Tube clogging	1 (6.6)	0	1
Aspiration pneumonia	0	0	-
Exacerbation of pain	5 (33.3)	2 (20)	0.68
Vomiting	2 (13.3)	1 (10)	1
Diarrhoea	5 (33.3)	3 (30)	1
Amylase increase > 10%	0	0	-
Lipase increase > 10%	1 (6.6)	0	1
CRP increase > 10%	2 (13.3)	2 (20)	1
Need to switch to TPN	4 (26.6)	0	0.27
Energetic target reached	14 (93.3)	8 (80)	1
Days to caloric target, mean (95% CI)	5.6 (3.8-7.4)	4.3 (3.1-5.6)	0.3

TPN: Total parenteral nutrition.

Safety and tolerability of nutrition

There were no differences regarding complications of the feeding tube positioning, such as malpositioning, epistaxis or aspiration pneumonia between patients with a NG or a NI tube. Moreover, after EN start, there was no significant difference between the NG and the NI tube groups in terms of exacerbation of pain, biochemical changes (amylase, lipase and C-reactive protein), side effects or need to switch to TPN. A similar high percentage of patients reached the energetic target (2000 Kcal) in both groups without significant time difference (Table 3).

Clinical outcome

As detailed in Table 4, there was no significant difference in the clinical outcome between the two groups, although more complications occurred in the NG group.

DISCUSSION

In the present study spontaneous migration of the EN tube beyond the stomach occurred in 40% of predicted SAP patients, and a higher CT severity index was associated with the tube being retained in the stomach. However, EN was successfully delivered in some 90% of cases, even in those patients in which tube migration beyond the ligament of Treitz was unsuccessful. Similar results in terms of safety and tolerability were observed in patients with

Table 4 Clinical outcomes of patients according to tube position *n* (%)

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Mortality	0	0	-
Need of surgery	0	0	-
Complications			
Infected pancreatic necrosis	3 (20)	1 (10)	1
Renal failure	1 (6.6)	0	1
Respiratory failure	2 (13.3)	0	0.1
Bleeding	1 (6.6)	0	1
Any of the above complications	4 (26.6)	1 (10)	0.6
Total hospital stay, mean (95% CI)	30.6 (18.1-43)	21.2 (17.7-24.6)	0.1

an "NG" or an "NI" tube. Furthermore, both approaches were equally effective in providing the nutritional support needed, caloric goals were reached in similar time intervals and length of hospital stay was not different.

A first interesting result regards the rate of spontaneous tube migration after bedside positioning, without endoscopic or radiologic assistance. The feeding tube migrated to a NI position in 40% of cases, and patients with the NG tube had a significantly higher CT scan severity index. Bedside tube positioning caused only few mild complications without differences between the two groups, but no cases of aspiration pneumonia occurred. This finding is relevant, as although delivery of feeding into the small bowel should be associated with a lower risk of aspiration, there are few data supporting this view[18].

The rate of spontaneous distal tube migration with unguided probing is considered to be around 50% in patients admitted to intensive care units (ICU) for different diseases[19]. Few studies have reported these data specifically for patients with predicted SAP, with success rates of spontaneous migration ranging from 60% to 80%[20,21]. Our results are in agreement with a recent French study reporting an overall successful migration in 61% of patients with either mild or severe AP, with this rate being reduced to 48% in SAP patients having a CT severity index score $\geqslant 4$[21]. Similarly, in our experience, in all patients with extensive pancreatic necrosis (CT severity index > 6), the tube did not migrate beyond the pylorus, and the CT index seemed a risk factor associated with failed spontaneous migration. These data may be explained by an impaired transpyloric migration due to gastroparesis, or to mechanical obstacles caused by local oedematous reactions and/or fluid collections present in the most advanced SAP cases.

As far as tolerability and safety are concerned, we have not observed any significant difference between patients receiving EN either by NG or NI tube. The nutritional goal was reached in 93% of NG patients and 80% of NI. Our findings seem to be in agreement with those published by Eatock *et al*[15], who had randomized 49 SAP patients into two groups, administering EN through NG tube in 27 cases and through NI tube in the remaining 22. Patients had been monitored daily by severity index and pain score to evaluate changes in AP severity due to enteral feeding, and during hospital stay groups behaved similarly, no matter the kind of EN used. Another two randomized clinical trials[16,17] dealing with NG enteral feeding have supported the safety and efficacy of this nutritional route, and subsequent meta-analyses confirmed the lack of difference between the two approaches, although the paucity of available data was underlined as a factor limiting the evaluation[22,23].

Regarding clinical outcomes, we have not found any significant difference in terms of complications, mortality and length of hospital stay between the two groups, although most complications occurred in patients receiving NG feeding (Table 4). This small, not significant gap is probably due to a higher prevalence of extensive necrosis in the NG group, accordingly to the significant higher CT scores of these patients at entry. However, since our group of predicted SAP patients did not experience prolonged organ failure which is a key event in discriminating patients with more severe forms[24], and we observed absence of mortality in both groups, the findings obtained in our study may not apply to patients with SAP and prolonged multiple organ failure.

This is the first study of its kind observing the outcome of EN in SAP patients in a "real world" clinical setting, with the study protocol driven by the need to have more solid grounds in making clinical decisions about everyday medical care circumstances. Both the proximal and the distal enteral approaches result to be feasible, safe and effective in most patients.

The working hypothesis we wanted to test, and that seems to be confirmed by our results, was that when spontaneous tube migration fails EN can be safely administered through NG tube. This issue has a relevant impact on everyday clinical practice as the main limit to EN usage in AP is the technical difficulty in obtaining small bowel access, as reported by 72% of ICUs joining a national survey in Canada[25].

Of course, the present non-randomized study design cannot highlight the potential benefits of NG nutrition, such as the possibility of immediate start of EN after tube positioning, but only the potential harms caused by stimulation of pancreatic function. However our observation may support the need for further clinical research aimed at clarifying this issue. Furthermore, as the rate of spontaneous distal migration of the nutrition tube, and factors related with it, was one of the results the study was aimed at identifying, the design could not imply a randomization between NG and NI, nor a power calculation. As a consequence, it is possible that differences between groups have not been appreciated due to underpowered samples. In this view, the ongoing multicentre trial on gastric *vs* mid-jejunal feeding funded by the National Institutes of Health will probably provide further important information (http://clinicaltrials.gov Identifier: NCT00580749).

In conclusion, spontaneous distal tube migration in patients with predicted SAP is successful in 40% of patients, and CT severity index is higher in patients with failed distal migration of the nutrition tube. EN administered by NG or NI tubes seems to provide equal safety,

tolerability and efficacy, even if more results are necessary to validate the routinely use of NG tubes in SAP patients.

COMMENTS

Background

Severe acute pancreatitis (SAP) requires an adequate nutritional support. Enteral nutrition (EN) should be preferred to total parenteral nutrition in patients with SAP, as it is associated with reduced mortality and complications. However, in clinical practice EN is employed far less frequently than it should. The main obstacle to EN diffusion is that it is considered complicated, as to ensure full pancreatic rest, nutrition tubes should be placed in the jejunum, requiring often troublesome procedures. In the past few years, it has been proposed that EN through nasogastric (NG) tubes may be a simple, safe and equally valid alternative to nasojejunal tubes.

Research frontiers

The authors speculated that a pragmatic possibility in real-world clinical practice would be to employ NG feeding whenever tube migration to the jejunum of bedside inserted feeding tubes does not occur spontaneously. They therefore aimed at assessing the rate of spontaneous distal migration of EN tubes in patients with predicted SAP, to identify possible factors associated with it, and to compare the safety and tolerability of EN with an elemental formula in patients who started nutrition with a "proximal", NG or a "distal", naso-intestinal (NI) tube, depending on the success of spontaneous tube migration.

Innovations and breakthroughs

The authors found that spontaneous tube migration to a NI site occurred in 10/25 (40%) prospectively enrolled SAP patients, while in 15 (60%) nutrition was started with a NG tube. Groups were similar for demographics and pancreatitis aetiology but computed tomography (CT) severity index was higher in NG tube patients than in NI (mean 6.2 *vs* 4.7, $P = 0.04$). The CT index seemed a risk factor for failed obtainment of spontaneous distal migration. EN trough NG or NI tube were similar in terms of tolerability, safety, clinical goals, complications and hospital stay.

Applications

This is the first study of its kind observing the outcome of EN in SAP patients in a "real world" clinical setting, with the study protocol driven by the need to have more solid grounds in making clinical decisions about everyday medical care circumstances. Both the proximal and the distal enteral approaches resulted to be feasible, safe and effective in most patients. This issue has a relevant impact on everyday clinical practice as the main limit to EN usage in AP is the technical difficulty in obtaining small bowel access.

Peer review

NG tube insertion, a simpler approach, will probably replace total parenteral nutrition and nasojujunal feeding in the near future. Therefore, despite the small number of patients, this paper is suitable for publication after revision.

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S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3697

World J Gastroenterol 2010 August 7; 16(29): 3697-3703
ISSN 1007-9327 (print)

BRIEF ARTICLE

Approach to early-onset colorectal cancer: Clinicopathological, familial, molecular and immunohistochemical characteristics

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Supported by The Instituto de Salud Carlos Ⅲ, Ministerio de Sanidad y Consumo (FIS01-04, project P047-04)
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Received: March 20, 2010 Revised: May 4, 2010
Accepted: May 11, 2010
Published online: August 7, 2010

Abstract

AIM: To characterize clinicopathological and familial features of early-onset colorectal cancer (CRC) and compare features of tumors with and without microsatellite instability (MSI).

METHODS: Forty-five patients with CRC aged 45 or younger were included in the study. Clinical information, a three-generation family history, and tumor samples were obtained. MSI status was analyzed and mismatch repair genes were examined in the MSI families. Tumors were included in a tissue microarray and an immunohistochemical study was carried out with a panel of selected antibodies.

RESULTS: Early onset CRC is characterized by advanced stage at diagnosis, right colon location, low-grade of differentiation, mucin production, and presence of polyps. Hereditary forms represent at least 21% of cases. Eighty-one percent of patients who died during follow-up showed a lack of expression of cyclin E, which could be a marker of poor prognosis. β-catenin expression was normal in a high percentage of tumors.

CONCLUSION: Early-onset CRC has an important familial component, with a high proportion of tumors showing microsatellite stable. Cyclin E might be a poor prognosis factor.

Key words: Early onset colorectal cancer; Microsatellite instability; Lynch syndrome; Microsatellite stable colorectal cancer

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Perea J, Alvaro E, Rodríguez Y, Gravalos C, Sánchez-Tomé E, Rivera B, Colina F, Carbonell P, González-Sarmiento R, Hidalgo M, Urioste M. Approach to early-onset colorectal cancer: Clinicopathological, familial, molecular and immunohistochemical characteristics. *World J Gastroenterol* 2010; 16(29): 3697-3703 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3697.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3697

INTRODUCTION

The prevalence of colorectal cancer (CRC) has been increasing during recent years. In 2004, it had the second highest incidence of all cancers and was the second most common cause of cancer-related death in the European Union[1]. Early onset CRC is infrequent, with an incidence of 2%-8% of all CRCs. In the United States, between 1992 and 2005, the incidence of CRC in young individuals (ages 20-49 years) increased at a rate of 1.5% per year in men and 1.6% per year in women[2].

Early onset of cancer is an indicator that a hereditary component is more likely. The most frequent hereditary form of CRC is Lynch syndrome. It is estimated to represent about 2%-5% of all CRCs, and is characterized by the development of CRC (and other types of cancer) at a mean age of 43 years[3-6]. Its molecular basis is a DNA mismatch repair (MMR) gene defect, which leads to changes in the length of repetitive DNA sequences, known as microsatellite instability (MSI).

The proportion of MSI tumors found in young patients varies between 19.7% and 41%, depending on the age of onset[7-9]. On the other hand, Lynch syndrome is estimated to cause about 1/3 of the CRC cases occurring at a young age[4,10,11]. There are some controversial aspects to the natural history and prognosis of early onset CRC, and some clinical and pathological differences compared to CRC in elderly patients[8]. Early onset CRCs are localized mostly in the right colon, are frequently poorly differentiated, show mucin production, and can develop synchronous and metachronous tumors[12]. These differences are more marked in cases with a family history suggestive of Lynch syndrome, or with molecular characteristics like MSI[8,12-14].

There is little information about microsatellite stable (MSS) forms of CRC in young adults, not only regarding their anatomoclinical features but also regarding their molecular characteristics. For example, there is an increased proportion of MSS tumors in young patients with rectal cancer[15]. Furthermore, several studies show that some alterations in molecular markers typical of MSS early onset CRCs also occur in sporadic cases of CRC, such as modified expression of APC, β-catenin and p53[9].

The aim of our study was to characterize early onset CRC by analyzing its clinical, pathological, familial, molecular, and immunohistochemical (IHC) features. We have determined the proportion of Lynch syndrome in our series, and have compared the characteristics of the MSS and MSI groups.

MATERIALS AND METHODS

Families, samples and data collection

A total of 45 individuals diagnosed with CRC at an age of 45 or younger were collected from two different Spanish institutions (Gregorio Marañón Hospital in Madrid, and Segovia General Hospital). All patients, or a first degree relative in case of death of the index case, provided written consent.

A full three-generation family medical history and colorectal paraffin-embedded tumors were obtained from each proband.

Personal and tumor clinicopathological information was obtained regarding age of onset, gender, location of the CRC (right/left colon or rectum), grade of cell differentiation (low, medium, or high), mucin production, modified Astler-Coller stage, the existence of polyps, and the presence of synchronous or metachronous CRCs. Mean follow-up was 60 mo.

To analyze the familial cancer history of each index case, we divided the neoplasms into two groups: Lynch syndrome-related tumors, and Lynch syndrome-unrelated tumors.

DNA extraction

A tissue specimen was obtained from the index case. Prior to DNA extraction, tumor and normal areas of the paraffin-embedded samples were selected *via* microscopic inspection. The proportion of tumor cells in the material used for DNA extraction exceeded 70% in all cases. DNA was extracted using proteinase K digestion, phenol-chloroform extraction, Phase Lock Gel™ Light (Eppendorf AG, Germany), and EtOH protocol precipitation.

MSI and MMR immunohistochemistry analyses

Microsatellite status was assessed using the BAT-26 mononucleotide marker, based on its high sensitivity[16-18]. However, in order to discard false negative results, all BAT-26 MSS cases fulfilling the Amsterdam I criteria[19], were analyzed using the Bethesda panel. BAT-26 was PCR amplified and fragments were evaluated using an ABI automated sequencer and GeneScan Software. For analysis of the Bethesda panel, we used the HNPCC Microsatellite Instability Test kit (Roche, Mannheim, Germany).

IHC staining for markers of the following processes was performed: MMR; apoptosis; cell adhesion; cell cycle; proliferation, and others. Markers and clones used are shown in Table 1. All samples were fixed onto a tissue microarray, on which the IHC analysis was carried out.

Scoring of tumor staining was done without any knowledge of the patients' family history or results of mutation analyses.

Detection of mutations and large deletions

Cases showing MSI and/or lack of expression of MMR proteins in tumors were screened for germline mutations in the DNA MMR genes *MLH1* and *MSH2*, by denaturing gradient-gel electrophoresis (DGGE) on a DCode System (BioRad), using primers and denaturing conditions previously reported, with slight modifications[20]. Some samples were analyzed by denaturing high-performance liquid chromatography (dHPLC) (http://insertion.stanford.edu/melt.html). When an anomalous band pattern was observed by DGGE or dHPLC, a new PCR product of the fragment was sequenced, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with a Genetic Analyzer ABI Prism 3130 (Applied Biosystems).

Table 1 Antibodies, suppliers and clones used for immunohistochemical analysis

Marker	Supplier	Clone
Mismatch repair system proteins		
Mlh1	BD PharMingen	G168-15
Msh2	Oncogene	FE11
Msh6	BD Transduction Lab.	44
Apoptosis		
Bcl-2	DAKO	124
Cell adhesion		
β-catenin	BD Transduction Lab.	14
E-cadherin	Zymed	4A2C7
Cell cycle markers		
Chk2	Novocastra	DCS 270.1
Cyclin A	Novocastra	6E6
Cyclin D1	NeoMarkers	SP4-rabbit
Cyclin D3	Novocastra	DCS-22
Cyclin E	Novocastra	13A3
p16	Santa Cruz	F-12
p21 (WAF1)	Oncogene	EA10
p27	BD Transduction Lab.	57
RB-P	Santa Cruz	Poli-rabbit
Skp2	Zymed	1G12E9
Cdk2	NeoMarkers	8D4
Proliferation		
p53	Novocastra	DO-7
Ki-67	DAKO	MIB-1
Others		
CK20	DAKO	Ks20.8
RAD50	Abcam	2C6
SMAD4	Santa Cruz	B8

Statistical analysis

Continuous variables were expressed as the median values plus/minus SD, and categorical variables were expressed as number of cases and their percentage. Differences were considered significant when $P < 0.05$. For associations between clinicopathological, familial and molecular features, and MSI status, statistical analyses were performed using Pearson's χ^2 test for parametric variables, and Fisher's exact test for non-parametric variables. When those features were continuous variables, Student's *t*-test was used, as well as for some familial features, to compare the differences between both groups. The SPSS v.11.5 for Windows (SPSS, Inc., Chicago, IL) statistical package was used.

RESULTS

Clinicopathological features

We studied a total of 45 subjects diagnosed with CRC at an age of 45 years old or younger. Clinicopathological characteristics of all cases are shown in Table 2. A high proportion of tumors were located in the right side of the colon (45%). The proportion of poorly differentiated tumors was also notable (16%). The proportion of mucin-producing tumors (mucinous tumors and "signet ring" cell tumors) was 33% of all cases. More than 50% of all tumors were in an advanced stage when diagnosed, with lymph-node involvement and/or distant metastasis. Other remarkable features were incidence of polyps found during follow-up (33%) and three cases (7%) of synchronous and metachronous CRCs. Adenomatous polyps were the most frequently observed type of polyp (13/15).

Median overall survival was 60 mo, while Median Free-Disease Survival was 48 mo. Twenty-three point nine per cent of the patients showed recurrence during follow-up, and overall mortality was 38% (17 patients).

Table 2 Clinical, pathological, and familial characteristics of the global group and the microsatellite stable and microsatellite instability groups *n* (%)

	Global	MSS	MSI	*P* (χ^2)
Patients	45 (100)	29 (69)[1]	13 (31)[1]	
Median age of onset (range, yr)	39 (25-45)	41 (25-45)	37 (32-42)	0.03[2]
Sex				
Male	23 (51)	13 (45)	7 (54)	
Female	22 (49)	16 (55)	6 (46)	NS
Location				
Right colon	20 (45)	11 (38)	9 (69)	
Left colon	15 (33)	11 (38)	2 (16)	
Rectum	10 (22)	7 (24)	2 (15)	NS
Tumor differentiation				
Well	2 (4)	2 (7)	0 (0)	
Moderate	36 (80)	23 (79)	10 (77)	
Poor	7 (16)	4 (14)	3 (23)	NS
Mucin production	15 (33)	5 (17)	5 (39)	NS
"Signet ring" cells	4 (9)	0/29 (0)	4/13 (30)	0.006
Modified Astler Coller stage				
A	3 (7)	2 (7)	0 (0)	
B	17 (38)	12 (41)	5 (39)	
C	15 (33)	7 (24)	6 (46)	
D	10 (22)	8 (28)	2 (15)	NS
Associated polyps during follow-up	15 (33)	10 (35)	4 (31)	NS
Synchronous or metachronous CRCs	3 (7)	1 (3)	1 (8)	NS
Familial history of cancer				
Lynch-related neoplasms in family	18 (40)	5 (17)	11 (85)	< 0.01
Lynch unrelated neoplasms	12 (27)	8 (28)	7 (54)	0.02
Sporadic cases	27 (60)	24 (83)	2 (15)	< 0.001

[1]Proportions were calculated for 42 tumors; [2]Statistical comparison between microsatellite stable (MSS) and microsatellite instability (MSI) groups was performed using Student´s *t* test. CRC: Colorectal cancer; NS: Not significant.

Familial features

Table 2 shows the familial cancer history data. One patient was a case with familial adenomatous polyposis (FAP); 27 (60%) were sporadic cases with Lynch syndrome (without Lynch syndrome-associated neoplasms in their families), while 20% showed familial aggregation, and eight fulfilled Amsterdam II criteria[6]. Apart from CRC, the other most frequent tumors were endometrial adenocarcinoma (six families) and gastric cancer (four families). Twelve families (27%) had other neoplasms not related to Lynch syndrome; the most frequent ones being breast cancer (four cases), larynx cancer (three cases), and leukemia (three cases).

Table 3 Immunohistochemical study of the global group[1] *n* (%)

	Normal expression	Lack of expression
Mismatch repair system proteins		
Mlh1	24 (86)	4 (14)
Msh2	24 (86)	4 (14)
Msh6	23 (88)	3 (12)
Apoptosis		
Bcl-2	7 (25)	21 (75)
Cell adhesion		
β-catenin		
Membrane	24 (86)	4 (14)
Nucleus	10 (36)	18 (64)
E-cadherin	19 (73)	7 (27)
Cell cycle markers		
Chk2	13 (54)	11 (46)
Cyclin A	13 (46)	15 (54)
Cyclin D1	12 (43)	16 (57)
Cyclin D3	12 (43)	16 (57)
Cyclin E	8 (30)	19 (70)
p16	13 (48)	14 (52)
p21	14 (56)	11 (44)
p27	12 (52)	11 (48)
RB-P	13 (48)	14 (52)
Skp2	12 (46)	14 (54)
Cdk2	13 (48)	14 (52)
Proliferation		
p53	15 (54)	13 (46)
Ki-67	17 (63)	6 (37)
Others		
CK20	10 (59)	7 (41)
RAD50	10 (63)	6 (37)
SMAD4	13 (54)	11 (46)

[1]Not all tumors could be studied by immunohistochemical.

Molecular features

Forty-two of the 45 cases were studied for MSI. The three excluded cases were: the FAP case, with an *APC* gene germline mutation (c.916delCT), and two additional cases, due to lack of paraffin embedded tumor tissue. Thirteen tumors (31%) showed MSI and the remaining 29 were therefore defined as MSS.

Blood samples were taken from the MSI index cases for *MLH1* and *MSH2* mutation screening. *MSH6* was not studied because none of the tumors showed lack of Msh6 protein alone in IHC analysis. Eight of the 13 analyzed cases (62%) showed a pathogenic germline mutation in one of the MMR genes: three cases had a mutation in *MLH1*, and five cases had a mutation in *MSH2*. None of the MSS tumors showed lack of expression of MMR proteins in the IHC study.

IHC studies with the remaining antibodies were carried out on 28 cases (Table 3). A remarkable finding was the high proportion of tumors lacking expression of cyclin E, especially in those patients who died during follow-up (9/11). Similarly, all six stage D CRCs included in the IHC study also showed lack of cyclin E expression. The lack of cyclin E expression is either an indicator of poor prognosis or a marker of advanced stage disease. β-catenin and E-cadherin, two proteins in the Wnt pathway, which plays an important role in CRC carcinogenesis, were normal in a high proportion of studied tumors.

Comparison between MSI and MSS tumors

Clinicopathological features: The comparison of the clinicopathological characteristics of the two defined groups (MSS and MSI) is shown in Table 2. Statistically significant differences were observed in the age of onset, which was earlier in the MSI cases, and in the presence of "signet-ring cell" tumors, which were absent in the MSS group. No statistically significant differences were found in the other variables analyzed, probably due to the small sample size. However, it is important to underline that the MSI CRCs were more frequently located proximally (69%), were poorly differentiated with higher mucin production, and were associated with other CRCs. The frequency of polyps during follow-up was the same in MSS and MSI tumors (35% and 31%, respectively). Another remarkable feature was that more than a half of all cases were diagnosed at an advanced stage (with lymph node involvement and/or distant metastasis, stages C or D): 52% for MSS and 61% for MSI.

There might be a trend towards a better prognosis for the MSI group when compared with the MSS group, but without reaching statistical significance: 62 mo *vs* 48 mo for median overall survival, and 62 mo *vs* 29 mo in terms of median disease-free survival. Mortality was higher in the MSS group (41%) than in the MSI group (31%).

Familial features: Table 2 shows familial cancer information and results obtained from the comparison of the MSS and MSI families. MSI cases are more frequently associated to Lynch-related neoplasia. On the other hand, the proportion of sporadic cases is very high in MSS tumors (83%).

Molecular features: The comparison of the IHC study in MSI and MSS tumors is shown in Table 4. None of the MSS tumors demonstrated lack of expression of MMR proteins in the IHC study. There was, as mentioned above, a good correlation between MSI and the IHC study of the MMR proteins.

Normal expression of β-catenin reached the same proportion (86%) in both types of tumors, indicative of the integrity of the Wnt signalling pathway in early age-of-onset CRCs. The high rate of MSI tumors with lack of expression of CK20 and RAD50 was also remarkable.

DISCUSSION

Early onset of CRC in young adults used to be considered to be rare, but many recent reports suggest not only that early onset CRC reaches 8% of all CRCs, but also that it might be increasing[1,2]. Similarly, it is a common belief that early onset CRC is mainly related to hereditary forms, especially to Lynch syndrome. In our study, the presence of a familial background of CRC is confirmed in 38% of the cases, 18% fulfilling Amsterdam criteria type Ⅱ for Lynch syndrome. Hereditary forms of CRC were confirmed in nine patients of the present series. One was a FAP case with an APC germline mutation, and the other eight cases were Lynch syndrome. The rate of MSI in

Table 4 Comparison of the immunohistochemical analyses of microsatellite stable and microsatellite instability groups *n* (%)

	IHC expression				*P*
	MSS		MSI		
	Normal	Lack	Normal	Lack	
MMR system proteins					
Mlh1	20 (100)	0 (0)	4 (50)	4 (50)	0.010
Msh2	21 (100)	0 (0)	3 (43)	4 (57)	0.002
Ms6	21 (100)	0 (0)	2 (40)	3 (60)	0.004
Apoptosis					
Bcl-2	5 (24)	16 (76)	2 (29)	5 (71)	NS
Cell adhesion					
β-catenin					
Membrane	18 (86)	3 (14)	6 (86)	1 (14)	NS
Nucleus	8 (38)	13 (62)	2 (29)	5 (71)	NS
E-cadherin	15 (75)	5 (25)	4 (60)	2 (40)	NS
Cell cycle markers					
Chk2	10 (53)	9 (47)	4 (60)	2 (40)	NS
Cyclin A	8 (38)	13 (62)	5 (71)	2 (29)	NS
Cyclin D1	7 (33)	14 (67)	5 (71)	2 (29)	NS
Cyclin D3	8 (38)	13 (62)	4 (57)	3 (43)	NS
Cyclin E	3 (14)	18 (86)	5 (83)	1 (17)	0.004
p16	9 (43)	12 (57)	4 (67)	2 (33)	NS
p21	10 (53)	9 (47)	4 (67)	2 (33)	NS
p27	8 (44)	10 (56)	4 (80)	1 (20)	NS
RB-P	11 (55)	9 (45)	2 (29)	5 (71)	NS
Skp2	9 (43)	12 (57)	3 (60)	2 (40)	NS
Cdk2	9 (43)	12 (57)	4 (67)	2 (33)	NS
Proliferation					
p53	11 (52)	10 (48)	5 (57)	3 (43)	NS
Ki-67	14 (67)	7 (33)	3 (50)	3 (50)	NS
Others					
CK20	9 (75)	3 (25)	1 (20)	4 (80)	NS
RAD50	9 (75)	3 (25)	1 (25)	3 (75)	NS
SMAD4	11 (55)	9 (45)	2 (50)	2 (50)	NS

IHC: Immunohistochemical; MSS: Microsatellite stable; MSI: Microsatellite instability; MMR: Mismatch repair; NS: Not significant.

our series is similar to that described in previous studies, ranging between 19.7% and 41%[7-9]. Twenty-nine cases showed MSS. This group showed a predominance of sporadic cases. Nevertheless, there were cases that showed a positive family history. Some of them might be associated to Familial CRC type X, namely cases with MSS tumors but fulfilling Amsterdam criteria for Lynch syndrome[21,22]. These findings, however, underline the important, but not unique role, of the known hereditary factors in this age group, prompting further searches for additional causative genes for inherited CRC.

The total sample of early age-of-onset CRCs is characterized by an important proportion of tumors that are localized in the right colon (44%), have a low-grade of differentiation (16%), produce mucin (33%), and have associated polyps (33%). Regarding pathological features, the strong trend towards low-grade differentiation and mucin production of tumors in this age group is described in the literature[7]. Other characteristics, such as synchronous and metachronous CRCs (7%) and the appearance of predominantly adenomatous polyps during the follow-up in a third of the cases, have rarely been studied previously[7,13].

Another finding is the advanced stage of the tumors at diagnosis, with more than half of the cases presenting with lymph node and/or distant metastasis in the pathological exam. This is the consequence of a delay in diagnosis, which is a characteristic of these early age-of-onset CRCs reported repeatedly in the literature[7,8,13].

The results obtained with the cyclin E antibody in the IHC study are quite remarkable. High levels of cyclin E, a G1/S phase transition controller, are found in many different tumors, including CRCs[23-25]. Cyclin E expression has only been evaluated in sporadic forms of CRC, with a variable value as a prognostic factor. Some publications suggest that lack of expression of cyclin E is associated with a faster growth of CRC, but others suggest the opposite[24,26,27]. Seventy per cent of our tumors showed lack of expression of this marker, especially the group of MSS CRCs, in which the proportion reached 86%, while the opposite occurred in the MSI group. The lack of expression of cyclin E might be associated with a poor prognosis, because expression was absent in most patients who died during follow-up (9/11), and all six stage D CRCs included in the IHC study also showed lack of expression of cyclin E. This was observed in both the MSS and the MSI groups, and in the latter group, the only case that showed lack of expression also died during follow-up. Although the sample size is small and the results must therefore be taken with caution, data related to cyclin E as a factor of poor prognosis must be validated in a larger series. In fact, it would be interesting to see if lack of expression of cyclin E corresponds with a subgroup of patients with apparently stable, near-diploid chromosomes and MSS (MACS); CRC in these patients shows an aggressive pattern and the MACS phenotype appears to be overrepresented in early-onset tumors[28,29].

Another important finding of the present study is related to the Wnt pathway. We found a high proportion of normal expression of β-catenin. This protein is an indicator of Wnt pathway dynamics[30], and the expression of β-catenin, mainly in the nucleus, is considered a good marker of the activity of the Wnt pathway[31,32]. The activation of this pathway often occurs in MSS tumors, but also in MSI tumors, independent of the age of onset[33]. Our findings, however, might indicate that the Wnt pathway is not involved in a substantial proportion of our early onset CRCs.

Early onset CRC is a heterogeneous group. To classify different subtypes with common etiology, the use of tools, such as MSI or IHC for MMR proteins, to identify an MMR system deficiency or an intact system (MSS) might be an appropriate and useful approach. All clinicopathological features analyzed in the global series tend to be more marked when the series is divided into MSI and MSS groups. MSI early onset CRCs showed characteristics similar to Lynch syndrome: earlier age of onset, predominant location in the right colon, and a high proportion of poorly differentiated tumors, in accordance with previous reports[8,13]. The same occurs with an elevated proportion of mucin-producing tumors (39%), and "signet-ring" cell tumors. MSS, on the contrary, are tumors that have an

older age of onset, are mainly located in the left colon, and have a low proportion of mucinous or "signet ring" cell tumors.

From the molecular point of view, our data must be confirmed in a larger series to reach more reliable conclusions. Nevertheless, some results should be emphasized. There is a good correlation between the lack of expression of MMR proteins and MSI. This is in agreement with published data, showing a positive prospective value of the IHC of 88%-100%[34,35]. Cyclin E is expressed in most of the MSI tumors, and the opposite occurs in the MSS, as published for sporadic CRC[27]. We found a normal expression of β-catenin in most MSI and MSS tumors. Our results contradict published findings for sporadic CRC, in which a high proportion of MSS tumors show an abnormal expression of β-catenin (90%), which decreases to 65% in MSI tumors[36,37]. There are few published studies focused on early onset CRC but in these the proportion of abnormal β-catenin expression is still significant (77.2% and 42.9% for MSS and MSI, respectively)[8]. Our results, however, should be confirmed by comparing them with control groups of CRCs, to exclude the possibility that technical differences or differences in interpretation of the staining patterns, might explain these contradictory findings.

Early onset CRC has an important proportion of hereditary forms of CRC. The apparent lack of involvement of the Wnt pathway is important, as is the possible value of cyclin E as a poor prognosis factor in early onset CRC. The advanced stage at diagnosis, as well as the still not fully understood group of MSS tumors, should promote a strong effort to diagnose these tumors at an earlier stage, providing a better understanding of MSS early onset CRC.

ACKNOWLEDGMENTS

We thank the Pathology Department of Gregorio Marañón University Hospital and Segovia Hospital for providing us with the paraffin-embedded tissues, and Ron Hartong for his help with the English redaction of the article.

COMMENTS

Background

Initially early-onset colorectal cancer (CRC) was thought to be a group mainly composed by hereditary forms of CRC (Lynch syndrome), as early-onset is a characteristic of hereditary forms of cancer, and because of that, most of the publications focused on the hereditary component of this group of CRC.

Research frontiers

There is a larger group of hereditary forms of CRC, compared with that arising in the older population. However, there is an important proportion of tumors that apparently do not show characteristics of the already known hereditary forms of CRC, and which are not well studied.

Innovations and breakthroughs

This is the first time that a complete approach (clinicopathological, familial, molecular, and immunohistochemical) to the early-onset CRC has been performed. The authors have identified certain characteristics that seem to be more frequent in the early-onset CRC. The proportion of hereditary forms, though, represents a relatively small amount of the cases, and some interesting findings are presented that allow prognozing of these patients.

Applications

This is the first step towards a deeper understanding of early-onset CRC, an entity that is increasing in an especially sensitive group of population.

Peer review

The authors present an in depth analysis of young patients (< 45 years) presenting with CRC. Though the sample is small (n = 45) they perform a comparative analysis between those cases that are microsatellite stable and those that show microsatellite instability.

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S- Editor Wang YR **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3704

World J Gastroenterol 2010 August 7; 16(29): 3704-3708
ISSN 1007-9327 (print)

BRIEF ARTICLE

Is autoimmune hepatitis a frequent finding among HCV patients with intense interface hepatitis?

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Supported by CAPES research support agency, Brazil
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Received: February 1, 2010 Revised: March 20, 2010
Accepted: March 27, 2010
Published online: August 7, 2010

Abstract

AIM: To evaluate the overlap of autoimmune hepatitis in hepatitis C virus (HCV)-infected patients with intense interface hepatitis.

METHODS: Among 1759 patients with hepatitis C submitted to liver biopsy, 92 (5.2%) presented intense interface hepatitis. These patients were evaluated regarding the presence of antinuclear antibody (ANA), anti-smooth muscle antibody (SMA) and anti-liver/kidney microsomal antibody (LKM-1), levels of γ-globulin and histological findings related to autoimmune hepatitis (plasma cell infiltrate and presence of rosettes).

RESULTS: Among patients with hepatitis C and intense interface hepatitis there was a low prevalence of autoantibodies (ANA = 12%, SMA = 5%, LKM-1 = 0%) and the median γ-globulin level was within the normal range. Typical histological findings of autoimmune disease were observed in only two cases (2%). After applying the score for diagnosis of autoimmune hepatitis, only one patient was classified with a definitive diagnosis of autoimmune hepatitis. Since overlap with autoimmune hepatitis was not the explanation for the intense necroinflammatory activity in patients with chronic hepatitis C we sought to identify the variables associated with this finding. The presence of intense interface hepatitis was associated with more advanced age, both at the time of infection and at the time of the biopsy, and higher prevalence of blood transfusion and alcohol abuse.

CONCLUSION: Although possible, overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis, an unusual presentation which seems to be related to other host variables.

Key words: Hepatitis C; Liver biopsy; Antinuclear antibody; Autoimmune hepatitis; Interface hepatitis

Peer reviewer: Dr. Eli Magen, Allergy and Clinical Immunology, Medicine B, Barzilai Medical Center, Ashdod 77456, Israel

Badiani RG, Becker V, Perez RM, Matos CAL, Lemos LB, Lanzoni VP, Andrade LEC, Dellavance A, Silva AEB, Ferraz MLG. Is autoimmune hepatitis a frequent finding among HCV patients with intense interface hepatitis? *World J Gastroenterol* 2010; 16(29): 3704-3708 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3704.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3704

INTRODUCTION

Hepatitis C is the main cause of liver-related morbidity and mortality and represents a worldwide public health problem[1]. An estimated 170 million individuals are infected with hepatitis C virus (HCV), corresponding to 3% of the world population[2].

Infection with HCV is characterized by a high chronicity rate (70% to 85%)[3-6], progression to cirrhosis in 20% to 30% of cases[1,6-8] and the development of hepatocarcinoma in 5% of patients[9]. In addition, this infection represents the most common indication for liver transplantation worldwide[10].

Histological analysis of patients chronically infected with HCV usually reveals some degree of fibrosis, generally associated with the presence of mild or moderate necroinflammatory activity[11]. However, a histological pattern demonstrating intense interface hepatitis has been reported[12,13]. In these cases a possible association with autoimmune hepatitis has been suggested, raising doubts regarding the correct diagnosis and the establishment of adequate treatment[14-16]. The objective of the present study was to evaluate the overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis.

MATERIALS AND METHODS

Patients

Patients chronically infected with HCV followed up at the Federal University of Sao Paulo between 1993 and 2006, who were submitted to a liver biopsy, were studied. The inclusion criteria were chronic infection with HCV (characterized by HCV-RNA positivity) and the presence of intense interface hepatitis upon histological analysis. Patients previously treated or who were HBsAg-positive were excluded.

A control group consisting of patients chronically infected with HCV, who presented absent, mild or moderate interface hepatitis, was included in order to evaluate if an eventual association of autoimmune hepatitis with hepatitis C was restricted to patients with intense necroinflammatory activity. In the absence of such association, a comparison with the control group was performed to evaluate other factors possibly related to intense interface hepatitis. This control group was randomly selected from the database of the Hepatitis Outpatient Clinic of the Federal University of Sao Paulo (1:1 ratio). The same exclusion criteria were adopted for the control group. For the comparative analysis, patients with associated diseases [human immunodeficiency virus (HIV), end-stage renal disease and kidney transplant] were excluded from both groups.

The study was approved by the local Ethical Committee.

Epidemiological characteristics

The patients were evaluated regarding gender, age, estimated duration of infection, age at the time of infection, abusive alcohol consumption (men > 40 g/d and women > 20 g/d), the presence of parenteral risk factors (intravenous drug use, hemodialysis or blood transfusion before 1992) and associated diseases (HIV, end-stage renal disease and kidney transplant). This information was recovered from charts where the data were systematically evaluated with a standardized questionnaire. The duration of infection was evaluated in patients with parenteral risk factors and was estimated from the first year of intravenous drug use or hemodialysis or from the year of first transfusion in patients who had received blood transfusions before 1992.

Laboratory tests

The liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) and alkaline phosphatase were assayed by an automated kinetic method and were expressed as the following index: value obtained/upper limit of normal. γ-globulins were assayed by electrophoretic fractionation on agarose gel and densitometry. All biochemical tests were performed within a period of 3 mo from the date of the liver biopsy.

Antinuclear antibody (ANA), anti-smooth muscle antibody (SMA), anti-liver/kidney microsomal antibody (anti-LKM) and anti-mitochondrial antibody were determined by indirect immunofluorescence and the titer was considered significant when higher than 1/40.

The patients were tested for the presence of HBsAg and anti-HIV-1/2 using commercial kits (Abbott Laboratories, Chicago, IL, USA). Anti-HCV was determined with a third-generation enzyme immunoassay (Abbott Laboratories, Chicago, IL, USA). Qualitative HCV-RNA was detected by PCR using the Amplicor® Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ, USA), with a detection limit of 50 IU/mL. HCV genotyping was performed by VERSANT HCV Genotype Assay - LiPA (Innogenetics N.V., Belgium).

Histological analysis

A liver biopsy was indicated in all patients, irrespective of ALT levels. Liver tissue fragments were obtained by percutaneous biopsy with a Tru-cut® needle. The liver biopsy slides were stained with hematoxylin-eosin, Masson's trichrome, Prussian blue (Perls' stain), and silver for reticular fibers (Gomori's stain), and were reviewed by a single pathologist who was unaware of the clinical data. Histological analysis included the determination of the grade of interface hepatitis and of the stage of fibrosis, which were assessed using a semiquantitative scoring system according to Ludwig[17]. Patients were classified as having intense interface hepatitis if they presented a score of periportal activity = 4, in a scale varying from 0 (no inflammation) to 4 (intense necroinflammatory activity).

In order to better characterize the presence of eventual histological components suggestive of autoimmune injury, the presence of plasma cell infiltrate and rosettes was also analyzed.

Scoring system for diagnosis of autoimmune hepatitis

All patients were evaluated regarding the reviewed interna-

Table 1 General characteristics of patients with intense interface hepatitis *n* (%)

	Intense interface hepatitis (*n* = 92)
Male gender	52 (57)
Age (mean ± SD, yr)	49.8 ± 10.5
Age at the time of infection (mean ± SD, yr)	29.5 ± 9.8
Parenteral risk factor	59 (64)
Duration of infection (mean ± SD, yr)	20.1 ± 8.7
History of blood transfusion	44 (48)
Intravenous drug use	13 (14)
Hemodialysis	2 (2)
Alcoholism	25 (27)
Human immunodeficiency virus-positive	5 (5)
Renal transplant	6 (7)
End-stage renal disease	2 (2)
Alanine aminotransferase (xULN)	4.1 (0.3-18.2)
Aspartate aminotransferase (xULN)	3.0 (0.9-10.4)
γ-glutamyltransferase (xULN)	4.1 (0.1-16.4)
Alkaline phosphatase (xULN)	0.9 (0.3-3.8)
γ-globulin (g/dL)	1.9 (0.73-5.74)
Antinuclear antibody	11 (12)[1]
Anti-smooth muscle antibody	5 (5)
Anti-liver/kidney microsomal antibody	0 (0)
Antimitochondrial antibody	1 (1)
Hepatitis C virus genotype	
Genotype 1	53/77 (69)
Genotype non-1	24/77 (31)
Cirrhosis	53 (58)
Parenchymatous activity ⩾ 3	50 (54)
Intense plasma cell infiltrate	2 (2)
Rosettes	26 (28)

[1]All antinuclear antibody positive patients presented the speckled pattern; titers varied from 1/80 to 1/640. xULN: Times the upper limit of normal.

tional diagnostic criteria for autoimmune hepatitis according to the International Autoimmune Hepatitis Group[18].

Statistical analysis

The χ^2 test and Fisher's exact test were used for statistical analysis of categorical variables. Numerical variables were compared between the two groups using the Student *t*-test and Mann-Whitney test. A level of significance of 0.05 (α = 5%) was adopted.

RESULTS

Among the 1759 patients chronically infected with HCV submitted to a liver biopsy during the study period, 92 presented intense interface hepatitis, corresponding to 5.2% of the initial sample. The characteristics of these patients are shown in Table 1.

Among patients presenting intense interface hepatitis, there was a low prevalence of autoantibodies and the median γ-globulin level was within the normal range. Typical histological findings of autoimmune disease were observed in only two cases (2%). After applying the scoring system for diagnosis of autoimmune hepatitis only one patient was classified as having a definitive diagnosis.

Since overlap with autoimmune hepatitis was not the explanation for the intense necroinflammatory activity in patients with chronic hepatitis C, we sought to identify the variables associated with this finding. Therefore, we compared epidemiological, laboratory and histological characteristics between patients with intense interface hepatitis and a randomly selected control group consisting of chronic HCV-infected patients with absent, mild or moderate interface hepatitis. For comparison between groups, 13 patients with associated disease were excluded from the group with intense interface hepatitis: 6 patients with kidney transplant, 5 with HIV co-infection and 2 with end-stage renal disease.

Table 2 Comparative analysis of general characteristics between groups *n* (%)

	Group 1 (*n* = 79)	Group 2 (*n* = 79)	*P*
Male gender	44 (56)	49 (62)	0.42
Mean age (mean ± SD, yr)	50.8 ± 10.6	43.9 ± 11.5	< 0.001
Alcoholism	22 (28)	10 (13)	0.02
Blood transfusion	37 (47)	23 (29)	0.02
Intravenous drug use	10 (13)	9 (11)	0.81
Duration of infection (mean ± SD, yr)	22.2 ± 7.9	20.9 ± 7.5	0.49
Age at the time of infection (mean ± SD, yr)	28.6 ± 9.6	23.0 ± 11.2	0.02
ALT (xULN)	4.2 (0.3-18.2)	1.8 (0.9-11.3)	< 0.001
AST (xULN)	3.1 (0.9-10.4)	1.4 (0.8-5.8)	< 0.001
GGT (xULN)	3.8 (0.1-16.4)	1.1 (0.1-10.6)	< 0.001
Alkaline phosphatase (xULN)	0.8 (0.3-2.1)	1.0 (0.1-3.0)	0.32
γ-globulin (g/dL)	1.9 (0.7-4.0)	1.7 (0.9-3.7)	0.19
ANA positive	9 (11)	7 (9)	0.59
Genotype 1	43/65 (66)	54/79 (68)	0.78
Cirrhosis	50 (63)	20 (25)	< 0.001
Parenchymatous activity ⩾ 3	43 (55)	3 (4)	< 0.001

Group 1: Patients with intense interface hepatitis; Group 2: Patients with absent, mild or moderate interface hepatitis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; xULN: Times the upper limit of normal; ANA: Antinuclear antibody.

In the group of patients with intense interface hepatitis, the subjects were older and the proportions of blood transfusion and abusive alcohol consumption were higher. In addition, these patients presented higher levels of ALT (4.2 *vs* 1.8, $P < 0.001$), AST (3.1 *vs* 1.4, $P < 0.001$) and GGT (3.8 *vs* 1.1, $P < 0.001$). No difference in the proportion of patients with reactive ANA or serum γ-globulin levels was observed between groups (Table 2).

Regarding liver biopsy, the mean number of portal tracts observed was 11. Histological aspects are presented in Table 2. The proportion of patients with moderate to intense lobular necroinflammatory activity and cirrhosis was higher in the group with intense interface hepatitis ($P < 0.001$).

DISCUSSION

Previous studies have demonstrated that the presence of intense interface hepatitis in patients chronically infected with HCV is rare[19,20]. When this finding is present, other liver diseases, especially autoimmune hepatitis, should be carefully ruled out. In the present study, 1759 patients

chronically infected with HCV were initially evaluated and in 92 of them (5.2%) a liver biopsy revealed intense interface hepatitis, indicating that, although uncommon, this finding might be a histological pattern of hepatitis C.

The main objective of the present study was to evaluate the overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis. In this sample only two patients (2%) had serological and histological evidence of autoimmunity in the group with intense interface hepatitis and only one patient had a definitive diagnosis of autoimmune hepatitis based on the International Autoimmune Hepatitis Group scoring system[18]. Although a 12% prevalence of ANA was found among the intense interface hepatitis patients, there was no difference in the proportion of patients with positive ANA when they were compared to patients with less intense necroinflammatory activity. In addition, the prevalence of SMA and anti-LKM was very low in the group with intense interface hepatitis.

No histological lesions typical of autoimmune hepatitis were identified in all except two patients and the proportion of cases presenting a significant plasma cell infiltrate was very low in patients with intense interface hepatitis. The high proportion of patients with rosettes observed in the group with intense interface hepatitis was not considered as suggestive of autoimmune injury, since it reflects hepatic regeneration activity as a consequence of greater necroinflammatory activity and can be observed in other etiologies of liver disease[21,22]. These findings support the suggestion that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis and raises the possibility that some mechanism related to the host-virus interaction might be responsible for the intense interface hepatitis observed.

Since overlap with autoimmune hepatitis was not found in association with intense necroinflammatory activity in patients with chronic hepatitis C we sought to identify other variables associated with this finding.

In comparison to the control group, the presence of intense interface hepatitis was associated with the following epidemiological characteristics: more advanced age both at the time of infection and at the time of the biopsy, and a higher prevalence of blood transfusion and alcohol abuse. With respect to age at the time of infection, a higher necroinflammatory hepatic activity was observed in patients with more advanced age at HCV infection[19,23]. However, the mechanisms related to this phenomenon are still unknown. One hypothesis is that the ability of the immune system to contain the pathological process triggered by the HCV infection declines with age. It is possible that the higher proportion of patients with a history of blood transfusion in the group with intense interface hepatitis, another association observed in this study, also reflects the association between more advanced age and intense interface hepatitis, since in this sample patients with a history of transfusion were older ($P = 0.025$).

Excessive alcohol consumption was another variable associated with intense interface hepatitis, suggesting that alcohol may modify the histological injury induced by HCV[23,24], rendering the disease more aggressive even in the absence of lesions characteristic of direct alcoholic hepatic disease. The mechanism whereby alcohol may aggravate the HCV-induced inflammatory process remains obscure.

Analysis of biochemical and histological characteristics demonstrated that patients with intense interface hepatitis present with more severe liver disease, including a high proportion of cirrhosis (63%). With respect to liver enzymes, significantly higher ALT, AST and GGT levels were observed, an expected finding since elevated aminotransferases[25] and GGT[26] levels have been shown to be associated with greater hepatic inflammatory activity.

Although an association between genotype 1 and more intense necroinflammatory activity has been demonstrated[27], no such association between HCV genotype and severity of liver disease was observed in the present study and in most of the studies reported in the literature[28-32].

Regarding histological findings, the histological variables associated with intense interface hepatitis were advanced fibrosis and more intense parenchymatous activity. Although the association between necroinflammatory activity and fibrosis is controversial, this finding supports the hypothesis that necroinflammatory activity influences the progression of hepatic fibrosis as demonstrated in other studies[33-36]. The parenchymatous activity was another variable independently associated with intense interface hepatitis. Although the interface hepatitis is the main histological lesion observed in chronic hepatitis C, whenever the necroinflammatory activity is intense, this process tends to involve all the compartments, and is not restricted to the portal tract.

In conclusion, the absence of elevated γ-globulin levels, the low prevalence of autoantibodies, the occurrence of typical histological findings of autoimmune disease in only two cases (2%), and a definitive diagnosis according to the autoimmune hepatitis score in only one case, suggest that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis. The uncommon histological presentation of hepatitis C with intense interface hepatitis seems to be related mainly to other host variables.

COMMENTS

Background

Previous studies have demonstrated that intense interface hepatitis is an uncommon finding in chronic hepatitis C. When this finding is present, it raises doubt regarding a possible association with autoimmune hepatitis.

Research frontiers

The main objective of the present study was to evaluate the overlap with autoimmune hepatitis in hepatitis C virus (HCV)-infected patients with intense interface hepatitis.

Innovations and breakthroughs

This study demonstrated that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis. This finding raises the possibility that some mechanism related to the host-virus interaction might be responsible for this histological pattern.

Applications

Considering that overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis is very uncommon, the best clinical approach for these patients should be antiviral therapy. These results reduce the dilemma of

whether immunosuppressive therapy is indicated for patients presenting with this histological finding.

Terminology

Interface hepatitis is a histological finding in liver biopsies observed in chronic hepatitis. It is also termed necroinflammatory periportal activity and was formerly known as piecemeal necrosis. Interface hepatitis is graded as mild, moderate or intense. In this study the authors aimed to evaluate HCV-infected patients with intense interface hepatitis.

Peer review

The paper is well written and represents timely research aimed at identifying a link between hepatitis C and autoimmune hepatitis.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3709

World J Gastroenterol 2010 August 7; 16(29): 3709-3715
ISSN 1007-9327 (print)

BRIEF ARTICLE

Capecitabine with radiation is an effective adjuvant therapy in gastric cancers

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Received: July 14, 2009 Revised: December 23, 2009
Accepted: December 30, 2009
Published online: August 7, 2010

Abstract

AIM: To analyze the outcome of patients who received concurrent capecitabine (Xeloda) and radiation (XRT) compared to the established concurrent 5-fluorouracil (5-FU) with radiation (5FU-RT) and fluoropyrimidine-based chemotherapy alone as adjuvant treatment in gastric cancers.

METHODS: All patients with gastric cancers who received adjuvant treatment at the National Cancer Centre Singapore between 1996 and 2006 were reviewed. Treatment outcomes of patients who received XRT were compared with those who had 5FU-RT or chemotherapy alone as adjuvant therapy for gastric cancers.

RESULTS: A total of 108 patients were reviewed. Median age at diagnosis was 60. The majority of the patients (64.8%) had advanced stage Ⅲ and Ⅳ disease (with no distant metastasis). All except 4 patients had D2 gastrectomy. Twenty one patients (19.4%) had positive surgical resection margins. Thirty three patients received XRT compared with 52 who had 5FU-RT and 23 who received chemotherapy alone. For the patients in the chemotherapy-only group, all had fluoropyrimidine-based therapy, with added cisplatin in 7 patients and epirubicin in 2 patients. Median recurrence-free survival was longer for the XRT group (52 mo) compared to the 5FU-RT (35 mo) and chemotherapy-only groups (25 mo) (P = 0.48). The patients in the XRT group achieved similar median overall survival (53 mo) as the 5FU-RT (54 mo) and the chemotherapy-only groups (44 mo) (P = 0.5).

CONCLUSION: Capecitabine with concurrent radiation was as effective as concurrent 5FU with radiation or fluoropyrimidine-based chemotherapy alone when used as adjuvant treatment in patients with gastric cancers.

Key words: Capecitabine; Radiation; Gastric cancer; Adjuvant chemotherapy

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Tham CK, Choo SP, Poon DYH, Toh HC, Ong SYK, Tan SH, Wang MLC, Foo KF. Capecitabine with radiation is an effective adjuvant therapy in gastric cancers. *World J Gastroenterol* 2010; 16(29): 3709-3715 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3709.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3709

INTRODUCTION

Surgery is the curative treatment for gastric cancer. However, the outcome of large T3-T4 tumors and those with lymph node involvement remains poor after surgical resection alone with high risk of local and distant recurrence[1]. Many attempts had been made to improve the prognosis of resected gastric cancers. These include postoperative adjuvant chemotherapy with or without radiation and perioperative chemotherapy.

Adjuvant therapy in gastric cancer is a field of ongoing active research. The different modalities in the adjuvant setting include chemoradiation and chemotherapy alone. There is currently no randomized phase Ⅲ trial that directly compared these various modalities. Hence, the optimal adjuvant therapy in gastric cancer remains to be determined.

Capecitabine (Xeloda), an oral fluoropyrimidine, was shown to have equivalent efficacy to continuous-infusional 5-fluorouracil (5-FU) in several phase Ⅲ trials in metastatic gastric cancers. The REAL-2 study compared capecitabine or 5-FU in combination with cisplatin and oxaliplatin and showed no differences in response rates or survival between all the 4 combination regimens[2]. The ML 17032 trial comparing the combination of capecitabine with cisplatin to 5-FU with cisplatin showed that capecitabine and cisplatin had a better response rate (41% *vs* 29%) and overall survival (10.5 mo *vs* 9.3 mo)[3]. Because of the convenient oral administration of capecitabine, its use in the adjuvant setting is an attractive option to explore. In fact, capecitabine with concurrent radiation as an adjuvant or neoadjuvant treatment in resected gastric cancer and other gastrointestinal malignancies, particularly rectal cancer, has been explored in several studies[4-6]. These studies demonstrated that capecitabine with concurrent radiation was feasible with manageable toxicities. However, the regimen has never been compared with the more established concurrent 5-FU and radiation (5FU-RT) or adjuvant chemotherapy alone.

Hence, in this study, we aimed to analyze the outcome of patients who received adjuvant concurrent capecitabine and radiation (XRT) compared to those who received concurrent FU-RT or fluoropyrimidine-based chemotherapy alone in the adjuvant setting in gastric cancers.

MATERIALS AND METHODS

All patients diagnosed with gastric cancer who received adjuvant treatment at National Cancer Center Singapore from 1996 to 2007 were reviewed. Clinical information was collected retrospectively and included age, gender, performance status, *Helicobacter pylori* status, surgical outcome, tumor histology, carcinoembryonic antigen, baseline hematologic and biochemical parameters, recurrence and survival data.

The patients were divided into 3 groups based on the adjuvant treatment received. These included 5FU-RT, XRT and chemotherapy alone for analysis. The patients in the XRT group received 825 mg/m^2 capecitabine daily with concurrent radiation followed by capecitabine 2000 mg/m^2 every 3 wk for 6 cycles. Patients in the 5FU-RT group received chemoradiation which consisted of 5 cycles of 5-FU 400 mg/m^2 plus leucovorin (20 mg/m^2) given every 28 d with 2 cycles given concurrently with radiation. The total radiation dose in both the XRT and 5FU-RT groups was 45 Gy delivered over a period of 5 wk. The patients in the chemotherapy-only group received fluoropyrimidine-based chemotherapy.

The patients receiving XRT were compared with those who had 5FU-RT and fluoropyrimidine-based chemotherapy in terms of recurrence-free survival and overall survival. We also compared the patients who received radiation as part of their adjuvant therapy to those who received chemotherapy alone.

Comparison of the median age at baseline between treatment groups was done using the Mann-Whitney *U* test. Other baseline characteristics, such as gender, performance status, stage and grade, as shown in Table 1, were performed using Fisher's exact test.

Recurrence-free survival duration was calculated from the date of diagnosis to the date of recurrence or death (whichever occurred first) or last follow-up. Overall survival duration was calculated from the date of diagnosis to the date of death or last follow-up. The Kaplan-Meier method was used to estimate and plot the recurrence-free survival and overall survival curves for the 3 treatment groups. The log-rank test was used to test if the survival function for the treatment groups were statistically different at the 5% significance level.

For recurrence-free survival and overall survival, the Cox proportional hazards model was used to estimate the crude and age-adjusted hazard ratios between treatment groups using the XRT arm as the reference treatment. Age at diagnosis was included in multivariate Cox regression analyses to estimate the adjusted hazard ratios. Subgroup analysis of the recurrence-free survival and overall survival hazard ratios between the chemotherapy-only group and chemoradiotherapy (XRT or 5FU-RT) were performed using the Cox proportional hazard model for patients with a positive surgical margin, Stage 3 or 4, and positive nodes, respectively.

Statistical analysis

The Cox proportional hazards model and log-rank test were also used to estimate the hazard ratios to assess if any of the baseline characteristics were associated with recurrence-free survival and overall survival in each of the 3 treatment groups. Normality of the variables in the Cox model was assessed using a Q-Q plot and proportionality assumption of the Cox model was assessed using the Schoenfeld test.

Two-sided *P*-values of less than 5% were considered as statistically significant. The software used for the analyses was STATA version 9.1.

RESULTS

A total of 108 patients had received adjuvant therapy for gastric cancer at our institution in the specified period. Median follow-up duration was 23 mo.

Table 1 Patient characteristics *n* (%)

	All patients (*n* = 108)	XRT (*n* = 33)	5FU-RT (*n* = 52)	Chemo alone (*n* = 23)	*P*-value[1] for XRT *vs*	
					5FU-RT	Chemo alone
Age (yr)						
Median	60	64	57	56	0.003[2]	0.03[2]
Inter-quartile range	49.5-66.0	57.7-68.8	48.0-63.7	49.3-65.7		
Gender						
Male	65 (60.2)	22 (66.7)	30 (57.7)	13 (56.5)	0.5	0.6
Female	43 (39.8)	11 (33.3)	22 (42.3)	10 (43.5)		
ECOG						
0	6 (5.6)	1 (3.0)	3 (5.8)	2 (8.7)	1.0	0.6
1	102 (94.4)	32 (97.0)	49 (94.2)	21 (91.3)		
Helicobacter pylori status						
Yes	36 (53.7)	16 (66.7)	15 (50.0)	5 (38.5)	0.3	0.2
No	31 (46.3)	8 (33.3)	15 (50.0)	8 (61.5)		
Surgical margins						
Positive	21 (19.4)	4 (12.1)	14 (26.9)	3 (13.0)	0.2	1.0
Negative	87 (80.6)	29 (87.9)	38 (73.1)	20 (87.0)		
Stage						
1 or 2	38 (35.2)	12 (36.4)	14 (26.9)	12 (52.2)	0.5	0.3
3 or 4	70 (64.8)	21 (63.6)	38 (73.1)	11 (47.8)		
Grade						
1 or 2	31 (28.7)	12 (36.4)	12 (23.1)	7 (30.4)	0.2	0.8
3	77 (71.3)	21 (63.6)	40 (76.9)	16 (69.6)		
Albumin						
< 30	12 (13.3)	4 (12.9)	4 (10.0)	4 (21.1)	0.7	0.5
⩾ 30	78 (86.7)	27 (87.1)	36 (90.0)	15 (78.9)		
CEA						
Normal	50 (90.9)	17 (89.5)	24 (92.3)	9 (90.0)	1.0	1.0
High	5 (9.1)	2 (10.5)	2 (7.7)	1 (10.0)		
Hemoglobin						
< 10	26 (25.7)	8 (24.2)	14 (29.2)	4 (20.0)	0.8	1.0
⩾ 10	75 (74.3)	25 (75.8)	34 (70.8)	16 (80.0)		

[1]All *P*-values calculated using Fisher's exact test unless otherwise stated; [2]*P*-value calculated using the Mann-Whitney *U* test. XRT: Capecitabine + radiation; 5FU-RT: 5-fluorouracil + radiation; ECOG: Eastern Cooperative Oncology Group status; CEA: Carcinoembryonic antigen.

Thirty-three of these patients received XRT, 52 received 5FU-RT and 23 received fluoropyrimidine-based chemotherapy. Of the patients who received chemotherapy alone, 11 had capecitabine alone, 6 had ECF (epirubicin, cisplatin and 5-FU), 4 had XELOX (capecitabine and oxaliplatin) and 2 had 5-FU alone. Ninety-one percent of the patients in the XRT group, 89% in the 5FU-RT and 91% in the chemotherapy-only group completed the full course of adjuvant treatment.

The characteristics of these patients are shown in Table 1. Median age at diagnosis was 60 years. Sixty-five percent of the patients had advanced stage Ⅲ and Ⅳ (with no distant metastasis) disease. All except 4 patients had D2 gastrectomy (2 in the XRT group, 2 in the chemotherapy-only group). Twenty-one of these patients (19.4%) had positive surgical resection margins (14 in the 5FU-RT group, 4 in the XRT group and 3 in the chemotherapy-only group). The only significant difference in characteristics among the 3 groups of patients was median age, with the median age of the patients in the XRT group being greater than that of the other 2 groups.

Median recurrence-free survival was longer for the XRT group (52 mo) compared with the 5FU-RT (35 mo) and chemotherapy-only groups (25 mo). However, the recurrence-free survival curves between the 3 groups were not statistically significant ($P = 0.5$, Figure 1A, Table 2). Patients with positive surgical margins were found to have a significantly poorer recurrence-free survival in both the XRT and 5FU-RT groups but not in the chemotherapy-only group (Table 3).

The overall survival times of the patients in the 3 treatment groups were not statistically different ($P = 0.5$), with the median overall survival of the patients in the XRT group at 53 mo, the 5FU-RT group at 54 mo and the chemotherapy-only group at 44 mo (Figure 1B). Patients with positive surgical margins were found to have poorer survival across all the treatment groups (Table 3).

When comparing patients who received radiation as part of their adjuvant therapy to those who received chemotherapy alone, there was no statistical difference in recurrence-free survival and overall survival between these 2 groups of patients (Figure 1C and D). Subgroup analyses of patients with positive surgical margins, lymph node positive and T3 or T4 disease did not show a statistically difference in survival between patients who received radiation as part of their adjuvant therapy and those who did not (Table 4).

Treatment was generally well tolerated in the 3 treatment groups. In total only 6 patients (5%) had grade 3 or 4 toxicities, 4 in the 5FU-RT group, one in the XRT group and one in the chemotherapy-only group. Diarrhea was the most common grade 3 or 4 adverse effect. One

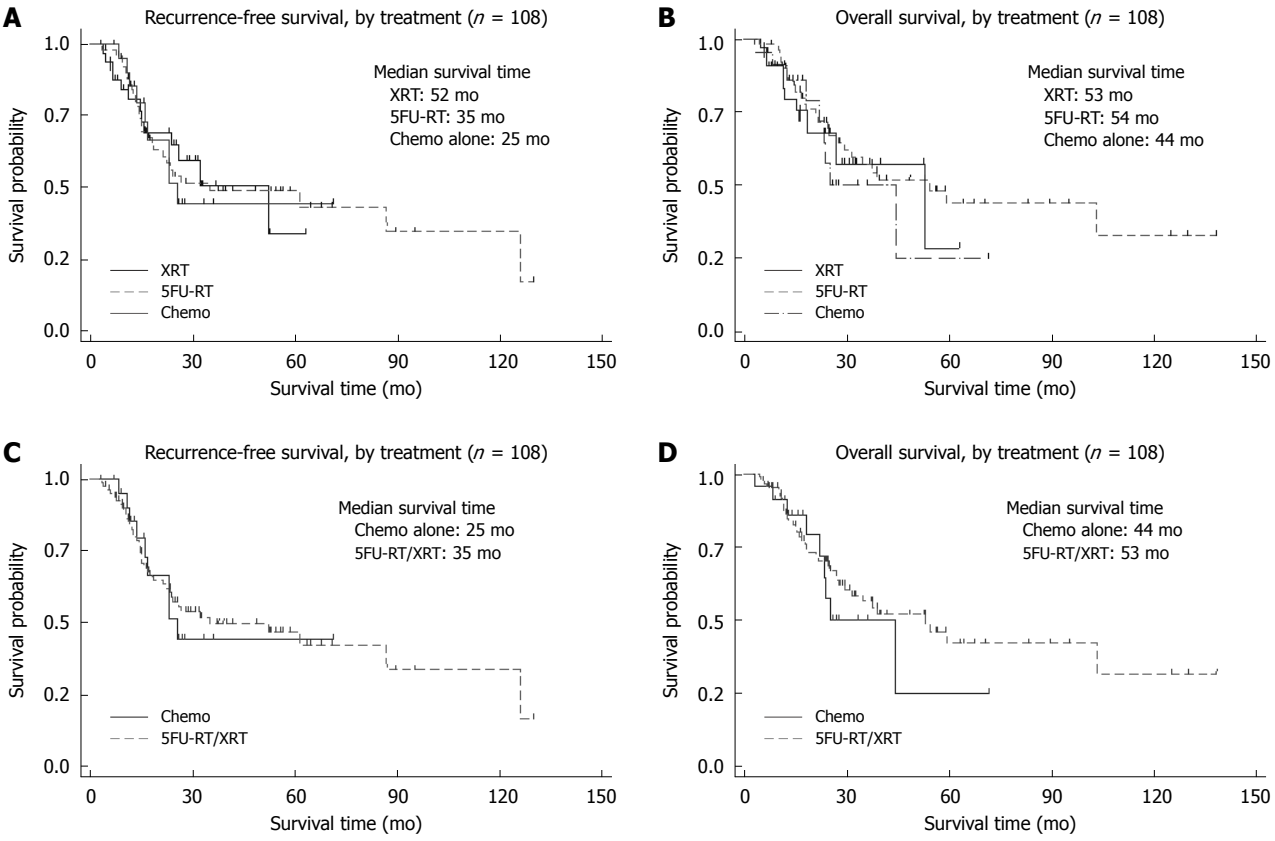

Figure 1 Kaplan-Meier estimates. A: Recurrence-free survival of capecitabine + radiation (XRT), 5-fluorouracil + radiation (5FU-RT) and chemotherapy alone; B: Overall Survival of XRT, 5FU-RT and chemotherapy alone; C: Recurrence-free survival of RT-containing regimen *vs* chemotherapy alone; D: Overall survival of RT-containing regimen *vs* chemotherapy alone.

Table 2 Recurrence-free survival and overall survival analysis

Variable	Categories/units	*n*	HR (95% CI)	*P*-value
Recurrence-free survival				
Treatment	XRT	108	1	
	5FU-RT		1.00 (0.508-1.959)	1.0
	Chemo only		1.05 (0.448-2.462)	0.9
Treatment	XRT	108	1	
	5FU-RT		0.97[1] (0.477-1.966)	0.9
	Chemo only		1.03[1] (0.433-2.444)	0.9
Overall survival				
Treatment	XRT	108	1	
	5FU-RT		0.83 (0.411-1.681)	0.6
	Chemo only		1.13 (0.474-2.677)	0.8
Treatment	XRT	108	1	
	5FU-RT		0.89[1] (0.428-1.856)	0.8
	Chemo only		1.19[1] (0.494-2.882)	0.7

[1]Age-adjusted hazard ratio (HR). XRT: Capecitabine + radiation; 5FU-RT: 5-fluorouracil + radiation.

patient in the XRT group had acute myocardial infarction during the therapy period. There was one death from non-neutropenic sepsis during the adjuvant treatment in the 5FU-RT group.

DISCUSSION

Our study showed that XRT was as effective as 5FU-RT and fluoropyrimidine-based chemotherapy when given as adjuvant treatment for locally advanced gastric cancer. XRT had comparable recurrence-free and overall survival with the other 2 adjuvant regimens.

The Intergroup-0116 study has established the role of adjuvant chemoradiation for resected locally advanced gastric cancer in the United States. Compared to surgery alone, the addition of chemoradiation after resection leads to an increased local control (30 mo *vs* 19 mo, $P = 0.001$) and better median overall survival (36 mo *vs* 27 mo, $P = 0.005$)[7]. In our study, the median overall survival for patients receiving XRT or 5FU-RT was 53 and 54 mo, respectively. This outcome when compared to the Intergroup trial is encouraging as 66% of patients in our study population had T3 or T4 disease and 86% had lymph node-positive disease, similar to the study population in the Intergroup study (68% and 85%, respectively). This difference could be explained by the differences in surgical techniques. All except 2 of our patients underwent D2 gastrectomies compared to only 10% in the Intergroup study. This may suggest that the adjuvant treatment could be a measure to compensate for inadequate surgical treatment. Nevertheless, the extent of lymph node dissection remains an ongoing debate with trials from the Dutch Gastric Cancer Group[8] and Medical Research Council[9] showing a lack of survival benefit of D2 over D1 lymph node dissection. However, the role of adjuvant chemoradiation in D2-resected gastric cancer had been studied in a Korean prospective non-randomized trial involving 544 patients receiving postoperative 5FU-

Table 3 Recurrence-free survival and overall survival analysis

Variable	Categories/units	Chemo alone		5FU-RT		XRT	
		HR (95% CI)	*P*-value[1]	HR (95% CI)	*P*-value[1]	HR (95% CI)	*P*-value[1]
Recurrence-free survival							
ECOG	0	1	0.9	1	1.0	E/N = 0/1	0.5
	1	0.92 (0.115-7.422)		1.01 (0.238-4.326)		NE	
Gender	Male	1	0.9	1	0.2	1	0.5
	Female	0.91 (0.244-3.419)		1.58 (0.727-3.436)		0.65 (0.198-2.163)	
Surgical margins	Positive	1	0.5	1	< 0.001	1	0.03
	Negative	0.51 (0.059-4.351)		0.16 (0.070-0.384)		0.27 (0.081-0.918)	
Stage	1 or 2	1	0.1	1	0.2	1	0.06
	3 or 4	2.92 (0.724-11.773)		1.86 (0.749-4.633)		3.87 (0.853-17.586)	
Grade of tumor	1, 2	1	0.3	1	0.09	1	0.3
	3	2.17 (0.444-10.636)		2.44 (0.836-7.099)		1.94 (0.515-7.288)	
Overall survival							
ECOG	0	1	0.8	1	0.9	E/N = 0/1	0.4
	1	0.78 (0.096-6.366)		0.95 (0.220-4.069)		NE	
Gender	Male	1	1.0	1	0.2	8/22	0.7
	Female	1.02 (0.253-4.148)		1.63 (0.740-3.598)		0.78 (0.234-2.597)	
Surgical margins	Positive	1	0.004	1	< 0.001	4/4	0.02
	Negative	0.10 (0.013-0.700)		0.26 (0.113-0.603)		0.25 (0.071-0.901)	
Stage	1 or 2	1	0.2	1	0.1	2/12	0.06
	3 or 4	2.59 (0.646-10.412)		2.08 (0.778-5.557)		3.80 (0.829-17.397)	
Grade of tumor	1, 2	1	0.4	1	0.06	3/12	0.4
	3	2.04 (0.418-9.944)		3.04 (0.903-10.239)		1.83 (0.482-6.980)	

[1]*P* -values were calculated using the Log-rank test. 5FU-RT: 5-fluorouracil + radiation; XRT: Capecitabine + radiation; ECOG: Eastern Cooperative Oncology Group status; E/N: Number of events/number of patients in the group; NE: Not estimable; HR: Hazard ratio.

Table 4 Subgroup analysis for overall and recurrence-free survival

Subgroup	Variable	Categories/units	*n*	Overall survival		Recurrence-free survival	
				HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Surgical margins = positive	Treatment	Chemo alone	21	1		1	
		5FU-RT/XRT		0.47 (0.100–2.191)	0.3	1.58 (0.206-12.154)	0.7
Stage T3/4	Treatment	Chemo alone	66	1		1	
		5FU-RT/XRT		0.65 (0.294–1.454)	0.3	0.78 (0.352-1.709)	0.5
Node positive	Treatment	Chemo alone	94	1		1	
		5FU-RT/XRT		0.92 (0.404–2.081)	0.8	1.10 (0.489-2.482)	0.8

5FU-RT: 5-fluorouracil + radiation; XRT: Capecitabine + radiation; HR: Hazard ratio.

radiation. This trial demonstrated significantly longer overall survival in the chemoradiation group compared to the surgery alone group (95.3 mo *vs* 62.6 mo, $P = 0.02$)[10]. Hence, there appears to be a role of adjuvant concurrent chemoradiation even in the setting of optimal surgical resection of gastric cancer.

Continuous infusion 5-FU was preferred over 5-FU bolus infusion in most of the gastrointestinal malignancies, especially colorectal cancer, and was also found to be more effective than the bolus 5-FU[11,12]. Continuous 5-FU infusion with radiation was used extensively in the neoadjuvant and adjuvant treatment of rectal cancer[13,14]. Oral capecitabine, in the metastatic and adjuvant setting, has been shown to be as effective as continuous 5-FU in gastrointestinal malignancies[2,12,15,16]. The ease of oral administration of capecitabine compared with the continuous infusion of 5-FU, which requires the placement of a central venous catheter, makes the use of capecitabine concurrent with radiotherapy an attractive option. Our results, albeit retrospective, showed an equivalent survival between XRT and 5FU-RT. Hence, XRT could be a reasonable alternative to 5FU-RT as adjuvant treatment in resected stomach cancer. Several phase Ⅰ / Ⅱ studies have already explored the addition of capecitabine with concurrent radiation[4,5] in adjuvant stomach cancer and shown it to be safe and tolerable.

Our analysis has shown that there was no survival difference between those who had radiation as part of their adjuvant therapy compared to those who did not. Even in patients with a positive surgical margin, the addition of radiation did not appear to significantly improve survival compared to adjuvant chemotherapy alone. The role of adjuvant chemotherapy had been studied in many phase Ⅲ Western trials but the results were inconsistent in showing a survival benefit of adjuvant chemotherapy over surgery alone[17-20]. Meta-analyses of these trials suggest a potential absolute increase in 5-year survival of 2% to 4% with adjuvant chemotherapy in resected gastric

cancer[21,22]. The Asian adjuvant trials had demonstrated more favorable results with a recent Japanese study on adjuvant S1 in resected stage Ⅱ or Ⅲ gastric cancer showing a significantly higher 3-year overall survival rate in the S1 group compared to the observation arm (80.1% *vs* 70.1%, $P = 0.003$)[23]. Hence, fluoropyrimidine-based chemotherapy may have a role in adjuvant treatment. However, there is currently no phase Ⅲ trial that compared adjuvant chemoradiation and chemotherapy alone. Our study has shown that there is no survival difference between adjuvant chemoradiation and chemotherapy, suggesting that adjuvant chemotherapy alone may be a reasonable option of adjuvant therapy in resected gastric cancer. A study involving capecitabine alone for adjuvant therapy in gastric cancer will be an interesting follow-up study.

In conclusion, XRT as an adjuvant therapy in resected gastric cancer can achieve similar outcomes to that of 5FU-RT or chemotherapy. The result from our hypothesis-generating study provides the basis for a further prospective study in evaluating the role of radiation with concurrent capecitabine as adjuvant therapy in resected gastric cancers.

COMMENTS

Background

Gastric cancer is a major cause of cancer deaths in the world. The outcome of large gastric tumors and those with lymph node involvement remains poor after surgical resection. The optimal adjuvant therapy after surgical resection remains to be determined.

Research frontiers

The most common strategies in the adjuvant treatment of gastric cancers include fluoropyrimidine-based chemotherapy with or without radiation. The introduction of capecitabine has largely replaced continuous-infusion 5-fluorouracil (5-FU) owing to its ease of administration. However, its efficacy is not proven in randomized phase Ⅲ trials involving gastric cancers. In this retrospective review study, the authors examined the role of capecitabine with radiation and compared its efficacy to the 5-FU with radiation regimen and fluoropyrimidine-based chemotherapy alone.

Innovations and breakthroughs

This study showed that capecitabine with concurrent radiation was as effective as 5-FU with radiation or fluoropyrimidine-based chemotherapy alone without radiation when given as adjuvant treatment for locally advanced gastric cancer.

Applications

This hypothesis-generating study will provide the platform for a larger randomized study to be conducted using capecitabine as one of the study regimens in adjuvant gastric cancer trials.

Terminology

Capecitabine and 5-FU are fluoropyrimidine-based chemotherapy commonly used in the treatment of gastrointestinal cancers.

Peer review

This is a retrospective clinical study that looked at the effects of chemoradiation therapy by using capecitabine in patients undergoing gastric surgery for gastric cancer. The results are well presented and the discussion is well organized. The conclusions are supported by the data and the tables contain appropriate information.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3716

World J Gastroenterol 2010 August 7; 16(29): 3716-3722
ISSN 1007-9327 (print)

BRIEF ARTICLE

Stent-grafts placement for treatment of massive hemorrhage from ruptured hepatic artery after pancreaticoduodenectomy

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Supported by Chinese PLA Scientific Foundation of the Eleventh Five-Year Plan (06MA263)
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Received: February 23, 2010 Revised: April 16, 2010
Accepted: April 23, 2010
Published online: August 7, 2010

Abstract

AIM: To present a series of cases with life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm after pancreaticoduodenectomy (PD) treated with placement of stent-grafts.

METHODS: Massive hemorrhage from ruptured hepatic artery pseudoaneurysm after PD in 9 patients (6 men, 3 women) at the age of 23-75 years (mean 48 years), were treated with placement of percutaneous endovascular balloon-expandable coronary stent-grafts. All patients were not suitable for embolization because of a non-patent portal vein. One or more stent-grafts, ranging 3-6 mm in diameter and 16-55 mm in length, were placed to exclude ruptured pseudoaneurysm. Follow-up data, including clinical condition, liver function tests, and Doppler ultrasound examination, were recorded at the outpatient clinic.

RESULTS: Immediate technical success was achieved in all the 9 patients. All stent-grafts were deployed in the intended position for immediate cessation of bleeding and preservation of satisfactory hepatic arterial blood flow. No significant procedure-related complications occurred. Recurrent bleeding occurred in 2 patients at 16 and 24 h, respectively, after placement of stent-grafts and treated with surgical revision. One patient died of sepsis 12 d after the interventional procedure. The remaining 6 patients were survived when they were discharged. The mean follow-up time was 10.5 mo (range 4-16 mo). No patient had recurrent bleeding after discharge. Doppler ultrasound examination verified the patency of hepatic artery and stent-grafts during the follow-up.

CONCLUSION: Placement of stent-grafts is an effective and safe procedure for acute life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm.

Key words: Pancreaticoduodenectomy; Hemorrhage; Hepatic artery; Pseudoaneurysm; Stent-graft

Peer reviewers: Luis Grande, Professor, Department of Surgery, Hospital del Mar, Passeig Marítim 25-29, Barcelona 08003, Spain; Laura Lladó, PhD, Department of Surgery, Liver Transplant Unit, Hospital Universitari de Bellvitge, IDIBELL, 08907 Barcelona, Spain

Wang MQ, Liu FY, Duan F, Wang ZJ, Song P, Fan QS. Stent-grafts placement for treatment of massive hemorrhage from ruptured hepatic artery after pancreaticoduodenectomy. *World J Gastroenterol* 2010; 16(29): 3716-3722 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3716.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3716

INTRODUCTION

Delayed hepatic arterial hemorrhage after pancreaticodu-

odenectomy (PD) is not a common, but a potentially fatal complication[1-4]. Massive hepatic arterial bleeding occurs as a result of inflammatory vascular erosion related to pancreatic juice or bile leaking from an insufficient anastomosis and/or due to local infection. Treatment options include re-operation or endovascular catheter techniques such as coil embolization of the bleeding vessels. Surgical exploration and identification of the bleeding vessel may be difficult in acute situations and hazardous because of adhesions and surrounding postsurgical tissue friability[5,6]. Embolization of the bleeding artery has an immediate impact on patient survival but often excludes the distal circulation, which may be a risk factor for hepatic ischemia and even fatal hepatic necrosis, particularly in patients with portal vein stenosis or thrombosis[3,7-9].

Recently the use of stent-grafts to exclude aneurysms in splenic and other visceral arteries has been presented in single case reports[10-15]. We report our experience in 9 patients with delayed massive hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, demonstrating the potential of interventional radiology in initial treatment of hemorrhage, instead of re-operation and coil embolization, using transluminal stent graft placement for emergency vessel repair retaining patency of hepatic artery.

MATERIALS AND METHODS

Patients

From March 2003 to December 2009, 9 patients (6 men, 3 women) at the age of 23-75 years (mean 48 years), referred to our department for delayed massive hemorrhage from ruptured hepatic artery pseudoaneurysm occurring 6 or more days after PD, were treated with percutaneous endovascular placement of balloon-expandable stent-grafts. All medical records, radiological reports, and images of the patients were retrospectively reviewed. Delayed massive hemorrhage occurring 5 or more days after PD in patients with stable hemodynamics was defined as a potentially life-threatening bleeding leading to hemorrhagic shock needing blood transfusions as previously described[2,3].

The indications for PD are listed in Table 1. Classic resection procedures were performed in all the 9 patients, including Whipple procedure (n = 6) and pylorus preserving pancreatoduodenectomy (n = 3). Pancreatic fistula (PF) after PD, diagnosed by routine assay of drainage fluid amylase levels, was present in 7 patients with local infection. PF was defined as drain output of any measurable volume of fluid on or after postoperative day 3 with amylase content 3 times greater than that of serum amylase activity[4]. Embolization of splenic artery was performed in 2 patients (Patients No. 1 and No. 2) due to bleeding from the splenic artery.

All the 9 patients presented with an unstable clinical condition with their heart rate higher than 90 beats per min, blood pressure lower than 80/40 mmHg, hemoglobin lower than 6.5 g/dL (reference range 13-18 g/dL), hematocrit lower than 40% (reference range 40%-54%), and blood transfusion greater than 5 U. Angiography revealed active hemorrhage from the hepatic artery pseudoaneurysm, including bleeding from the abdominal drain (n = 4), gastrointestinal tract (n = 2), or both (n = 3). Bleeding occurred in 7 patients (78%) before they were discharged and in 2 patients (Patients No. 5 and No. 7) after they were discharged following an uneventful postoperative course. The mean time between PD and onset of massive bleeding was 15.3 d (range 6-38 d).

All patients received an average blood transfusion of 10.6 U (9-12 U) before the interventional procedure. Of the 9 patients, 5 needed an average of 1520 mL (1200-1800 mL) fresh frozen plasma and 4 required intubation.

Emergency endoscopy performed in 3 patients showed mixed old and fresh blood in gastric lumen but no bleeding site. Computed tomography (CT) and ultrasonography were not performed because of the emergency situation.

Informed consent was obtained from the patients or their guardians before the interventional procedure.

Indication for stent graft placement

Instead of coil embolization, stent-graft was placed to exclude hepatic pseudoaneurysm because the patients had an obstructed portal vein prior to massive pseudoaneurysm bleeding, including thrombosis of the portal vein in 5 patients, tumor infiltration of the portal vein in 3 patients, and ligation of the portal vein during the surgical procedure in 1 patient. In patients with a non-patent portal vein, embolization of the common or proper hepatic artery was a high risk factor for hepatic ischemia and even fatal hepatic necrosis as previously described[9,16].

Endovascular techniques

After restoration of hemodynamic stability by aggressive resuscitation with intravenous fluids and administration of blood products, the patients underwent emergency abdominal angiography with standard Seldinger technique. All procedures were performed under local anesthesia (2% lidocaine). The patients were monitored by electrocardiogram and blood pressure measurements.

A pigtail catheter (4 Fr, Cordis, the Netherlands) was inserted into the abdominal aorta at level of the 12th thoracic vertebra, and an abdominal aortography was performed with digital subtraction angiography technique. The celiac trunk, hepatic artery, splenic artery, and superior mesenteric artery were selectively catheterized using a 4 Fr Cobra catheter (Cordis). Both arterial and portal venous phases were assessed, in order to detect the bleeding site and exclude the portal vein thrombosis.

Diameter of the affected artery at the located bleeding site was measured. A 0.35-inch guide-wire (Terumo Co., Tokyo, Japan) was passed into a distal branch of the right or left hepatic artery through the 4 Fr Cobra catheter under fluoroscopic guidance. The femoral 4 Fr introducer was replaced with an 8 Fr introducer with a 60 cm long vascular sheath (Arrow, Arrow International, USA).

A stent-graft (Jostent, Graftmaster, Coronary stent graft, Germany) was advanced into the distal extravasation site through the 8 Fr Arrow sheath over a 0.014 inch guide-wire (Table 2). The sheath was then pulled back to

Table 1 Clinical data about the patients with bleeding hepatic artery pseudoaneurysm following pancreaticoduodenectomy treated with stent grafts placement

Patient No.	Age (yr)/sex	Indication for surgery	PF	Bleeding at POD	Initial presentation of bleeding	Transfusion of units of blood	Stent-graft
1	75/F	Carcinoma of pancreatic head	Yes	14	Abdominal drain	9, 9 FFP	Jostent 4 mm × 19 mm × 2 pieces
2	23/F	Pancreatic trauma	Yes	9	Abdominal drain	12	Jostent 4 mm × 19 mm × 2 pieces
3	42/M	Distal common bile cholangiocarcinoma	Yes	15	Abdominal drain	11, 6 FFP	Jostent 4 mm × 19 mm × 3 pieces
4	56/M	Carcinoma of pancreatic head	Yes	7	Abdominal drain and hematemesis	11, 7 FFP	Jostent 3.5 mm × 19 mm × 1 piece 4 mm × 19 mm × 1 piece
5	62/M	Carcinoma of pancreatic head	No	35	Hematemesis and melena	9	Jostent 4 mm × 19 mm × 1 piece 4 mm × 16 mm × 1 piece
6	67/M	Pancreatic carcinoma	Yes	6	Surgical drainage, nasogastric tube	11	Jostent 6 mm × 55 mm × 1 piece
7	53/M	Periampullary cancer	No	38	Hematemesis and melena	9	Jostent 4 mm × 19 mm × 3 pieces
8	68/M	Pancreatic carcinoma	Yes	8	Abdominal drain	11, 9 FFP	Jostent 3.5 mm × 19 mm × 1 piece 4 mm × 16 mm × 1 piece
9	50/F	Carcinoma of pancreatic head	Yes	6	Surgical drainage, nasogastric tube	12, 7 FFP	Jostent 4 mm × 19 mm × 2 pieces

PD: Pancreaticoduodenectomy; POD: Postoperative day; FFP: Fresh frozen plasma; PF: Pancreatic fistula.

Table 2 Outcome of the patients enrolled in this study after placement of stent grafts

Patient No.	Immediate technical success	Intensive care unit/ length of stay	Clinical outcome	Length of hospital stay (d)	Follow-up
1	Yes	Yes/19 d	Bleeding stopped, no further hemorrhage	48	4 mo exitus, AMI
2	Yes	Yes/7 d	Bleeding stopped, no further bleeding	28	16 mo, clinical and laboratory findings normal
3	Yes	Yes/10 d	Bleeding stopped, no further bleeding	38	10 mo exitus, underlying malignancy
4	Yes	Yes/15 d	Recurrent bleeding 16 h later, underwent surgical revision	32	2 d exitus, uncontrolled bleeding
5	Yes	No	Cessation of bleeding, no further hemorrhage	11	14 mo exitus, underlying malignancy
6	Yes	Yes/8 d	Bleeding stopped, no further hemorrhage	36	8 mo exitus, underlying malignancy
7	Yes	No	Bleeding stopped, no further hemorrhage	12	11 mo, clinical and laboratory findings normal
8	Yes	Yes/21 d	Recurrent bleeding 24 h later, underwent surgical revision	40	3 d exitus, multiorgan failure
9	Yes	Yes/24 d	Bleeding stopped, no further hemorrhage	43	12 d exitus, abdominal sepsis

AMI: Acute myocardial infarction.

the proximal extravasation site to allow initial exposure of the stent-graft. The stent-graft was then deployed by inflating the balloon to a pressure of 8 atmospheres. A 6/20-mm balloon catheter (Abbott Lab, IL) was then used to post-dilate the stent-graft. If placement of one stent-graft failed to completely exclude the pseudoaneurysm, a second or even a third stent-graft was placed in a coaxial overlapping manner. Control angiography was repeated to confirm the exclusion of pseudoaneurysm, no sign of contrast medium extravasation, and patency of the hepatic artery.

Post-procedural management

All individuals were given antibiotics to prevent infection with aerobic and non-aerobic bacteria but no anticoagulation or anti-platelet drugs immediately after the procedure due to the emergent hemorrhagic conditions.

Six patients were survived when they were discharged and aspirin (100 mg/d) was given lifelong after discharge. Follow-up data, including clinical condition and laboratory (liver function test) findings, were recorded at the outpatient clinic. Doppler ultrasound studies were performed every day for 1 wk after placement of stent-grafts, then every 2-3 d, followed by every month on an outpatient basis.

Outcome parameters

Technical success was defined as the successful deploy-

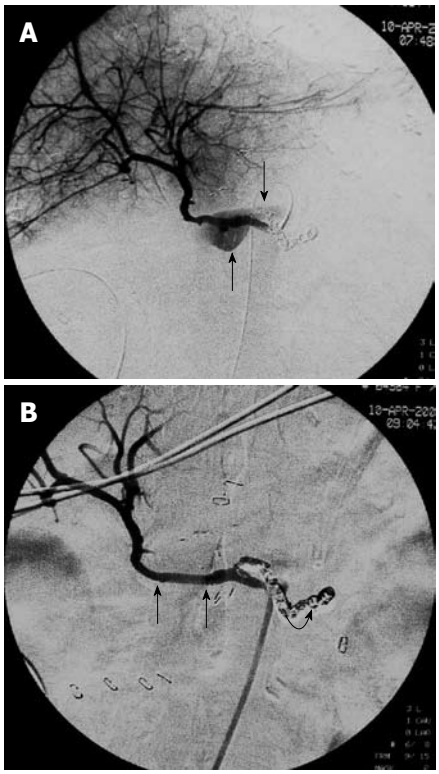

Figure 1 A 75-year-old female with pancreatic head malignancy underwent a pylorus-preserving pancreaticoduodenectomy (Case 1). A: Selective angiography of the common hepatic artery shows a large ruptured hepatic artery pseudoaneurysm with extravasation of contrast medium (arrows); B: Angiography after two stent-grafts placement demonstrates exclusion of the aneurysm and preserved hepatic artery blood flow through the stent-grafts (arrows). Note the splenic artery was embolized 4 d ago due to bleeding from the splenic artery (curved arrow).

ment of stent-graft within the intended artery, exclusion of pseudoaneurysm without evidence for contrast extravasation, cessation of immediate hemorrhage, and preservation of hepatic arterial flow. Clinical success was defined as the disappearance of signs or symptoms and improvement in laboratory findings. Re-bleeding from the same arterial focus after treatment was defined as clinical failure.

RESULTS

Immediate technical success

The selective angiography of celiac axis demonstrated active bleeding (extravasation of contrast agent) from the ruptured hepatic artery pseudoaneurysm in all patients (Figure 1A). A non-patent main portal vein was observed at the delayed phase in all patients.

Stent-graft placement was technically successful in all patients with the bleeding pseudoaneurysm completely excluded and stable hemodynamically achieved immediately (Figure 1B). The control angiography demonstrated patency of the hepatic artery in all individuals (Figure 2A and B). Each patient needed one or more stent-grafts to exclude his or her ruptured hepatic pseudoaneurysm. A total of 19 stent-grafts were implanted in 9 patients (Table 1).

The median time of interventional procedure, including diagnostic angiography, was 65 min (range 35-100 min).

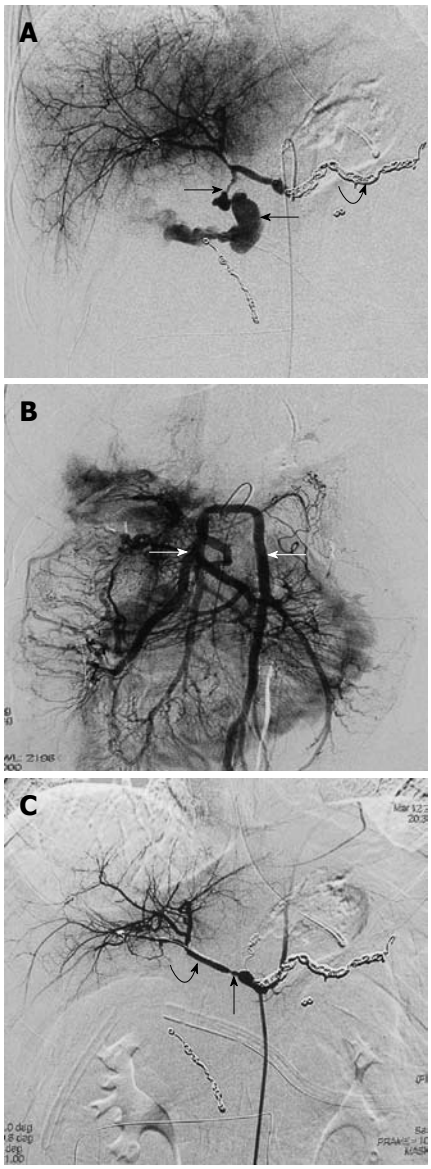

Figure 2 A 23-year-old female with pancreatic trauma underwent a pylorus-preserving pancreaticoduodenectomy (Case 2). A: Emergent selective angiography of the celiac trunk demonstrates massive extravasation of contrast medium into the abdominal cavity from the ruptured hepatic artery pseudoaneurysm (arrows). Note the splenic artery was embolized 6 d ago due to bleeding from the splenic artery (curved arrow); B: Digital substraction superior mesenteric artery angiogram with delayed phase demonstrates no visualization of the portal vein, and remarkable pooling of contrast medium at the branches of the SMV (arrows); C: Angiography after two pieces of stent-grafts placement shows exclusion of the bleeding hepatic pseudoaneurysm and good hepatic arterial blood flow through the stent-grafts (curved arrow). Note the spasm of the proximal hepatic artery (arrow).

No vascular adverse event occurred during the procedure. Control angiography showed vasospasm of the proximal and distal hepatic artery during and immediately after the procedure as expected because of the intra-arterial guide wire manipulation (Figure 2C), which did not affect the outcome of intervention. No major complication of the procedure was observed.

Clinical outcome

Seven patients were transferred to intensive care unit after

placement of stent-grafts with a mean stay time of 13.7 d (7-24 d). Four patients required intubation.

Hemodynamically stabilization was achieved in 7 of the 9 patients after the procedure without evidence of further bleeding (Table 2). Laboratory findings (liver function tests) were within the normal range 1, 3 and 7 d after the procedures and at the time when the patients were discharged.

Two patients (Patients No. 4 and No. 8) with clinical failure underwent surgical revision. In patient No. 4, the stent-graft was successfully placed, but his hemoglobin level dropped again 16 h after the procedure due to bleeding from the same artery probably due to the dislodgment of stent-grafts. This patient underwent surgical revision and died of uncontrolled bleeding 2 d after the procedure. In patient No. 8, recurrent bleeding occurred 24 h after the placement of stent-grafts due to bleeding from the same artery. He underwent surgical revision with haemostasis achieved. The patient died of multiorgan failure 3 d after the procedure.

Of the 7 patients with their bleeding completely controlled, one (Patient No. 9) died of intra-abdominal sepsis 12 d after the interventional procedure with no active bleeding until her death. Four patients were subjected to CT-guided interventional placement of additional drains close to the pancreaticojejunostomy due to considerable fluid collections. Two patients underwent re-operation for persistent abscess and PF.

Follow-up

Six patients were survived when they were discharged (Table 2). Their mean hospital stay time was 24.5 d (range 11-48 d). The mean follow-up time was 10.5 mo (range 4-16 mo). No further bleeding was seen during the follow-up. Clinical and laboratory follow-up findings were unremarkable. Doppler ultrasound examination verified the patency of hepatic artery and stent-grafts during the follow-up.

DISCUSSION

It was reported that approximately two thirds of delayed arterial hemorrhage cases after PD have an underlying collection or anastomotic leak, with pseudoaneurysm formation[1,2,4,5,9]. In our study, delayed hepatic arterial hemorrhage occurred in 7 of the 9 patients due to the local complication (PF).

Transcatheter arterial embolization (TAE) has been advocated as the first-line treatment modality for late-onset bleeding after PD[17-20], with a success rate of 83%-100% and a mortality of 0%-20%. In fact, the liver can tolerate embolization of the main hepatic artery without major consequences, since it has a dual blood supply from the portal and arterial circulations. Collateral arterial blood flow to the liver can also be expected. However, the number of collaterals is less than that of normal blood vessels after PD because of lymphadenectomy and skeletonization of the vasculature[3]. Embolization of the hepatic artery may result in liver failure and necrosis as well as intrahepatic abscesses[5,21,22].

In this study, all patients had an obstructed portal vein. Embolization of the hepatic artery itself, proximal and distal to the bleeding pseudoaneurysm, may influence the liver perfusion with an undesirable effect[9,16]. Thus, for preserving hepatic arterial blood flow, implantation of stent-grafts (covered stent) may be better than TAE[10-12].

In recent years, stent-grafts have been increasingly used in endovascular repair of thoracic and abdominal aneurysm, repair of traumatic subclavian artery and iatrogenic vascular injuries, and in exclusion of peripheral arterial aneurysms[23,24]. However, conventional peripheral vascular stent-grafts are 6-8 mm in diameter and 6 mm in length, making implantation difficult through the tortuous celiac arterial system. Moreover, stent-graft itself is less flexible and seldom used in the celiac system[25,26]. The Jostent, used in our patients for sealing perforation of coronary artery, is a covered stent, ranging 3.5-4.0 mm in diameter and 16-19 mm in length[27]. In the present study, placement of coronary stent-grafts was a useful procedure for the repair of ruptured hepatic artery pseudoaneurysm. However, angiography follow-up was not performed, but hepatic arterial flow was confirmed by Doppler ultrasound examination.

In this study, a high immediate technical success rate was archived using stent-grafts for bleeding hepatic artery pseudoaneurysm, which is consistent with the reported findings[10-15,22]. Bleeding was immediately controlled after placement of stent-grafts and all patients remained stable after the procedure. However, recurrent bleeding occurred in 2 patients, at 16 and 24 h, respectively, after the interventional procedure, possibly due to the dislodgment of stent-grafts.

Of the 7 patients with their bleeding successfully controlled, one died of intra-abdominal sepsis 12 d after the interventional procedure. Consequently, the patency of hepatic artery and stent-grafts without recurrent bleeding was achieved in 6 patients during a mean follow-up time of 10.5 mo (range 4-16 mo).

Placement of stent-grafts with preservation of organ arterial flow, if technically possible, may represent the best treatment option with the following advantages[10-15]. First, it permits immediate and effective control of hemorrhage, thus avoiding emergency surgery, and a second operation can be performed if hemodynamics is stable. Second, placement of stent-grafts is a minimally invasive technique with a low morbidity. Third, placement of stent-grafts preserves end-organ perfusion in the acute stage, thus reducing the risk of organ failure or infarction.

Placement of stent-grafts for hemorrhage from ruptured hepatic artery pseudoaneurysm has several limitations. First, although technical success has been achieved in most published case reports, stent-graft implantation in branches of the celiac trunk is not always possible[10,12,28]. Second, the procedure may lead to rupture of artery because of its eroded and fragile vascular wall, thus requiring emergency surgery. Third, placement of stent-grafts may

lead to in-stent-graft stenosis and occlusion[23,24,27]. It has been recommended that antiplatelet medication should be given after stent-graft deployment in order to prevent in-stent-graft stenosis.

In addition, placement of stent-grafts can control hemorrhage but cannot treat other potential complications[5,16]. To reduce the risk of recurrent hemorrhage, antibiotic therapy for peripancreatic infection and surgical revision of pancreatic or biliary anastomotic leakage may be necessary. Alternatively, pancreatic or biliary leakage can be treated by percutaneous drainage in some patients. In the present study, 6 patients recovered after placement of stent-grafts. Of the 6 patients, 4 underwent CT-guided placement of additional drains, and 2 underwent re-operation due to persistent abscess and PF.

This study has the following limitations: lack of a control group, randomization, and uniformity of evaluation and treatment. In fact, it is almost impossible to perform a prospective randomized study and no statistically significant conclusion could be drawn because of the limited number of patients.

In summary, placement of stent-grafts for acute life-threatening bleeding from hepatic artery pseudoaneurysm is a valuable alternative to embolization and surgical intervention. If technically possible, this technique should be considered the first-line treatment for bleeding from the common and proper hepatic artery, particularly in patients with a non-portal vein. Further data are required to evaluate its technical success rate, complications, and long-term outcome in a larger number of patients.

COMMENTS

Background

Delayed hepatic arterial hemorrhage after pancreaticoduodenectomy (PD) is not a common but a fatal complication, occurring in 7% of all patients. Its ideal management remains unclear and controversial.

Research frontiers

There are many diagnostic and therapeutic options for massive hepatic arterial hemorrhage after PD but no established guidelines are available. Traditional treatment modalities for massive bleeding include re-operation or endovascular catheter techniques such as coil embolization. Surgical exploration and identification of the bleeding vessel can be difficult in acute situations and hazardous because of adhesions and surrounding postsurgical tissue friability. Embolization of the bleeding artery has an immediate impact on patient survival but often excludes the distal circulation, which may have a risk of hepatic ischemia and even fatal hepatic necrosis, particularly in patients with portal vein stenosis or thrombosis. Placement of stent-grafts is a new procedure for control of bleeding without interruption of the distal circulation.

Innovations and breakthroughs

The authors reported the clinical outcome of 9 patients with life-threatening hemorrhage from a ruptured hepatic artery pseudoaneurysm after PD after treatment with a new interventional technique, namely placement of stent-grafts. This technique provides a good alternative option for the control of hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, especially in those who cannot undergo embolization. Although the number of patients was small, the procedure demonstrated a lower mortality than conventional surgical intervention.

Applications

Instead of coil embolization, the authors used stent-graft placement to exclude ruptured hepatic pseudoaneurysm because the patients had an obstructed portal vein prior to massive bleeding. In patients with a non-patent portal vein, embolization of the common or proper hepatic artery may have a high risk of hepatic ischemia and even fatal hepatic necrosis. Placement of stent-grafts in bleeding hepatic artery can immediately and effectively stop the hemorrhage, thus avoiding emergency surgery, and a second operation can be performed if the hemodynamics is stable. Placement of stent-grafts is a minimally invasive technique with a low morbidity. Placement of stent-grafts preserves end-organ perfusion in the acute stage, thus reducing the risk of liver ischemia or failure.

Peer review

The authors describe a small number of patients with life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, who were treated with implantation of endovascular stent-grafts. The study is quite interesting and innovative. Placement of stent-grafts is a good procedure for the control of massive bleeding from hepatic artery, especially in those who cannot undergo embolization. Although the number of patients is small, the results can be considered satisfactory and encouraging.

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S- Editor Wang JL **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3723

World J Gastroenterol 2010 August 7; 16(29): 3723-3726
ISSN 1007-9327 (print)

CASE REPORT

Right anterior segmental hepatic duct emptying directly into the cystic duct in a living donor

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Received: January 11, 2010 Revised: February 18, 2010
Accepted: February 25, 2010
Published online: August 7, 2010

Abstract

A 35-year-old mother was scheduled to be the living donor for liver transplantation to her second son, who suffered from biliary atresia complicated with biliary cirrhosis at the age of 2 years. The operative plan was to recover the left lateral segment of the mother's liver for living donor transplantation. With the use of cholangiography at the time of surgery, we found the right anterior segmental duct (RASD) emptying directly into the cystic duct, and the catheter passed into the RASD. After repairing the incision in the cystic duct, transplantation was successfully performed. Her postoperative course was uneventful. Biliary anatomical variations were frequently encountered, however, this variation has very rarely been reported. If the RASD was divided, the repair would be very difficult because the duct will not dilate sufficiently in an otherwise healthy donor. Meticulous preoperative evaluation of the living donor's biliary anatomy, especially using magnetic resonance cholangiography and careful intraoperative techniques, is important to prevent bile duct injury and avoid the risk to the healthy donor.

Key words: Bile duct injury; Cystohepatic duct; Intraoperative cholangiography; Living liver transplantation; Bile duct injury; Magnetic resonance cholangiography

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INTRODUCTION

Living donor liver transplantation (LDLT) is accepted around the world with the shortage of suitable deceased donors[1-4]. The safety of the living donor, an otherwise healthy individual, is important in the ethical considerations of performing LDLT[5]. Morbidity in living donors is not rare[3,6,7]. Preoperative imaging studies are needed not only to evaluate the donor's condition but also to exclude unsuitable candidates. The liver volume, condition of the liver parenchyma, and vascular anatomy are relatively easy to assess through imaging studies especially using multi-detector row computed tomography (MDCT). Variations in biliary anatomy are common,

and in order to avoid intraoperative biliary injury, precise preoperative imaging studies are needed. However, it is difficult to evaluate biliary anatomy because of the complicated nature of various imaging modalities. There is no consensus for preoperative imaging to evaluate the donor's biliary anatomy. Even today intraoperative cholangiography (IOC) has a very important role in the assessment of a donor's biliary anatomy.

We identified a rare biliary anatomic variant during LDLT. The donor had the right anterior segmental duct (RASD) emptying directly into the cystic duct. In this report, we present this rare but important anatomic variant and discuss preoperative biliary imaging and the procedure for IOC.

CASE REPORT

A 35-year-old mother was scheduled to be the living donor for liver transplantation to her second son, who suffered from biliary atresia complicated with biliary cirrhosis at the age of 2 years. There were no remarkable findings in her family or past medical history. On physical examination, she had a healed operative scar in the lower abdomen as a result of two caesarean section procedures. A preoperative medical evaluation was performed and her liver volume, condition of the liver parenchyma, and hepatic vascular anatomy made her a suitable living donor. Congenital absence of the right kidney and slight prolongation of the activated partial thromboplastin time were found, which were not considered as contraindications for donor surgery. At that time, preoperative imaging of the biliary system was not performed routinely in our institution. LDLT was then performed, using an upper midline incision to recover the left lateral segment. After intraoperative ultrasound examination, cholecystectomy was performed. Before the gallbladder was removed, IOC was attempted through the incision in the cystic duct based on the conventional practice. However, the catheter would not pass easily into the cystic duct. Instead, it passed through a small hole in the side wall of the cystic duct. Using cholangiography, we found that the RASD emptied directly into the cystic duct with the catheter inserted into the RASD (Figure 1A), and the right posterior segmental duct joined the left hepatic duct. The incision in the cystic duct was repaired carefully to prevent stenosis. After removing the left lateral segment, another intraoperative cholangiogram was performed through the stump of the left hepatic duct and no stenosis at the confluence of the RASD and cystic duct was seen (Figure 1B). Her postoperative course was uneventful.

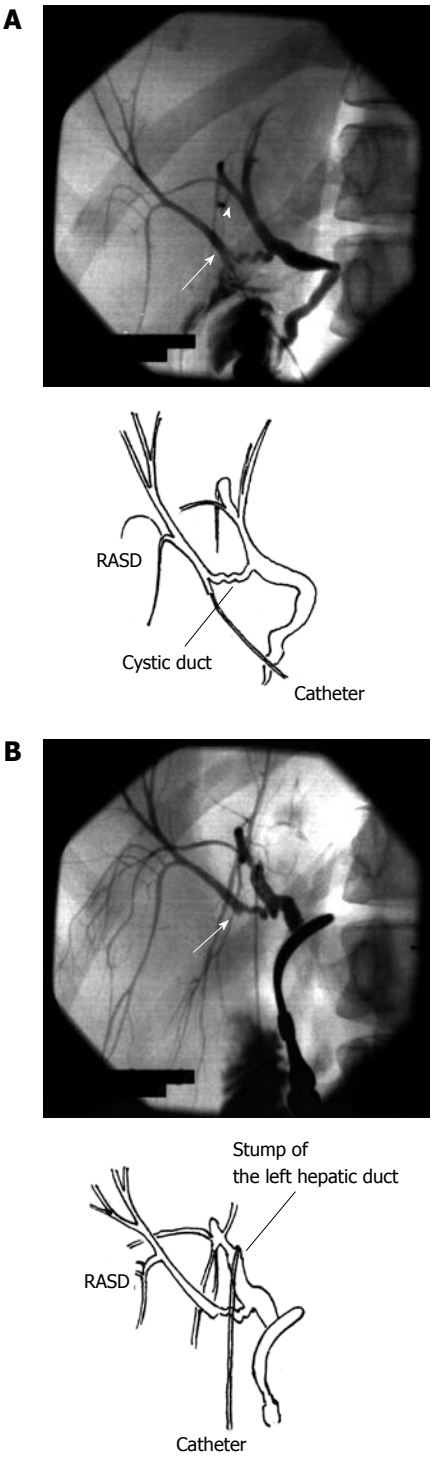

Figure 1 Intraoperative cholangiography. A: The right anterior segmental duct (RASD) emptying into the cystic duct is seen (arrow). The catheter is in the RASD. The right posterior segmental duct joining the left hepatic duct is also shown (arrowhead); B: After the left lateral segment was recovered, no stenosis was seen at the site of the repaired cystic duct (arrow). Cholangiography was performed through the stump of the left hepatic duct.

DISCUSSION

Biliary anatomical variants are frequently encountered[8], however, the variation reported in this case is very rare[9-12]. Champetier *et al*[13] stated that "The cystohepatic ducts drain the entirety of a hepatic territory of variable extent into the cystic duct or gallbladder", and they also emphasized the danger of these variants when performing a cholecystectomy.

The possibility of very rare biliary variations and the risks for the donor must be discussed in detail when ob-

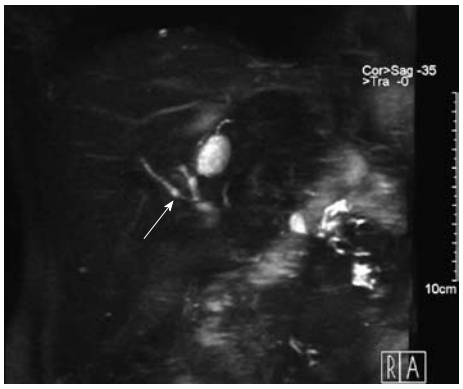

Figure 2 Magnetic resonance cholangiography was performed 3 mo postoperatively. The right anterior segmental duct and cystic duct are clearly seen (arrow).

taining informed consent. The living donor will be at risk, but continued efforts must be made to reduce the donor's morbidity as much as possible. In this case, we encountered a rare variant of biliary duct anatomy, but avoided injury to the RASD.

One of the ethical obligations of LDLT is to carefully protect the safety of the donor[5]. If the RASD was divided, the repair would be very difficult because the duct may not dilate sufficiently in an otherwise healthy donor. The donor might then suffer severe morbidities. To reduce the donor's risk, meticulous preoperative evaluation of the living donor's biliary anatomy is important. The transplant surgeons must be aware of biliary anatomic variants to prevent any intraoperative bile duct injury and minimize the risk to the donor. However, there is no single examination to demonstrate all biliary conditions expeditiously. Endoscopic retrograde cholangiography has some risks, such as the development of biliary injury, pancreatitis, and other conditions. MDCT is a breakthrough not only for demonstrating vascular anatomy but also to clearly depict the anatomy of the biliary system using biliary contrast material[14,15]. This modality also carries the risk of an allergic reaction. The possibility of a severe allergic reaction using vascular contrast material is only 0.04%[16]. On the other hand, biliary contrast materials have more risks of severe allergic reactions[17] and occur in up to 0.2% of patients according to the information from Japanese manufacturers. Dual enhanced MDCT is thought to be a very useful and accurate modality[14,15], but contains the relatively higher risk of an allergic reaction. Magnetic resonance cholangiography (MRC) is used in the preoperative examination of donors in some institutions[18,19]. However, MRC has relatively lower spatial resolution and a thin cystohepatic duct might not be accurately depicted. Postoperatively, the donor reported in this study was examined by MRC and the RASD was observed (Figure 2). The performance of this procedure was influenced by the intraoperative findings in this patient, but also led us to use MRC routinely in the preoperative evaluation of the donor's biliary anatomy to maximize the safety of the donors.

Careful intraoperative technique is also needed to improve the donor patient's safety, by assuring appropriate management of aberrant biliary branches. IOC is an essential modality to complete the donor transplantation procedure. In many cases, IOC is performed through an incision in the cystic duct; however, it may be hazardous to incise the cystic duct in a case as reported here. Therefore, we recommend using a modified IOC, incising the area of Hartmann's pouch. We believe that this method can reduce the possibility of injuring a very small but important biliary duct that might be not seen on preoperative imaging studies. It has been reported that IOC has some risks of allergic reactions[20], we believe that IOC itself is safe because little contrast material enters the systemic circulation. Using the knowledge gained from preoperative imaging and careful IOC, the safety of the donor is maximized.

In conclusion, to prevent injury of the bile ducts during the donor surgery of LDLT, preoperative delineation of the biliary anatomy is essential. Considering the donor's likelihood of suffering an allergic reaction, and the spatial resolution of various imaging modalities, we recommend MRC as the ideal preoperative biliary imaging study for the donors. IOC also plays an important role in the management of these donors. To perform IOC more carefully, we recommend that Hartmann's pouch should be incised first. By doing this we can help avoid transection of fine biliary branches that are not revealed during preoperative imaging, thus minimizing the likelihood of this avoidable complication.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i29.3727

World J Gastroenterol 2010 August 7; 16(29): 3727-3730
ISSN 1007-9327 (print)

CASE REPORT

Ultrasonography of sclerosing angiomatoid nodular transformation in the spleen

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Received: March 27, 2010 Revised: May 8, 2010
Accepted: May 15, 2010
Published online: August 7, 2010

Abstract

We report three rare cases of sclerosing angiomatoid nodular transformation (SANT) in the spleen. We compared the conventional and contrast-enhanced ultrasonographic appearance. The conventional sonographic examinations exhibited solitary lesions without common respects, while contrast-enhanced ultrasonography (CEUS) revealed nodular appearance mimicking its pathologic characteristics. It suggests that CEUS can provide morphologic information for diagnosing SANT.

Key words: Sclerosing angiomatoid nodular transformation; Contrast-enhanced ultrasonography; Ultrasound

Peer reviewer: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Cao JY, Zhang H, Wang WP. Ultrasonography of sclerosing angiomatoid nodular transformation in the spleen. *World J Gastroenterol* 2010; 16(29): 3727-3730 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3727.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3727

INTRODUCTION

Sclerosing angiomatoid nodular transformation (SANT) is a rare benign lesion. Martel named it for its pathological characteristics[1]. Its particular morphologic appearance, immunophenotype, and benign clinical course indicate that it is a distinctive non-neoplastic vascular lesion in the spleen. We report the ultrasonographic images in three cases of SANT confirmed by splenectomy. Two of them also underwent contrast-enhanced ultrasonography (CEUS). We used a second-generation contrast agent, SonoVue (Bracco, Italy) at a dose of 1.2 mL[2].

CASE REPORT

Case 1

A 36-year-old man was found to have a splenic mass by a routine medical examination without any symptoms. Physical examination found nothing remarkable. No cervical or inguinal lymphadenopathy was found. The laboratory test results were as follows: hemoglobin 130 g/L, Ery 4.1 T/L, WBC 14.5 g/L, neutrophils 12.0 g/L and platelets 174 g/L, and all other data were within normal limits. Abdominal ultrasonography (US) was done using a 3.5C ultrasound transducer attached to a LOGIQ 5 Expert; it demonstrated a 4.2 cm × 3.7 cm well-demarcated, hypoechoic lesion in the spleen (Figure 1). The patient also underwent an MRI examination. On T1- and T2-weighted images, heterogeneous hypo-signal intensity with peripheral and septa enhancement, especially at the delayed phase, was displayed. The patient underwent splenectomy. In the surgery, the mass was found to have an integrated envelope and a heterogeneous cut surface. A few days after the surgery, his WBC and neutrophils became normal.

Case 2

A 37-year-old woman was referred to abdominal ultrasound examination because of pain in the left upper quadrant. Lesions were found both in her liver and spleen. Pro-

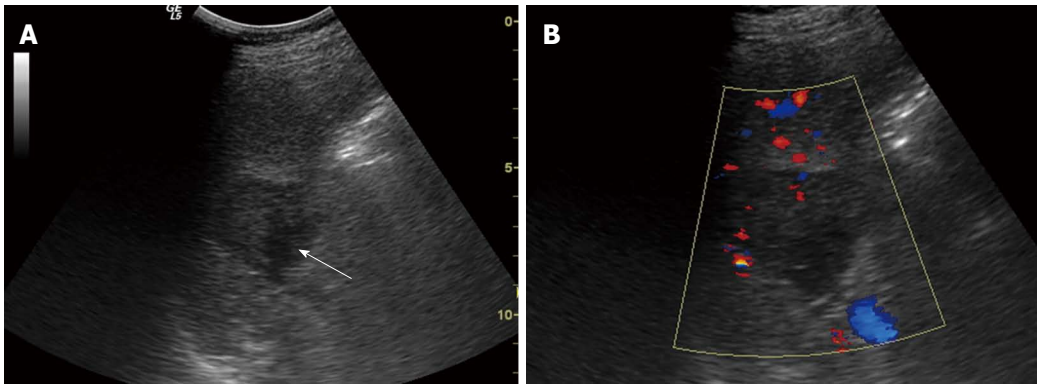

Figure 1 Conventional sonographic findings of sclerosing angiomatoid nodular transformation in a 36-year-old man. A: B mode ultrasonography demonstrates a hypoechoic lesion (arrow) in the spleen; B: Color Doppler flow imaging shows a low color-flow signal in the lesion.

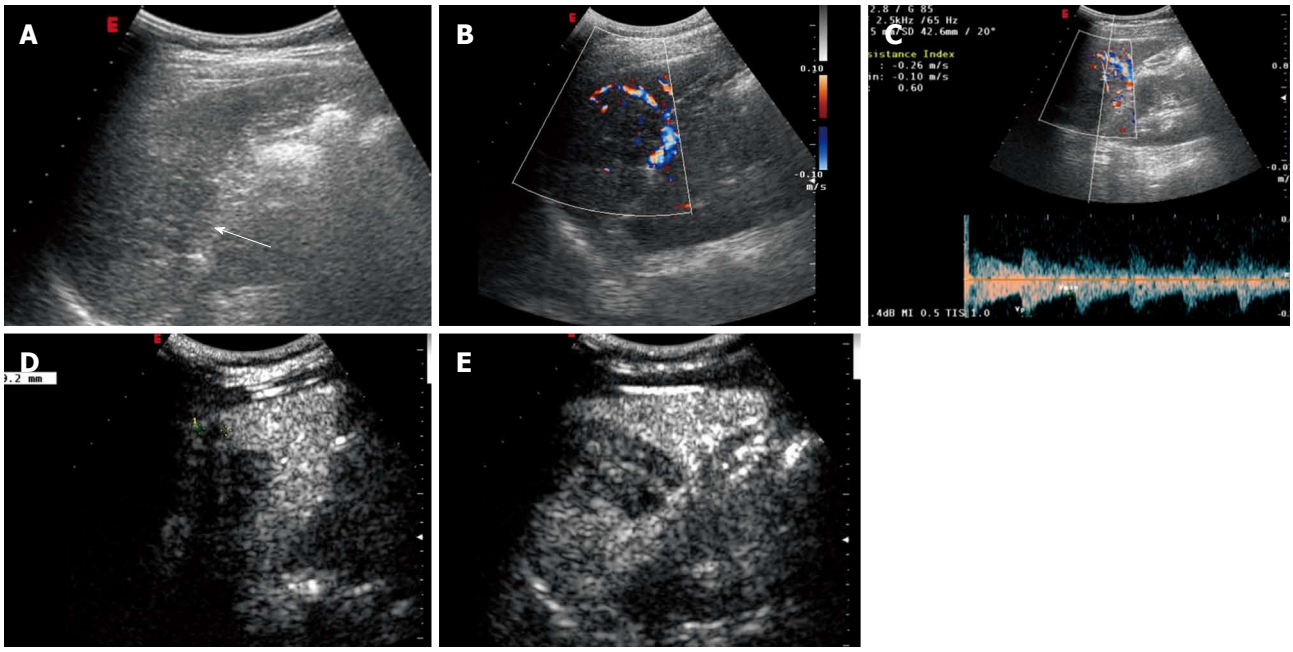

Figure 2 Conventional ultrasonography and contrast-enhanced ultrasonography findings of sclerosing angiomatoid nodular transformation in a 37-year-old woman with pain in the left upper quadrant. A-C: A heterogeneous hypoechoic lesion (arrow) in the spleen, with blood-flow signals in the peripheral area; D: There was a 0.9 cm homologous nodule attaching to the side of the lesion that became evident after being enhanced (2 min 48 s after injection); E: The lesion was enhanced homogeneously.

liferative disease of metastasis was taken into consideration during the differentiation process. Physical examination found nothing remarkable. No cervical or inguinal lymphadenopathy was observed. The laboratory tests yielded the following results: hemoglobin 120 g/L, Ery 3.88 T/L, WBC 4.6 g/L, neutrophils 3.2 g/L, platelets 214 g/L, CEA 1.16 ng/mL, AFP 2.4 ng/mL and CA19-9 6.79 U/mL. Other data were all within normal limits. Abdominal ultrasonography was done using a CA430E5-2 ultrasound transducer attached to a Technos ESAOTE DU 8; it demonstrated a 5.2 cm × 3.6 cm heterogeneous hypoechoic lesion in the spleen with an unclear margin. Color Doppler flow imaging (CDFI) showed blood-flow signals in the peripheral area of the lesion and resistance index (RI) was 0.66 (Figure 2A-C). The lesion was enhanced in the diffuse pattern from 11 s after injection, and appeared to be homogeneously hyperechoic in comparison with the splenic parenchyma in 21 s. It turned out to be isoechoic in 4 s and hypoechoic in 30 s after the injection (Figure 2E). The lesion showed a persistent enhancement up to about 7 min. Another 0.9 cm × 0.9 cm homologous nodule was found attaching to the lesion which became evident after being enhanced (Figure 2D). In order to reach a final diagnosis, the patient underwent surgical excision of the spleen and part of the liver. In the surgery, a splenic mass was firm with a clear margin. The pathological result is a SANT of the spleen and hemangiomas in the liver.

Case 3

A 39-year-old man was admitted to the hospital because of a left upper quadrant mass of unknown cause. Splenomegaly was suspected by physical examination. No cervi-

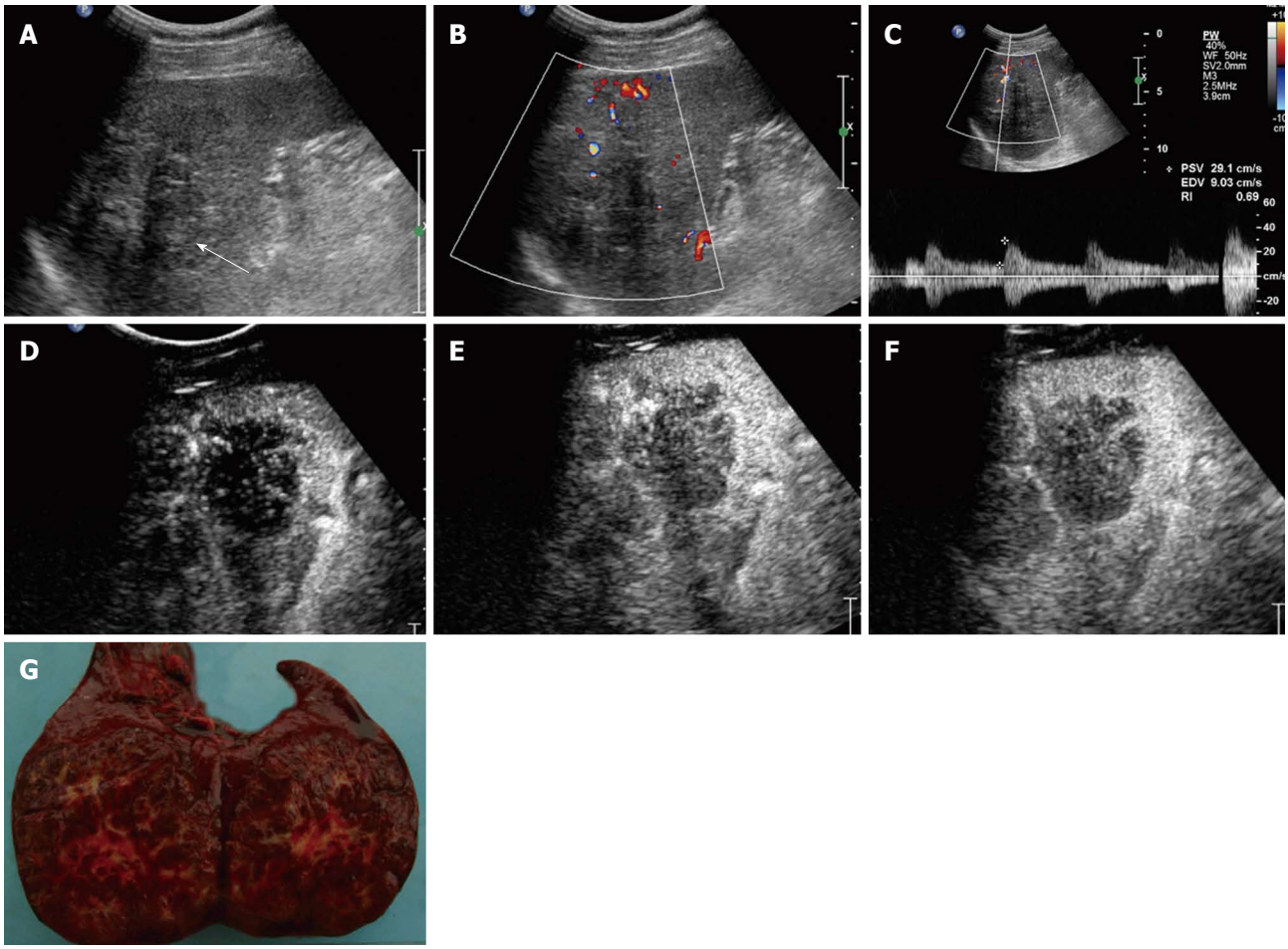

Figure 3 Conventional ultrasonography and contrast-enhanced ultrasonography findings of sclerosing angiomatoid nodular transformation in a 39-year-old man. A: A hypoechoic lesion (arrow) in the spleen; B and C: Color Doppler flow imaging shows branch-shaped and wire-like blood-flow signals inside and spot-shaped blood-flow signals in the periphery of the lesion; D: The lesion was enhanced in the branch-shaped diffuse pattern (15 s after injection); E: It presented heterogeneous lobular appearance when reaching its peak enhancement; F: Lots of septa inside the lesion; G: Surface of the lesion found during the operation.

cal or inguinal lymphadenopathy was found. The laboratory test results were: hemoglobin 123 g/L, Ery 4.23 T/L, WBC 6.7 g/L, neutrophils 4.6 g/L, platelets 167 g/L, CEA 0.56 ng/mL, AFP 2.4 ng/mL and CA19-9 3.6 U/mL. All other laboratory tests were within normal limits. Abdominal ultrasonography was done using a C5-2 ultrasound transducer attached to a Philips IU22; it demonstrated a 7.0 cm × 6.2 cm well-demarcated, hypoechoic lesion in the spleen. CDFI showed branch-shaped and wire-like blood-flow signals inside and spot-shaped blood-flow signals in the periphery of the lesion. RI was 0.50-0.66 (Figure 3A-C). The lesion was enhanced in the branch-shaped diffuse pattern from 12 s after injection (Figure 3D). It presented heterogeneous lobular appearance when reaching its peak enhancement in 23 s (Figure 3E). We also detected lots of septa inside the lesion (Figure 3F). It became hypoechoic in 37 s in comparison with the splenic parenchyma. The lesion showed a persistent enhancement up to about 3 min. Its enhancement intensity was less than that of the spleen during the whole enhancement process. Its structure and margin were clearer than those of conventional ultrasonographic images. The patient also received CT examination, which revealed a low-density mass with mildly septa enhancement. The final diagnosis of SANT was confirmed by operation (Figure 3G).

DISCUSSION

SANT is a rare mild vascular lesion in the spleen. The age of our patients ranged from 36 to 39 years. Two of them were asymptomatic (Case 1 and Case 3). The lesions were usually found incidentally. Few patients presented with abdominal discomfort or splenomegaly. Its etiology is still unclear. Concurrent diseases included chronic lymphocytic leukemia, lung squamous cell carcinoma, colonic carcinoma and some other diseases[1]. We cannot confirm whether hepatic hemangioma as shown in Case 2 is related to SANT. The lesion presented as a solitary mass measuring 3-17 cm in greatest diameter as reported before[1]. And usually it has a clear margin with a normal splenic parenchyma. The gross section shows splenic angiomatoid nodules as altered red pulp tissue is entrapped by a non-neoplastic stromal proliferative process[3]. SANT is composed of small blood vessels of three typically distinct immunophenotypes: cord capillaries (CD34+/CD8-/CD31+), sinusoids (CD34-/CD8+/

CD31+), and small veins (CD34-/CD8-/CD31+). It can be differentiated from other vascular tumors or tumor-like lesions, including hemangioma, littoral cell angioma (LCA), inflammatory pseudotumor (IPT) and follicular dendritic cell tumor (FDC), by immunohistochemical examinations.

Sonographic findings of our cases are described as follows: All the lesions were solitary and heterogeneous. Their greatest diameters were 3-7 cm. Two of them were hypoechoic and the other lesion was hyperechoic. Linear and slit-like hyperechoic septa was presented in one lesion (Case 3). The ratio of having a clear and unclear margin is 2:1. The color-Doppler showed arterial flow signals in all the lesions and their RIs were low. The contrast-enhanced images in the two lesions had something in common. The contrast media entered the lesions 1-2 s later than the splenic parenchyma. And they came out of the lesions earlier than that of the spleen. The lesions were both enhanced in diffuse pattern. One lesion (Case 3) presented a heterogeneous lobule with lots of septa inside at the peak enhancement. An extra 0.9 cm × 0.9 cm nodule attaching to the previously-found lesion was detected in Case 2 during the enhancement process.

Usually, we consider hyperechoic lesions in the spleen as benign ones on US images such as hemangioma. Malignant lesions are mostly hypoechoic and occur most frequently in lymphomas (80% of focal lesions)[4]. On conventional US images, the three lesions presented with different echogenicity. They were lack of consistency in their boundaries and shapes. An assessment of conventional US images does not provide any differential diagnostic clues. Additionally, color-Doppler technique is only capable of detecting some obvious color flow signals in big solid masses in the spleen. The images of conventional US examination are not characteristic.

We found few cases reporting the behavior of SANT with ultrasonography in the literature. Gutzeit reported a case of SANT[5], which had a hypoechoic halo, predominantly vascular on conventional US images and a "spoke wheel" pattern on CEUS images. In our cases, the heterogeneous lobule and lots of septa inside at the peak enhancement in Case 3 are similar to the "spoke wheel" pattern, but no vascular halo was present. The angiomatoid nodules are frequently delimited by concentric bands of collagen fibers, and there is always an inflamed fibrocellular to sclerotic stroma between the nodules at low magnification. Some exhibited fibrin deposition in the peripheral zone of the nodules. CEUS has shown to be useful in observing nodules whose contrast to the spleen parenchyma was enhanced after injection of SonoVue. Because of its multinodular angiomatoid appearance, SANT was described by Rosai[6] as the term "multinodular hemangioma". In conclusion, CEUS is a new imaging technique that provides direct visualization of vessel structure and morphologic characteristics of the lesions.

Pathologically, SANT has been thought to be a variant of IPT. SANT has fewer inflammatory cells and myofibroblasts than those in IPT. Martel *et al*[1] reported one case of fever and three cases of high erythrocyte sedimentation among 25 cases of SANT. One patient (Case 1) also had high WBC and neutro phils before the operation. Consequently, the US appearances of the lesions reported above are between those of IPT and hemangioma. It can explain the diversity of its echogenicity, morphology and boundary.

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World J Gastroenterol 2010 August 7; 16(29): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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ISSN 1007-9327 (print)

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March 09-12
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March 12-14
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Dubrovnik, Croatia
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May 06-08
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World J Gastroenterol 2010 August 7; 16(29): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

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Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

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World Journal of Gastroenterology®

Volume 16 Number 30
August 14, 2010

Published by Baishideng
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

ISSN 1007-9327
30

World Journal of Gastroenterology www.wjgnet.com Volume 16 Number 30 Aug 14 2010

ISSN 1007-9327
CN 14-1219/R

WJG

World Journal of Gastroenterology®

Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 30
August 14, 2010
World J Gastroenterol
2010 August 14; 16(30): 3731-3858
Online Submissions
www.wjgnet.com/1007-9327office
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 30 August 14, 2010

EDITORIAL

3731 AMPK-associated signaling to bridge the gap between fuel metabolism and hepatocyte viability
Yang YM, Han CY, Kim YJ, Kim SG

3743 Gastroesophageal reflux disease: From heartburn to cancer
Patti MG, Waxman I

TOPIC HIGHLIGHT

3745 Gastroesophageal reflux disease: From pathophysiology to treatment
Herbella FA, Patti MG

3750 Advances in diagnostic testing for gastroesophageal reflux disease
Gawron AJ, Hirano I

3757 Gastroesophageal reflux disease and severe obesity: Fundoplication or bariatric surgery?
Prachand VN, Alverdy JC

3762 Pathophysiology and treatment of Barrett's esophagus
Oh DS, DeMeester SR

3773 Effect of medical and surgical treatment of Barrett's metaplasia
Wassenaar EB, Oelschlager BK

3780 Endoscopic treatment of Barrett's esophagus: From metaplasia to intramucosal carcinoma
Chennat J, Waxman I

3786 Esophageal resection for high-grade dysplasia and intramucosal carcinoma: When and how?
Konda VJA, Ferguson MK

3793 Neoadjuvant treatment of esophageal cancer
Campbell NP, Villaflor VM

Contents

World Journal of Gastroenterology
Volume 16 Number 30 August 14, 2010

3804 Transhiatal *versus* transthoracic esophagectomy for esophageal cancer
Barreto JC, Posner MC

3811 Minimally invasive esophagectomy
Herbella FA, Patti MG

ORIGINAL ARTICLE

3816 Portal vein ligation accelerates tumor growth in ligated, but not contralateral lobes
Sakai N, Clarke CN, Schuster R, Blanchard J, Tevar AD, Edwards MJ, Lentsch AB

3827 Decreased IgA+ plasma cells and IgA expression in acute liver necrosis mice
Fu JL, Wang ZH, Li GZ, Wang YR, Liu P

BRIEF ARTICLE

3834 Early ileocolonoscopy with biopsy for the evaluation of persistent post-transplantation diarrhea
Bamias G, Boletis J, Argyropoulos T, Skalioti C, Siakavellas SI, Delladetsima I, Zouboulis-Vafiadis I, Daikos GL, Ladas SD

3841 *Helicobacter* infection concomitant with metabolic syndrome further increase risk of colorectal adenomas
Lin YL, Chiang JK, Lin SM, Tseng CE

3847 Germline mutation analysis of *hPMS2* gene in Chinese families with hereditary nonpolyposis colorectal cancer
Sheng X, Zhou HH, Zhou XY, Du X, Zhang TM, Cai SJ, Sheng WQ, Shi DR

CASE REPORT

3853 Poorly differentiated endocrine carcinoma of the pancreas responded to gemcitabine: Case report
Nakazuru S, Yoshio T, Suemura S, Itoh M, Araki M, Yoshioka C, Ohta M, Sueyoshi Y, Ohta T, Hasegawa H, Morita K, Toyama T, Kuzushita N, Kodama Y, Mano M, Mita E

LETTERS TO THE EDITOR

3857 Reply to "Application of contrast-enhanced intraoperative ultrasonography in the decision-making about hepatocellular carcinoma operation"
Donadon M, Torzilli G

World Journal of Gastroenterology
Volume 16 Number 30 August 14, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
August 14, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3731

World J Gastroenterol 2010 August 14; 16(30): 3731-3742
ISSN 1007-9327 (print)

EDITORIAL

AMPK-associated signaling to bridge the gap between fuel metabolism and hepatocyte viability

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Supported by The National Research Foundation of Korea Grant, Funded by the Korea Government (MEST), No. 2010-0001706, South Korea
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Received: February 16, 2010 Revised: March 25, 2010
Accepted: April 2, 2010
Published online: August 14, 2010

Abstract

The adenosine monophosphate-activated protein kinase (AMPK) and p70 ribosomal S6 kinase-1 pathway may serve as a key signaling flow that regulates energy metabolism; thus, this pathway becomes an attractive target for the treatment of liver diseases that result from metabolic derangements. In addition, AMPK emerges as a kinase that controls the redox-state and mitochondrial function, whose activity may be modulated by antioxidants. A close link exists between fuel metabolism and mitochondrial biogenesis. The relationship between fuel metabolism and cell survival strongly implies the existence of a shared signaling network, by which hepatocytes respond to challenges of external stimuli. The AMPK pathway may belong to this network. A series of drugs and therapeutic candidates enable hepatocytes to protect mitochondria from radical stress and increase cell viability, which may be associated with the activation of AMPK, liver kinase B1, and other molecules or components. Consequently, the components downstream of AMPK may contribute to stabilizing mitochondrial membrane potential for hepatocyte survival. In this review, we discuss the role of the AMPK pathway in hepatic energy metabolism and hepatocyte viability. This information may help identify ways to prevent and/or treat hepatic diseases caused by the metabolic syndrome. Moreover, clinical drugs and experimental therapeutic candidates that directly or indirectly modulate the AMPK pathway in distinct manners are discussed here with particular emphasis on their effects on fuel metabolism and mitochondrial function.

Key words: Adenosine monophosphate-activated protein kinase; Cell survival; Energy metabolism; Fatty liver; Insulin resistance; Glycogen synthase kinase 3β; p70 ribosomal S6 kinase-1

Peer reviewers: Hui-Kang Liu, PhD, Assistant Research Fellow, National Research Institute of Chinese Medicine, 155-1, Li-nung Street Section 2, Taipei 112, Taiwan, China; Vance Matthews, PhD, BS, Cellular and Molecular Metabolism Laboratory, Baker University of Texas Medical Branch, IDI, PO Box 6492, St Kilda Road Central, VIC 8008, Melbourne, Australia

Yang YM, Han CY, Kim YJ, Kim SG. AMPK-associated signaling to bridge the gap between fuel metabolism and hepatocyte viability. *World J Gastroenterol* 2010; 16(30): 3731-3742 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3731.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3731

INTRODUCTION

Metabolic regulation of carbohydrate, lipid and protein, and synthesis of proteins and lipids are the principal functions of the liver, as well as xenobiotic detoxification. The

function and survival of organisms are dependent on the dynamic control of energy metabolism. The regulation of fuel metabolic processes can be mediated by hormones and other endogenous ligands in response to changes in energy status. Diverse signaling pathways contribute to the regulation of energy metabolism, which is associated with the activation of cell surface and nuclear receptors in hepatocytes. Thus, the modulation of specific pathways can provide therapeutic strategies for hepatic diseases that result from metabolic derangements[1].

In a variety of hepatic diseases, abnormal fat accumulation in the liver is often a prerequisite metabolic event for further pathogenesis[2]. Lipotoxicity can lead to the generation of oxidative stress and inflammation, ultimately causing apoptosis[3]. Programmed cell death is elicited by cell surface death receptors, the caspase cascade, deranged mitochondrial metabolism, and energy deficiency. Mitochondria, cytoplasmic organelles in eukaryotic cells, play a key role in energy utilization such as oxidative phosphorylation; dysfunction of mitochondria is closely related with apoptosis[4].

The relationship between fuel metabolism and cell survival strongly implies the existence of a shared signaling network, which is responsible for the regulation of both phenomena. Emerging evidence indicates that the adenosine monophosphate (AMP)-activated protein kinase (AMPK) and p70 ribosomal S6 kinase-1 (S6K1) pathway serves as a key pathway that regulates fuel energy metabolism. In addition, it has been suggested that AMPK controls the redox-state and mitochondrial function. In this review, we focus on the role of the AMPK pathway in hepatic fuel metabolism in conjunction with cell survival. Moreover, clinical drugs and experimental therapeutic candidates that activate the AMPK-S6K1 pathway in distinct manners are discussed here with particular reference to their roles in mitochondrial function and energy metabolism.

FUEL METABOLISM AND SIGNALING PATHWAYS IN THE LIVER

The liver plays a central role in fuel metabolism, and thus regulates dynamic catabolic and anabolic processes to maintain energy homeostasis of organisms. Breakdown products of carbohydrate and lipid (i.e. glucose and fatty acids) are common energy sources which are converted to adenosine-triphosphate (ATP) in mitochondria. In addition, mitochondria have many other metabolic functions, such as regulation of membrane potential, cellular metabolism, calcium signaling (including calcium-induced apoptosis), and apoptosis. During the process of catabolism, the mitochondrion serves as the main source of energy for the cell because it converts nutrients into energy *via* cellular respiration[5]. Most of the oxygen delivered to cells or organs is consumed by mitochondria for ATP generation. When the energy is excessive in the cell, mitochondrial energy production is inhibited so that glucose and free fatty acids can be stored as glycogen and fat through anabolic processes.

AMPK signaling pathways for fuel metabolism

AMPK: AMPK is a heterotrimer complex that consists of a catalytic subunit (α1/2) and two regulatory subunits (β1/2 and γ1/2/3), and functions as a serine/threonine protein kinase[6]; AMPK activation is mediated by phosphorylation of threonine-172 in the catalytic domain of the α subunit[7]. The activity of AMPK can be regulated by upstream kinases, which include liver kinase B1 (LKB1)[8], Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) β[9], and transforming growth factor β-activated kinase-1[10]. Both LKB1 and CaMKK increase the AMPK activity through direct phosphorylation of threonine-172 in the α subunit. In addition, LKB1 is constitutively active as a major upstream kinase. The upstream signaling molecules of LKB1 may include protein kinase C (PKC)-ζ[11], protein kinase A[12], and p90 kDa ribosomal S6 kinase[13]. The fact that the calcium/calmodulin complex regulates CaMKK suggests that AMPK may be involved in Ca^{2+} modulation in cells.

AMPK regulates energy homeostasis in various organs through response to hormones and nutrient signals. AMPK physiologically responds to the change in the AMP:ATP ratio, and thus serves as an intracellular sensor for energy homeostasis[7]. In addition to ATP production with switching off from anabolic processes in tissues, the activation of AMPK affects whole body fuel utilization and induces fatty acid oxidation and glucose uptake in skeletal muscle and heart, but inhibits lipogenesis and adipocyte differentiation[6-7]. In the liver, AMPK inhibits gluconeogenesis and synthesis of glycogen, fatty acid and cholesterol. Since AMPK plays a key role in metabolic regulation, it is recognized as an important target for metabolic disorders such as obesity, diabetes, and metabolic liver diseases.

S6K1: S6K1 is a mitogen-activated serine/threonine protein kinase that is associated with growth and cell cycle progression. In translational processes, S6K1 phosphorylates the S6 protein of the 40S ribosomal subunit. Phosphoinositide-3 kinase (PI3K)-the mammalian target of rapamycin (mTOR) regulates S6K1 as a distinct pathway from the Ras/mitogen-activated protein kinase cascade[14]. S6K1 signaling suppresses catabolic events such as lipolysis in adipose tissue and fatty acid oxidation in muscle, both of which stimulate ATP generation[15]. Since S6K1 is sensitive to nutrients including amino acids, nutrients negatively regulate insulin signaling by phosphorylating insulin receptor substrate-1 (IRS1) through S6K1 activation. Thus, S6K1 may also affect the regulation of nutrient and hormone signaling pathways under normal and pathological conditions (e.g. obesity, diabetes, and cancer). Moreover, S6K1 may play a role in the balance between survival and death in tissues including the liver. It is noteworthy that AMPK activation leads to inhibition of the mTOR/S6K1 pathway through tuberous sclerosis protein 2 (TSC2) phosphorylation[16]. The regulation of S6K1 by AMPK is now recognized as an important regulatory step, by which cells maintain energy metabolism.

AMPK as a target for metabolic diseases

Nonalcoholic fatty liver disease (NAFLD) is defined as a common liver disease ranging from steatosis to nonalcoholic steatohepatitis, and cirrhosis[2]. Moreover, NAFLD is considered as a main hepatic component of metabolic syndrome[17]. The characteristics of metabolic syndrome are obesity, insulin resistance, and cardiovascular disorders. In obese people mostly with insulin resistance, excessive fat is deposited in the liver and the raised hepatic lipid amount is closely associated with pathogenic processes of the syndrome[18,19].

Hepatic steatosis by liver X receptor-α-sterol regulatory element, binding protein-1c: A variety of conditions such as excess delivery of fatty acids, decreased oxidation of hepatic fatty acid and/or impaired synthesis or secretion of very low-density lipoprotein increase the sources of hepatic lipids, leading to fatty liver disease. The amount of accumulated fat is also increased by lipogenesis; emerging evidence supports the importance of *de novo* lipogenesis in abnormal hepatic fat accumulation in NAFLD patients[20,21]. Lipogenesis is transcriptionally regulated by the membrane-bound sterol regulatory element, binding protein-1c (SREBP-1c), which belongs to the basic helix-loop-helix-leucine zipper family. In the nucleus, SREBP-1c activates transcription of genes involved in lipogenesis, as supported by the finding that the overexpression of SREBP-1c in transgenic mice promotes the development of fatty liver. In animal models of insulin-resistant diabetes and obesity, the increased synthesis of fatty acids contributes to the development of hepatic steatosis.

Liver X receptor-α (LXRα), a transcriptional nuclear receptor, is a key regulator of lipogenic genes encoding for the enzymes that promote hepatic fat accumulation (e.g. fatty acid synthase, FAS; acetyl-CoA carboxylase, ACC; and stearoyl-CoA desaturase-1, SCD-1)[22,23]. Ligand activation of LXRα promotes induction of the lipogenic genes through SREBP-1c, causing increases in fatty acid synthesis and progression to steatosis, hypertriglyceridemia, and steatohepatitis[22]. Thus, SREBP-1c is an important target gene of LXRα. Since the LXRα-SREBP-1c pathway activates lipogenesis in the liver, it is an attractive target for the treatment of hepatic steatosis and hepatitis. In clinical situations, the expression of SREBP-1c and lipogenic genes including ACC and FAS is enhanced in NAFLD patients[24,25]. In addition, increases in LXRα target gene expression (e.g. ACC and FAS) were observed in the patients with fatty liver, which was accompanied by SREBP-1c activation, but not activation of carbohydrate responsive element-binding protein[26].

The AMPK-S6K1 pathway is involved in the regulation of LXRα-SREBP-1c and thus in LXRα-induced lipogenesis; chemical activation of AMPK in conjunction with its inhibition of S6K1 leads to the intervention of hepatic steatosis (Figure 1)[27]. As an example, AMPK activation by oltipraz treatment inhibits S6K1 activity, which inhibits the activity of LXRα[27] and prevents the ability of activated LXRα to bind the LXR binding site upstream

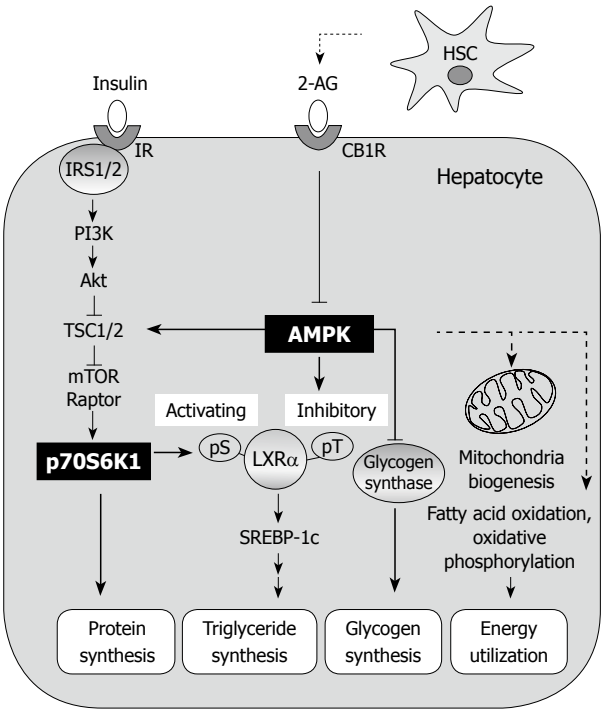

Figure 1 Adenosine monophosphate-activated protein kinase pathway in hepatic fuel metabolism. Adenosine monophosphate-activated protein kinase (AMPK), a metabolic energy sensor, negatively regulates protein synthesis through inhibition of the mammalian target of rapamycin (mTOR)-S6 kinase-1 (S6K1) pathway. The inhibitory effect of AMPK on liver X receptor-α (LXRα)-dependent triglyceride synthesis is opposed by the action of S6K1. AMPK also shuts down glycogen synthesis via inhibitory phosphorylation of glycogen synthase. AMPK as a fuel sensor induces glucose transport and fat oxidation in response to metabolic stress such as energy deprivation, and also increases mitochondrial biogenesis. AMPK counteracts energy depletion by stimulating energy production and limiting energy utilization. Endocannabinoids such as 2-arachidonoylglycerol derived from hepatic stellate cells decrease AMPK phosphorylation resulting in downregulation of lipogenic action. 2-AG: 2-arachidonoylglycerol; CB1R: Cannabinoid 1 receptor; HSC: Hepatic stellate cell; IR: Insulin receptor; Raptor: Regulatory-associated protein of mTOR; IRS1: Insulin receptor substrate-1; PI3K: Phosphoinositide-3 kinase; TSC1: Tuberous sclerosis complex 1; pS: Phospho-serine; pT: Phospho-threonine; SREBP-1c: Sterol regulatory element, binding protein-1c.

of the genes including SREBP-1c and CYP7A1. Therefore, the consequent repression of SREBP-1c expression contributes to decreased synthesis of fat in the liver[27].

Repeated alcohol consumption decreases the production of adiponectin secreted from adipocytes[28]. Adiponectin increases hepatic fatty acid oxidation through AMPK activation[29]. Therefore, it is tempting to speculate that AMPK activity is repressed as hepatic function deteriorates in alcoholic patients. Similarly, AMPK activity was decreased in animals which consumed alcohol for 4 wk[30]. As a compensatory response, alcohol consumption increased lipogenesis in the liver, which may also result from the reduced rate of fatty acid oxidation. Thus, pharmacological activation of AMPK may be of help in treating hepatic steatosis. Peroxisome proliferator-activated receptors (PPARs) play a role in sensing nutrient levels and regulating lipid and glucose metabolism[31]. Thiazolidinediones (TZDs) and fibrates that activate PPARγ and PPARα, respectively, are prescribed for patients with diabetes and/or

dyslipidemia. In those taking PPARγ agonists, insulin-mediated adipose tissue uptake and storage of free fatty acids are augmented with the inhibition of hepatic fatty acid synthesis, which may result in part from indirect activation of AMPK[32,33].

Hepatic insulin resistance: Insulin signaling is important in maintaining homeostasis of glucose, lipid, and protein metabolism, and thus induces anabolism in tissues. In addition, it has effects on normal growth and development. Insulin receptor and its associated protein IRS1 relay signal transmission to the PI3K-Akt pathway, which consequently increases mTOR-S6K activity. Activation of the mTOR-S6K1 pathway by insulin may lead to fat accumulation in adipose tissue, hypertrophy of skeletal muscle, growth of pancreatic β cells, and protein synthesis[15]. Therefore, the control of insulin signaling is tightly regulated by a negative feedback mechanism. In fact, the downstream components of the insulin receptor give inhibitory autoregulatory signals to upstream molecules along the insulin-signaling pathway or through unrelated pathways that cause insulin resistance. In particular, phosphorylation of IRS proteins on serine residues is a key step in the processes of physiological and pathological conditions. So, the kinases that phosphorylate IRS1/2 have been extensively studied.

Hepatic steatosis alone, or to a greater degree in combination with endotoxin challenge, makes the liver susceptible to oxidative damage and thus facilitates the pathologic process of hepatitis. The cytokines produced by accumulated fat with or without endotoxin cause insulin resistance. In particular, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) lead to insulin resistance through multiple mechanisms. These include c-Jun N-terminal kinase 1 (JNK1)-mediated serine phosphorylation of IRS-1, IκB kinase-dependent nuclear factor-κB activation, and suppressors of cytokine signaling-3 (SOCS-3) induction[34-36]. Since TNFα increases insulin resistance in peripheral organs, inhibition of TNFα activity and/or its decreased expression would be of help to overcome insulin resistance. However, IL-6 displays pleiotropic functions in a tissue-specific and time-dependent manner. IL-6 confers insulin resistance in hepatocytes through activation of SOCS protein through the Jak/Stat pathway to inhibit tyrosine phosphorylation of IRS1[36], while IL-6 increases insulin sensitivity by stimulating basal glucose transport in 3T3-L1 adipocytes[37], smooth muscle[38] and chondrocytes[39]. Acute treatment with IL-6 increases insulin sensitivity due to AMPK activation[40], while chronically elevated IL-6 leads to impaired insulin signaling and cellular insulin resistance *via* activating SOCS-3[36] and reducing the expression of the adiponectin, GLUT4, IRS1 mRNA, IRS-1 protein and its tyrosine phosphorylation[41,42].

Glucose is overproduced in the liver of patients with type 2 diabetes[43]. Because AMPK serves as an energy-saving mechanism, its activation decreases hepatic gluconeogenesis. The experimental results using gene knockouts, pharmacological means, or adenoviral activation of AMPK support the role of AMPK in the regulation of glucose production in the liver. Consistently, hepatic glucose production increased to show hyperglycemia and glucose intolerance in liver-specific AMPKα2 deficient mice. Hence, it is highly likely that the hepatic AMPKα2 isoform is critical for repressing hepatic glucose production and maintaining fasting blood glucose levels in the physiological range[44]. Consistently, AMPK activation by adenovirus expressing a constitutively active form of AMPKα2 as well as by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, a direct AMPK activator) or metformin reduced glucose output[45-47].

Activation of S6K1 exerts a negative feedback action on insulin signaling. As an example, TNFα secreted by non-parenchymal cells activates S6K1 in pathologic states. The important role of S6K1 on insulin resistance was proven by a study using S6K1-null mice[48,49]. A key role for mTOR-S6K1 regulation of insulin resistance was also supported by the finding that rapamycin blocked IRS1 phosphorylation[50,51], confirming the importance of S6K1 activity in inducing insulin resistance. Hence, insulin resistance induced by abnormal conditions such as hyperinsulinemia, obesity and excess nutrient availability is accompanied by an increase in S6K1 activity[48,52]. The result of a study using a knockout model proved the critical role of S6K1 and its physiological feedback importance to IRS1/2 and PI3K for insulin resistance. In an experimental model, the inhibitory effect of high-fat diet consumption on the insulin receptor-PI3K pathway is also mediated by S6K1. In our laboratory, it was found that the inhibitory modulation of S6K1 activity by beneficial candidates reversed insulin resistance and hyperglycemia[50]. In particular, oltipraz treatment inhibited S6K1 through AMPK activation. Consistently, a dominant negative mutant of AMPK abrogated S6K1 phosphorylation[50]. AMPK activation by other drugs like metformin and rosiglitazone also contribute to insulin sensitivity enhancement[47,53]. Similarly, other agents that inhibit insulin resistance also antagonize S6K1 activation downstream of AMPK[50]. So, these agents have the effects of improving insulin sensitivity through a mechanism involving AMPK-mediated S6K1 inhibition in hepatocytes[50].

JNK1 is activated by various stress signals such as cytokines or oxidative stress, and thus the activity of JNK1 increases under prediabetic or diabetic conditions. This important kinase is also implicated in the phosphorylation of IRS1/2[54-56], interfering with insulin action. The JNK pathway is stimulated by oxidative stress conditions, increased flux of free fatty acids and TNFα production, which contributes to developing insulin resistance. The importance of JNK activation is supported by the finding that a deficiency of JNK1 prevented insulin resistance in an experimental model[54]. Moreover, JNK mediates dysfunction of insulin secretion from β cells[57]. Hence, inhibition of JNK by chemical means may help improve insulin resistance and ameliorate hepatic energy metabolism[54,58]. For example, isoliquiritigenin from various natural herbs including licorice has a JNK-inhibitory effect. Thus, isoliquiritigenin is capable of repressing lipogenesis in the liver and protecting hepatocytes from oxidative

injury inflicted by fat accumulation through a novel JNK-dependent pathway that acts as an upstream component of LXRα (unpublished data).

CYTOPROTECTIVE EFFECT OF AMPK

An energy flux is a crucial factor for cell viability. To keep the energy supply constant, eukaryotic cells use AMPK as a mechanism to monitor ATP production and expenditure. As a consequence of its sensitivity to AMP levels, AMPK is activated by treatment with drugs including metformin and TZDs as well as by conditions of metabolic stress that repress ATP production (e.g. hypoxia or glucose deprivation). Thus, AMPK activation causes upregulation of ATP-producing catabolic pathways. However, AMPK inhibits ATP-consuming pathways including synthesis of fatty acids, cholesterol, glycogen, and proteins[59]. Although AMPK signaling is intricately tied to energy metabolism and homeostasis, it is also critical for various physiological processes including inflammation, and proliferation[60,61]. It is noteworthy that the AMPK-associated pathway may suppress apoptosis induced by glucocorticoids[62], hyperglycemia[63], hepatic ischemia-reperfusion[64] and oxidative stress[65-69]. AMPK activation has a beneficial effect on cell viability *via* protection of mitochondria from apoptosis: phosphorylation of glycogen synthase kinase 3β (GSK3β)[66], and phosphorylation of Bad, which leads to inhibition of cytochrome *c* release and attenuation of caspase-3 activity[70]. AMPK is also implicated in other pathophysiological responses in various cell types: a decrease in endoplasmic reticulum (ER) stress[71], DNA damage repair[72,73], autophagy[74,75], and the antioxidant defense system[65-69]. This review focuses on the role of AMPK in hepatocyte viability.

Regulation of autophagy and cell survival

Regulation of cellular balance between biosynthesis and turnover is crucial for the maintenance of metabolic homeostasis. Autophagy is an evolutionally conserved pathway for self-digesting of cytoplasmic components and organelles by lysosomal degradation[76]. Autophagy contributes to cell survival *via* removal of long-lived proteins and damaged organelles, thus this event plays a role in adaptive protection upon starved conditions[77]. In addition, a recent study showed that autophagy regulates lipid metabolism by inducing lipid utilization in hepatocytes, implicating a possible link with metabolic diseases[78]. The autophagic processes are regulated by several signal transduction mechanisms. Among them, AMPK activation induces autophagy in response to diverse stress conditions including energy depletion, ER stress, and hypoxia. The action of AMPK is mediated by the inhibition of mTOR-dependent signaling, which is a central inhibitory pathway of autophagy[79]. The AMPK-induced autophagy exerts a cytoprotective effect, which can be regulated by upstream kinases such as LKB1[74,75]. However, the role of S6K1 inhibition by AMPK in the modulation of autophagy is unclear. Despite these primarily defensive effects, autophagy mediates cell death under certain conditions[77], thus further study would help understand the role of AMPK in autophagy-associated cell viability.

Protection of mitochondria from external stress

Insulin resistance has been associated with a reduction in mitochondrial oxidative phosphorylation and ATP production, and thus downregulates the expression of genes encoding for oxidative metabolism[80-82]. Thus, mitochondrial dysfunction is frequently observed in the metabolic syndrome[82]. Under mitochondrial dysfunction caused by several endogenous or exogenous stimulants, it is difficult to maintain redox-homeostasis. In this situation, changes in mitochondrial membrane permeability (MMP) cause the release of proapoptotic mediators that can damage DNA and lead to apoptosis[83,84]. Oxidative stress inhibits endoplasmic reticulum calcium pumps, releasing calcium into the cytoplasm from endoplasmic reticulum. The cytoplasmic calcium is taken up by mitochondria, which makes the mitochondrial permeability transition pore (mPTP)[85,86]. In basal conditions, the mPTP is closed but opens in response to stress, allowing passage of small molecules. Opening of the mPTP causes MMP transition and cytochrome *c* release, inducing apoptosis. A number of studies have shown that chemical inhibitors of the mPTP have the ability to prevent the release of cytochrome *c* and protect cells from death[87]. Excess reactive oxygen species may enhance the opening of the mPTP, and cause mitochondrial depolarization and cytochrome *c* leakage[88,89]; the release of cytochrome *c* from mitochondria to cytoplasm activates procaspase-9 and Apaf-1, and stimulates apoptosome formation and caspase-3 activation so that it induces cell death[90].

AMPK-associated signaling mediates hepatocyte viability

AMPK: AMPK responds to external stress as a modulator of cell viability or death. In many cases, AMPK activation exerts a cytoprotective effect[62-64,66]. Chemical activation of AMPK protected cells from arachidonic acid-induced apoptosis and restored MMP. In this model, cell viability depended on mitochondrial function; treatment of the AMPK activator (e.g. oltipraz and resveratrol) protected cells from mitochondrial injury. Thus, the direct or indirect AMPK activators have the ability to protect cells from mitochondrial oxidative stress. This mitochondrial protective effect could be reversed by either compound C treatment or overexpression of the dominant negative mutant of AMPKα. In our laboratory, the AMPK-dependent antioxidant and cytoprotective effects had been tested with AICAR. Cellular H_2O_2 production increased by arachidonic acid treatment impairs mitochondrial function, and promotes apoptosis. Thus, arachidonic acid propagates apoptotic signals due to oxidative stress alone or in combination with an increase in mitochondrial Ca^{2+} uptake[91]. In this model, AICAR exhibited a cytoprotective effect against injury caused by arachidonic acid so that it abolished reactive oxygen species production in the cell. The data showing that

compound C treatment induced MMP transition indicate that AMPK is necessary for MMP regulation. AMPK increases its activity through TSC2 phosphorylation, which leads to translational suppression and cell size reduction under the situation of energy deprivation. Moreover, the phosphorylation of TSC2 protects cells from apoptosis induced by energy deprivation[16], suggesting that the downstream components of AMPK may be responsible for MMP regulation.

In a recent study, resveratrol, a polyphenolic component found in grapes and red wine, was shown to protect mitochondria from oxidative stress in an AMPK-dependent manner. AMPK activation by resveratrol depended on LKB1, but not CaMKK. Thus, LKB1 activation protects cells from apoptosis under the condition of energy stress[92]. The importance of LKB1 for AMPK-dependent cytoprotection is also supported by the result of the sauchinone study: sauchinone exerted a protective effect against MMP transition *via* LKB1 activation[69]. The upstream components that activate LKB1 include SIRT1[93], nitric oxide synthase[94], and protein kinase A[12]. In addition, we identified the formation of poly (ADP-ribose) (PAR) as the upstream event, by which resveratrol activates LKB1[66]. PAR polymerase (PARP) represents a nuclear enzyme that plays a role in DNA damage repair through PAR formation. In an energetic process, PAR causes rapid depletion of NAD^+, decreases ATP production, and thus leads to cell death[75]. In contrast, PARP prevents cell death through LKB1-AMPK-mediated autophagy activation[75], which may be associated with LKB1. Sometimes, AMPK activation may cause apoptosis; sustained AMPK activation (>10 h) triggered hepatocyte death through JNK and caspase-3 activation. In this process, p53, Bax and Fas ligand are upregulated or activated by activated JNK[95]. Hence, AMPK-dependent cell survival may rely on cell type, environmental conditions and on the duration of this kinase activation[95].

Mn-superoxide dismutase (Mn-SOD) as a mitochondrial enzyme converts the superoxide anion to hydrogen peroxide, and plays a role in cytoprotection[96]. Pro-oxidants like paraquat and dinitrophenol induce Mn-SOD in the liver[97,98]. Treatment with metformin or AICAR, an AMPK activator, increases the expression of MnSOD mRNA, suggesting that Mn-SOD induction may be coupled to the AMPK-associated pathway.

GSK3β: GSK3β is a constitutively activated serine/threonine kinase in normal state. This enzyme is well known as a regulator of glycogen metabolism, gene expression, and cell cycle progression[99]. GSK3β is inactivated by serine 9 phosphorylation[100], enabling cells to suppress mPTP opening[101] and prevent apoptosis of hepatocytes[66]. Hence, this kinase may contribute to cell viability against external stress (e.g. ischemia/reperfusion injury). It has also been recognized that inhibitory phosphorylation of GSK3β prevents phosphorylation of voltage-activated anion channel, and promotes binding of GSK3β with adenine nucleotide translocase. In our study, GSK3β inhibition protected mitochondria from mPTP opening and contributed to cell survival against severe oxidative stress[66], as also supported by other reports[102,103]. This contention is supported by the finding that treatment by a direct AMPK activator (i.e. AICAR) leads to GSK3β inhibition (Figure 2), as mediated with Raf1/ERK/p90 kDa ribosomal S6 kinase[104]. Some other cytoprotective compounds also act as AMPK activators, which include resveratrol and isoliquiritigenin, and cause GSK3β inhibition[66]. Thus, GSK3β phosphorylation may lie downstream of AMPK.

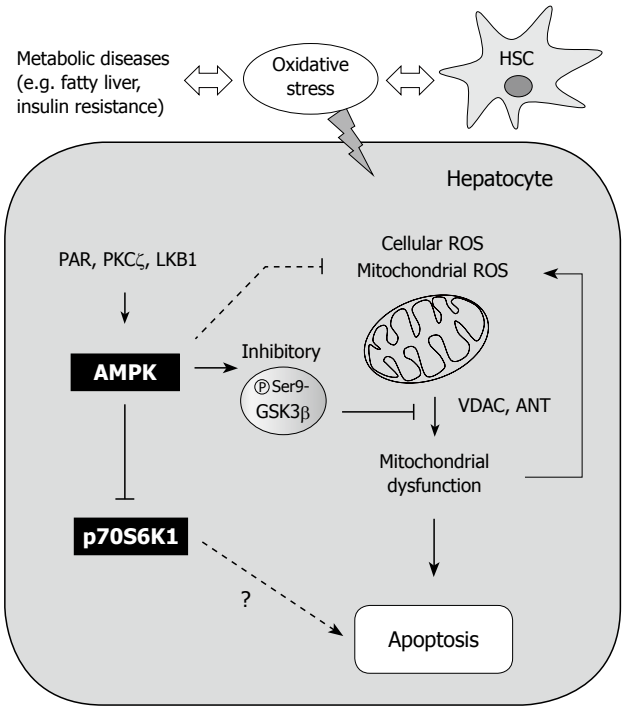

Figure 2 Adenosine monophosphate-activated protein kinase regulation of cell viability. Adenosine monophosphate-activated protein kinase (AMPK) protects cells from oxidative stress-induced H_2O_2 production and mitochondrial dysfunction, which results in part from the inhibitory phosphorylation of glycogen synthase kinase 3β (GSK3β). GSK3β inhibits mitochondrial function through voltage-activated anion channel (VDAC) phosphorylation. AMPK activation contributed to cell survival, whereas the regulatory role of S6 kinase-1 (S6K1) in apoptosis is still unclear. HSC: Hepatic stellate cell; PAR: Proliferator-activated receptor; PKC: Protein kinase C; LKB1: Liver kinase B1; ROS: Reactive oxygen species; ANT: Adenine nucleotide translocator.

PKC: In certain situations, necrosis may also be programmed through specific pathways. Hepatocytes undergo necrosis several hours after H_2O_2 treatment in association with PKC activation and/or AMPK inhibition, as evidenced by a decrease in cell death by PKC inhibitor treatment. Interestingly, PKC inhibition results in AMPK upregulation, suggesting that these two pathways are inversely coupled to each other[105]. Apparently, these pathways are linked to a cytoprotective effect, as shown by decreased H_2O_2-induced necrosis after treatment with PKC inhibitor or AMPK activator. Consistently, compound C treatment (an AMPK inhibitor) abrogated the ability of PKC inhibitor to protect cells, suggesting that PKC inhibitors have a cytoprotective effect through AMPK upregulation.

Table 1 Effects of candidate compounds on the adenosine monophosphate-activated protein kinase-S6 kinase-1 pathway and liver function

Chemicals	AMPK	S6K1	NAFLD	Hepatic insulin resistance	Cyto-protection in the liver	Effective conc.	Ref.
A class of synthetic dithiolethiones							
Oltipraz	↑	↓	+	+	+	30 μmol/L, 30 mg/kg	[27,50,67,107]
CJ11764	↑	↓	+	+	+	30 μmol/L	[27,50,67]
CJ12064	↑	↓	+	+	+	30 μmol/L	[27,50,67,107]
CJ11842	↑	↓	+	+	+	30 μmol/L	[27,50,67,107]
CJ11840	↑	↓	+	+	+	30 μmol/L	[27,50,67]
CJ11792	↑	↓	+	+	+	30 μmol/L	[27,50,67,107]
CJ11788	↑	↓	+	+	+	30 μmol/L	[27,50,67,107]
CJ11766	↑	↓	ND	+	+	30 μmol/L	[67,107]
CJ12073	↑	-	+	+	+	30 μmol/L	[27,67,107]
CJ11780	↑	ND	+	ND	ND	30 μmol/L	[27]
Metabolites of oltipraz							
M1	↑	↓	ND	+	+	30 μmol/L	[50,68]
M2	↑	ND	ND	ND	+	30 μmol/L	[68]
Phytochemicals							
Resveratrol	↑	ND	+	+	+	30 μmol/L	[66,108,109]
Isoliquiritigenin (*Glycyrrhizae radix*)	↑	-	+	ND	+	20 μmol/L, 30 mg/kg	[65], UD
Liquiritigenin (*Glycyrrhizae radix*)	↑	-	+	ND	+	100 μmol/L, 30 mg/kg	[65], UD
Sauchinone (*Saururus chinensis*)	↑	-	+	ND	+	30 μmol/L, 30 mg/kg	[69,110]
Baicalin (*Scutellaria baicalensis*)	↑	ND	+	ND	ND	10 μmol/L, 80 mg/kg	[111]

ND: Not done; UD: Unpublished data; ↑: Activation; -: No change; ↓: Inhibition; +: Beneficial effect against nonalcoholic fatty liver disease (NAFLD) or insulin resistance, or cytoprotection in the liver; AMPK: Adenosine monophosphate-activated protein kinase; S6K1: S6 kinase-1.

S6K1: In S6K1-/- hepatocytes, caspase-8 and Bid (a pro-apoptotic protein) were both down-regulated relative to control. A deficiency of S6K1 was not sensitive to the cascades of death receptor activation, as shown by no caspase-8 activation or FLIP$_L$ degradation in hepatocytes challenged by TNF-α or anti-Fas antibody treatment. The finding that Bid cleavage, cytochrome *c* release, caspase-3 activation, and DNA laddering were all attenuated by a deficiency of S6K1 raises the importance of S6K1 in the apoptotic process. Consistently, the lack of S6K1 did not diminish the BclxL/Bim ratio in cells deprived of serum, and thus prevented cytochrome *c* release and DNA fragmentation[106]. In an animal model, S6K1 deficiency enabled hepatocytes to survive against concanavalin A-induced apoptosis[106]. Inhibition of S6K1 may activate survival pathways through PI3K/Akt and ERK pathways. However, hepatocytes deficient in S6K1 underwent apoptosis on serum withdrawal when combined with PI3K or ERK inhibitor treatment[106]. In this sense, S6K1 inhibition along with Akt and ERK inhibitors, would enhance the efficacy of cancer chemotherapy for hepatocarcinoma[106]. In our oxidative stress model, rapamycin, an inhibitor of mTOR-S6K1 activity that causes dissociation of raptor from mTOR by binding FK506 binding protein 12, had no effect on apoptosis elicited by arachidonic acid + iron, suggesting that the inhibition of S6K1 alone may not be sufficient to promote cell viability. Overall, the inhibition of S6K1 may contribute to protecting hepatocytes from liver failure, and if so, it might result from improvement in insulin signaling.

AMPK REGULATION OF ENERGY METABOLISM AND CELL SURVIVAL

A series of beneficial compounds with the abilities of AMPK activation are listed in Table 1 and Figure 3, which may have liver-protective effects against external stimuli. Thus, the compounds that have modulating activities on metabolism may also have cytoprotective effects (Figure 4). In these actions, LKB1-dependent AMPK activation may be one of the key molecular pathways for cell survival. A number of studies have shown how AMPK responds to an increase in AMP as an energy sensing enzyme. In this way, it integrates diverse signal inputs, controls a number of metabolic enzymes in various cell types, and adapts cellular processes to the energy status. Since AMPK activation may not always be on the side of cell survival, the specific AMPK pathways responsible for cell viability still remain to be elucidated.

CLINICAL IMPLICATIONS

Metformin is a major drug used in the treatment of type 2 diabetes. AMPK activation by metformin suppresses hepatic glucose production and lowers blood glucose levels[47,112]. In addition, metformin has been shown to reverse fatty liver disease in humans[113,114]. TZDs belong to another important class of antidiabetic drugs that augment systemic insulin sensitivity. In diabetic patients, pioglitazone decreases hepatic fat content and increases splanchnic glucose uptake presumably through AMPK[115]. In addition, these medications may prevent simple hepatic steatosis from progressing to steatohepatitis. Although the molecular mechanism of AMPK activation by TZDs is unclear, AMPK activation is attributed to their ability to increase plasma adiponectin levels[53].

Hepatic ischemia-reperfusion injury, usually in association with liver transplantation and hepatic resection, is an important clinical issue. Ischemic preconditioning may

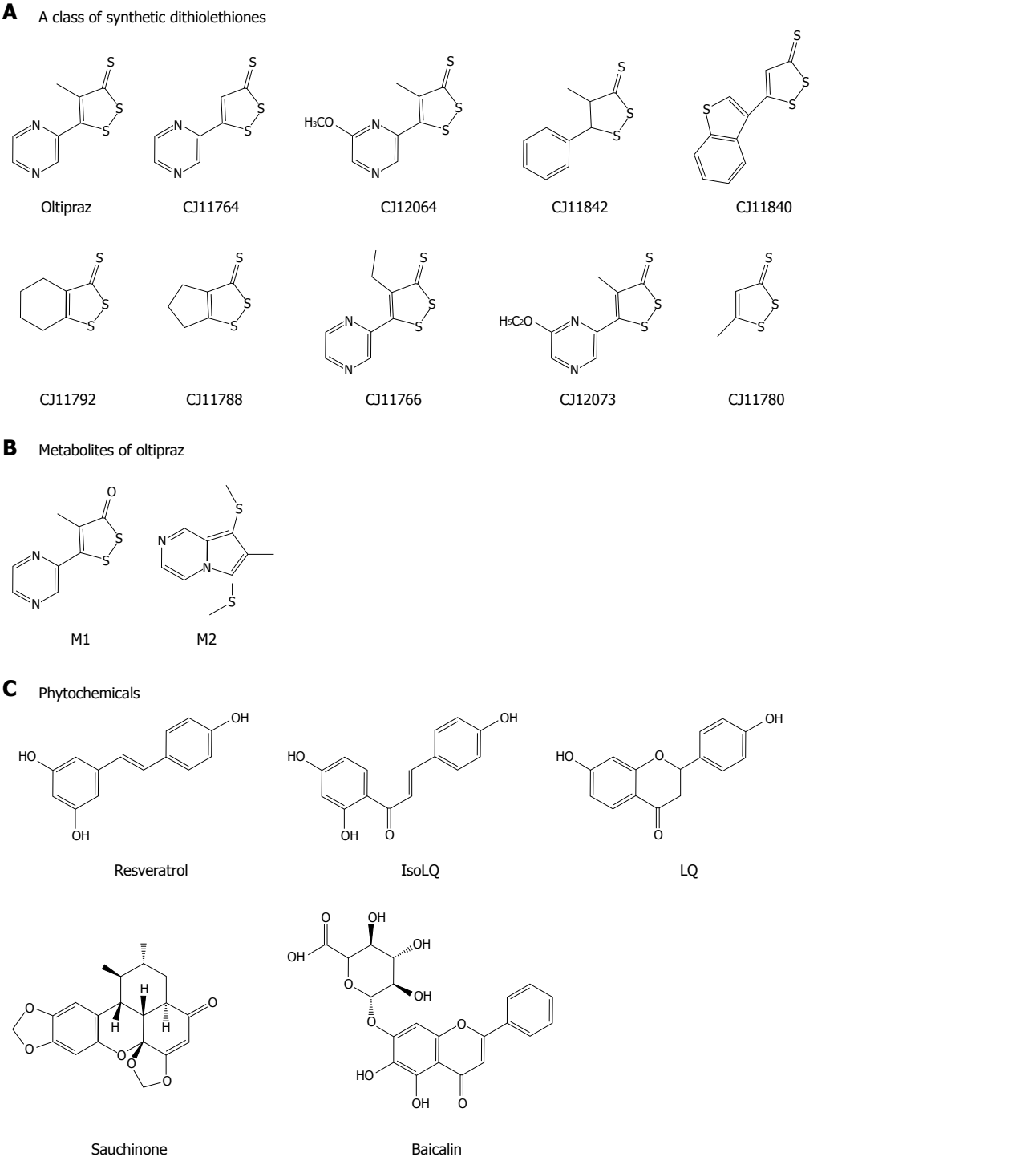

Figure 3 Chemical structures of adenosine monophosphate-activated protein kinase activators. A: Dithiolethione derivatives: Oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-thione], CJ11764 (5-pyrazinyl-1,2-dithiole-3-thione), CJ12064 [5-(6-methoxypyrzinyl)-4-methyl-1,2-dithiole-3-thione], CJ11842 (4-methyl-5-phenyl-1,2-dithiole-3-thione), CJ11840 (5-benzo[b]thiophene-3-yl-1,2-dithiole-3-thione), CJ11792 (4,5,6,7-tetrahydrobenzo-1,2-dithiole-3-thione), CJ11788 (5,6-dihydro-4H-cyclopenta-1,2-dithiole-3-thione), CJ11766 (4-ethyl-5-pyrazin-2-yl-1,2-dithiole-3-thione), CJ12073 [5-(6-Ethoxypyrazin-2-yl)-4-methyl-1,2-dithiole-3-thione], CJ11780 (5-methyl-1,2-dithiole-3-thione); B: Metabolites of oltipraz: First, oxidative desulfuration of the thione among approximately 1% of oltipraz to yield M1 [4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-one], which is not metabolized further; and secondly, desulfuration, methylation, and molecular rearrangement among a large amount of oltipraz to yield M2 [7-methyl-6,8-bis(methylthio)H-pyrrolo(1,2-a)-pyrazine], which is metabolized to other oxidized forms; C: Phytochemicals: Resveratrol (flavonoid found in the skin of red grapes and red wine), isoLQ (a flavonoid aglycone of isoliquiritin from licorice), LQ (a flavonoid aglycone of liquiritin from licorice), sauchinone (a lignan in *Saururus chinensis*), baicalin (the major flavonoid compound in *Scutellaria baicalensis*).

be beneficial to patients with hepatic resections in which long periods of ischemia are necessary. Ischemic preconditioning prevents ATP degradation and intracellular accumulation of AMP induced by subsequent ischemia[116]. Increases in AMP levels during ischemia activate AMPK, while AMPK inhibition abolishes the effect of preconditioning, indicating that AMPK plays a role in this effect[64]. So, hepatic preconditioning may allow the liver to

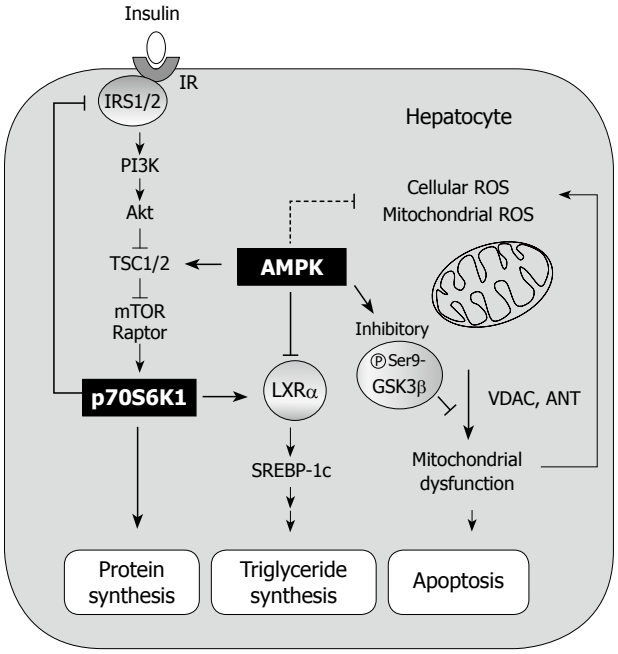

Figure 4 Dual regulation of fuel metabolism and cell viability by adenosine monophosphate-activated protein kinase. The adenosine monophosphate-activated protein kinase (AMPK)-S6 kinase-1 (S6K1) pathway regulates metabolic signaling flow. In addition, AMPK controls the redox-state and mitochondrial function, therefore its activation protects cells from apoptosis. AMPK is the key molecule to bridge the gap between fuel metabolism and hepatocyte viability. IR: Insulin receptor; IRS1: Insulin receptor substrate-1; PI3K: Phosphoinositide-3 kinase; TSC1: Tuberous sclerosis complex 1; ROS: Reactive oxygen species; mTOR: Mammalian target of rapamycin; LXRα: Liver X receptor-α; GSK3β: Glycogen synthase kinase 3β; SREBP-1c: Sterol regulatory element, binding protein-1c; VDAC: Voltage-activated anion channel; ANT: Adenine nucleotide translocator.

preserve energy metabolism during sustained ischemia[116]. Since AMPK activation by preconditioning may represent a new strategy to reduce the ischemia-reperfusion injury, modified preservation solutions containing AMPK activators may be of use, which should be evaluated in clinical settings.

CONCLUSION

As the mitochondrion plays a diverse role in essential cellular functions including energy production and homeostasis, redox cell signaling, and apoptosis, the chemical activators of AMPK protect hepatic mitochondria against toxic stress. The inhibition of S6K1 downstream of AMPK may also have a distinct role in liver biology. Thus, the AMPK pathway is associated with various pathological conditions, including metabolic syndrome and numerous apoptotic conditions. Because of the shared regulatory functions of AMPK in metabolism and cell viability, it becomes an advantageous target. In this review, we have proposed the concept that AMPK-associated signaling bridges the gap between fuel metabolism and hepatocyte viability, which may be of help in identifying valuable potential targets to prevent and/or treat derangement of metabolism and cell death in the liver.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3743

World J Gastroenterol 2010 August 14; 16(30): 3743-3744
ISSN 1007-9327 (print)

EDITORIAL

Gastroesophageal reflux disease: From heartburn to cancer

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

About 10%-15% of patients with gastroesophageal reflux disease develop Barrett's esophagus. This is considered a premalignant condition because it can progress from metaplasia to high-grade dysplasia, and eventually to adenocarcinoma. Recently, major advances have been made in the endoscopic treatment of Barrett's esophagus, therefore limiting the role of surgery in the treatment of this disease.

Key words: Gastroesophageal reflux disease; Barrett's esophagus; Esophageal adenocarcinoma; Laparoscopic fundoplication; Radiofrequency ablation; Esophageal endoscopic mucosal resection; Minimally invasive esophagectomy

Patti MG, Waxman I. Gastroesophageal reflux disease: From heartburn to cancer. *World J Gastroenterol* 2010; 16(30): 3743-3744 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3743.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3743

Gastroesophageal reflux disease affects an estimated 20% of the population in the United States. About 10%-15% of patients with gastroesophageal reflux disease develop Barrett's esophagus, which eventually can progress to adenocarcinoma, which is currently the fastest growing cancer in the United States. It is recognized that adenocarcinoma is in most cases the end stage of a sequence of events whereby the squamous esophageal epithelium is initially replaced by columnar epithelium without dysplasia. Subsequently, the metaplastic epithelium can progress to low- and high-grade dysplasia and eventually cancer[1-3].

This symposium addresses some key questions in the treatment of this disease process. The pathophysiology and diagnosis of the disease are reviewed, particularly in morbidly obese patients[4-10]. Based on the pathophysiology, the treatment of metaplasia is discussed. Special attention has been placed on new treatment modalities such as radiofrequency ablation and endoscopic mucosal resection, which have revolutionized the treatment of high-grade dysplasia and intramucosal carcinoma[11-16]. The remaining indications for esophagectomy in these cases are discussed[17]. Finally, we have reviewed what to do when invasive cancer is present, discussing the role of neoadjuvant therapy[18-20], the type of esophageal resection (transhiatal versus trans-thoracic)[21,22], and the current data available about minimally invasive esophagectomy[23,24]. The authors are both experts dedicated to the treatment of patients with esophageal disorders and have published extensively on these topics.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3745

World J Gastroenterol 2010 August 14; 16(30): 3745-3749
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Gastroesophageal reflux disease: From pathophysiology to treatment

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

This review focuses on the pathophysiology of gastroesophageal reflux disease (GERD) and its implications for treatment. The role of the natural anti-reflux mechanism (lower esophageal sphincter, esophageal peristalsis, diaphragm, and trans-diaphragmatic pressure gradient), mucosal damage, type of refluxate, presence and size of hiatal hernia, *Helicobacter pylori* infection, and Barrett's esophagus are reviewed. The conclusions drawn from this review are: (1) the pathophysiology of GERD is multifactorial; (2) because of the pathophysiology of the disease, surgical therapy for GERD is the most appropriate treatment; and (3) the genesis of esophageal adenocarcinoma is associated with GERD.

Key words: Gastroesophageal reflux disease; Pathophysiology; Acid reflux; Non-acid reflux; Esophageal manometry; Ambulatory pH; Barrett's esophagus; Esophageal adenocarcinoma

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Herbella FA, Patti MG. Gastroesophageal reflux disease: From pathophysiology to treatment. *World J Gastroenterol* 2010; 16(30): 3745-3749 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3745.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3745

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a very prevalent disease. Population studies have repeatedly shown GERD-related symptoms in a significant proportion of adults. The Montreal consensus conference defined GERD as "a condition which develops when the reflux of gastric contents causes troublesome symptoms and/or complications"[1]. However, this definition did not include details of the pathophysiology of the disease and its implications for treatment. The Brazilian consensus conference considered GERD to be "a chronic disorder related to the retrograde flow of gastro-duodenal contents into the esophagus and/or adjacent organs, resulting in a spectrum of symptoms, with or without tissue damage"[2]. This definition recognizes the chronic character of the disease, and acknowledges that the refluxate can be gastric and duodenal in origin, with important implications for the treatment of this disease.

This review focuses on the pathophysiology of GERD and its implications for treatment.

GERD - ROLE OF NATURAL ANTI-REFLUX MECHANISMS

Although all normal individuals experience some sort of "physiological" gastroesophageal reflux, a highly efficient barrier exists between the stomach and the esophagus. From the esophageal side, esophageal clearance is pro-

moted by peristalsis and salivary production. A valve mechanism exists between the esophagus and the stomach, formed by the lower esophageal sphincter (LES), the diaphragm, the His angle, the Gubaroff valve and the phrenoesophageal membrane.

Peristalsis

Esophageal peristalsis is an important component of the antireflux mechanism because it is the main determinant of esophageal clearance of the refluxate. Defective peristalsis is associated with severe GERD, both in terms of symptoms and of mucosal damage[3]. As matter of fact, the composite reflux score (DeMeester score)[4] includes in its calculation two indirect measurements of esophageal clearance (number of reflux episodes longer than 5 min and length of the longest episode). In addition, the average esophageal clearance time can be calculated by dividing the total minutes the pH is below 4 by the number of reflux episodes. This association also explains the high prevalence and severity of GERD in systemic diseases that affects peristalsis, such as connective tissue disorders[5].

It is known that 40%-50% of patients with GERD have abnormal peristalsis[3]. This dysmotility is particularly severe in about 20% of patients because of very low amplitude of peristalsis and/or abnormal propagation of the peristaltic waves (ineffective esophageal motility)[6]. Esophageal clearance is slower than normal, therefore, the refluxate is in contact with the esophageal mucosa for a longer period of time and it is able to reach more often the upper esophagus and pharynx. Thus, these patients are prone to severe mucosal injury (including Barrett's esophagus) and frequent extra-esophageal symptoms such as cough[6,7].

It is still unclear whether esophageal dysmotility is a primary condition that leads to GERD, or it is a consequence of esophageal inflammation. Medical therapy does not ameliorate esophageal peristalsis[8,9]. However it has been shown that effective fundoplication improves the abnormal peristalsis in most patients[10].

LES

Physiologically, the LES is a 3-4-cm-long segment of tonically contracted smooth muscle at the distal end of the esophagus[11]. It is intuitive that the LES creates a high pressure zone between the esophagus and the stomach that prevents reflux. An effective LES must have an adequate total and intra-abdominal length, and an adequate resting pressure[12]. However, a normal LES pressure does not exclude GERD, because abnormal transient relaxation might occur. Periodic relaxation of the LES in normal individuals has been termed transient lower esophageal sphincter relaxation (TLESR), to distinguish it from relaxation triggered by swallowing. TLESR accounts for the physiological reflux found in normal subjects. When it becomes more frequent and prolonged, TLESR can contribute to reflux disease, and this phenomenon appears to explain the reflux seen in the 40% of patients with GERD whose resting LES pressure is normal. What determines TLESR is unknown, but postprandial gastric distention is probably involved[11,13]. It has been shown that a mechanically incompetent LES is progressively associated with worse mucosal damage[7].

At the present time, there are no medications used in clinical practice that act on the LES. Some studies are presently conducted using inhibitors of the GABA type B receptor, especially baclofen, but the effect of this medication is still not clear. These data underline that an incompetent LES represents a mechanical and permanent defect of the gastroesophageal barrier. Only fundoplication can correct the functional and mechanical profile of the LES, therefore resulting in control of any type of reflux from the stomach into the esophagus.

Diaphragm

The crus of the diaphragm provides an extrinsic component to the gastroesophageal barrier. This pinchcock action of the diaphragm is particularly important as a protection against reflux induced by sudden increases in intra-abdominal pressure[13]. This mechanism is obviously disrupted by the presence and size of a hiatal hernia.

Increase of thoraco-abdominal pressure gradient

Abnormal gastric emptying might contribute to GERD by increasing intra-gastric pressure. Patients with suspected abnormal gastric emptying should be tested with nuclear markers[14] or ultrasound[15]. If slow emptying is diagnosed, appropriate therapy should be considered. Medication such as metoclopramide and Nissen fundoplication improve gastric emptying[16].

There is also strong evidence of a possible link between obesity and GERD. Specifically, it has been shown that there is a dose-response relationship between increasing body mass index (BMI) and prevalence of GERD and its complications[17-19]. Some studies have reported that morbidly obese patients with GERD have a higher incidence of incompetent LES, transient LES relaxation and impaired esophageal motility than non-obese patients with GERD[8,20,21]. However, a detailed mathematical analysis has shown that the severity of GERD (based on the DeMeester score) is associated with BMI[22], which suggests that obesity plays an independent role in the pathophysiology GERD, mainly through increased abdominal pressure[18,23].

The association of different pulmonary diseases and GERD has recently gained renewed interest[24]. It has been shown that patients with end-stage lung disease have a high prevalence of GERD; up to 70%[25]. Although in these patients pan-esophageal motor dysfunction is frequently found[25], a more negative thoracic pressure with an increase in the gradient between intra-gastric and intra-thoracic pressure might also contribute.

GERD: ROLE OF MUCOSAL DAMAGE

Increasing severity of esophagitis is associated with increasing acid exposure[26]; however, erosive esophagitis is present in only 50% of GERD patients[7]. Some experts believe that the erosive and non-erosive forms of the

disease might actually account for different subsets of the disease; others believe that they represent two different and progressive stages of the disease.

It is still unclear if mucosal inflammation is a cause or a consequence of GERD. Evidence has shown that esophagitis is associated with esophageal body dysmotility[7]. However, it is still unclear if it is the cause or the effect of the altered peristalsis. We do know that medical therapy for GERD does not ameliorate esophageal peristalsis[8,9], whereas surgical therapy clearly results in improvement[10].

GERD: ROLE OF THE REFLUXATE

As previously mentioned, gastric and duodenal contents can reflux into the esophagus and adjacent organs. Gastric hydrochloric acid has long been recognized as harmful to the esophagus[27]. However, gastro-esophageal refluxate contains a variety of other noxious agents, including pepsin[26]. Currently, it is recognized that this component of the refluxate (commonly called bile reflux and identified by the Bilitec bile reflux monitor using bilirubin as a marker) is composed of bile salts and pancreatic enzymes[26], and is also injurious to the esophageal mucosa. It causes symptoms[28], and could be linked to the development of Barrett's esophagus[29] and esophageal adenocarcinoma[30].

Besides the constituents of the refluxate, symptom perception and mucosal damage also appear to be linked to the patterns of esophageal exposure and the volume of the refluxate. Individuals are more likely to perceive a reflux event if the refluxate has a high proximal extent and a large volume[26].

Acid suppression is the main treatment for GERD. It has evolved from topical alkaline antacids to very effective proton pump inhibitors (PPIs). Several studies have shown the efficacy of PPIs in almost neutralizing gastric acid. These medications make the refluxate less aggressive, which leads to symptom amelioration and healing of esophagitis[31]. However, they do not stop reflux or cure GERD, as different studies with intraluminal impedance technology have shown that PPI therapy alters the pH of the refluxate but does not change the occurrence and number of reflux episodes[32,33]. Currently, there is no specific medication that controls non-acid reflux. On the other hand, fundoplication blocks any type of gastric refluxate because it restores the competence of the gastroesophageal junction.

GERD: ROLE OF HIATAL HERNIA

Hiatal hernia and GERD were once considered synonyms and hiatal hernia was considered a *sine qua non* condition for GERD to occur[34,35]. Currently, it is well known that both conditions can exist independently. However, it is recognized that hiatal hernia disrupts most of the natural antireflux mechanisms, and is considered an independent factor for GERD[26]. The simple presence of an abdominal portion of the esophagus is considered an antireflux mechanism, because it is submitted to positive abdominal pressure and acts as a valve[34]. In addition, TLESR seems to occur more frequently when a hiatal hernia is present. Not surprisingly, the presence and size of a hiatal hernia are associated with a more incompetent LES (the pinchcock action of the diaphragm is absent), defective peristalsis, more severe mucosal damage, and increased acid exposure[36].

Hiatal hernia is associated with early recurrence and failure of medical therapy for GERD[34]. The reduction of a hiatal hernia with narrowing of the esophageal hiatus is a key element in fundoplication and its omission or failure is a cause of recurrence of GERD.

GERD: ROLE OF *HELICOBACTER PYLORI*

The association of GERD and *Helicobacter pylori* (*H. pylori*) is very controversial. While some argue that the infection might play a role in the prevention of GERD by altering the nature of the refluxate (gastritis leading to achlorhydria), others find no link between the infection and esophageal diseases[37,38].

Prevalence studies seem to suggest that *H. pylori* infection is inversely associated with reflux esophagitis in some populations[37]. Eradication studies also suggest that *H. pylori* infection is protective with respect to GERD[37].

If *H. pylori* protects against GERD, a logical assumption would be that it also protects against adenocarcinoma development. Furthermore, adenocarcinoma incidence is rising worldwide; however, the increasing pace is slow in underdeveloped countries, exactly where *H. pylori* incidence is higher. Indeed, the majority of epidemiological studies have found a protective association, and the results of three recently published meta-analyses have shown that *H. pylori* colonization of the stomach is associated with a nearly 50% reduction in cancer risk[39].

GERD AND BARRETT'S ESOPHAGUS

The history of Barrett's esophagus has been complicated by different opinions on the genesis of the disease[40]. Currently, it is unquestionable that Barrett's esophagus is an acquired disease caused by GERD, although risk factors and innate predisposition are still been scrutinized. Also, it is believed that most, if not all, esophageal adenocarcinoma arises in Barrett's mucosa[41].

With regard to GERD pathophysiology, Barrett's esophagus represents an end stage form of the disease. It encompasses pan-esophageal motor dysfunction that is characterized by abnormalities in esophageal peristalsis, defective LES, and bile reflux[42]. Most authors consider this form of GERD to be a surgical disease[43], based on the aforementioned points.

FROM PATHOPHYSIOLOGY TO TREATMENT

The simultaneous use of intra-esophageal impedance and pH measurement of acid and non-acid gastroesophageal

reflux has clearly shown that treatment with PPIs only changes the pH of the refluxate, without stopping reflux through a functionally or mechanically incompetent LES[44]. For instance, using this technology, Vela *et al*[44] have shown that during treatment with omeprazole, postprandial reflux still occurs but it becomes predominantly non-acid. In a study in normal subjects, Vela and colleagues also have shown that baclofen, a GABA B antagonist, is able to reduce both acid and non-acid reflux by decreasing TLESR, the primary mechanism for both acid and non-acid reflux[45]. This study signals an important shift toward treatment focused on the competence of the LES rather than the pH of the refluxate alone. This goal can also be achieved by fundoplication; an operation that can be done laparoscopically with a short hospital stay, minimal postoperative discomfort, fast recovery time and excellent results[46-49]. Long-term studies have shown that fundoplication controls symptoms in 93% of patients after 5 years and in 89% after 10 years[46]. The operation controls reflux because it improves esophageal motility, both in terms of LES competence and quality of esophageal peristalsis[10]. Control of reflux is not influenced by the pattern of reflux, and is equally effective when reflux is upright, supine or bipositional[47]. In addition, the operation is equally safe and effective in young or elderly patients[48]. Concern has been raised about the presence of postoperative dysphagia. In our experience, this occurs in about 8% of patients, irrespective of the type of fundoplication, and it resolves spontaneously in all but a few patients in a few months, without requiring re-intervention[49].

It is important to select the best treatment for the individual patient based on a review of symptoms, age, sex, esophageal function, and type of refluxate. We feel that laparoscopic fundoplication is indicated in the following circumstances: when heartburn and regurgitation are not affected by medical treatment; when it is thought that cough is induced by reflux (Mainie *et al*[50] have shown that patients with a positive symptom index resistant to PPIs with non-acid or acid reflux demonstrated by multichannel intraluminal impedance-pH monitoring can be treated successfully by laparoscopic Nissen fundoplication); poor patient compliance; cost of medical therapy if more than one pill/day of PPI is needed (most insurance companies in the United States pay for one pill/day only); and postmenopausal women with osteoporosis. It has been shown that PPIs and histamine-2 receptor antagonists can increase the risk of hip and femur fractures[51]. Therefore, medical treatment is not advisable for young and very symptomatic patients.

Finally, in a recently published meta-analysis of medical *vs* surgical management for GERD, Wileman *et al*[52] have shown that, in adults, laparoscopic fundoplication is more effective than medical management for the treatment of GERD in the short to medium term. Surgery, however, carries some risk and its application should be individualized and the decision to undergo fundoplication should be based on patient and surgeon preference.

CONCLUSION

The pathophysiology of GERD is clearly multifactorial. While medical therapy can only affect gastric acid production, fundoplication restores the function of the LES and improves esophageal peristalsis. In addition, fundoplication stops any type of refluxate because it restores the competence of the gastroesophageal junction. It seems that fundoplication alone does not cause regression of Barrett's esophagus and does not prevent the development of adenocarcinoma. It will be important to study in patients with Barrett's esophagus the long-term effect of surgery in association with new treatment modalities such as radiofrequency ablation (RFA) and endoscopic mucosal resection (EMR). The combination should be more effective than monotherapy, because RFA and EMR eliminate the metaplastic or dysplastic epithelium, while fundoplication stops reflux, which is the original cause of Barrett's esophagus.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3750

World J Gastroenterol 2010 August 14; 16(30): 3750-3756
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Advances in diagnostic testing for gastroesophageal reflux disease

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

Gastroesophageal reflux disease (GERD) contributes substantially to morbidity and to costs in the United States health care system. The burden of this disease has resulted in attempts at improving diagnosis and characterizing patients. Numerous research and technical advances have enhanced our understanding of both the utility and limitations of a variety of diagnostic modalities. The purpose of this review is to highlight recent advances in GERD diagnostic testing and to discuss their implications for use in clinical practice. Topics addressed include esophageal pH monitoring, impedance testing, symptom association analyses, narrow-band imaging, and histopathology.

Key words: Gastroesophageal reflux disease; pH impedance; pH monitoring; Symptom association

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Gawron AJ, Hirano I. Advances in diagnostic testing for gastroesophageal reflux disease. *World J Gastroenterol* 2010; 16(30): 3750-3756 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3750.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3750

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common, chronic disease that affects up to 20% of the adult population in the United States[1]. It is the most frequent digestive system diagnosis in ambulatory care and at inpatient discharge[2]. GERD contributes in excess of $10 billion in annual direct health care costs, with the majority of cost attributed to proton pump inhibitors (PPIs)[2,3]. The substantial disease burden of GERD and recognition of PPI unresponsive patients has fostered numerous efforts to improve diagnostic and therapeutic monitoring modalities.

Research investigations have enhanced our understanding of both the utility and limitations of a variety of diagnostic modalities. Newer techniques for esophageal functional testing such as wireless pH capsule monitoring, duodenogastroesophageal (also referred to as alkaline or bile reflux) reflux detection, and esophageal impedance testing have been introduced over the past decade and are utilized in clinical practice. The American College of Gastroenterology, American Society for Gastrointestinal Endoscopy and American Gastroenterological Association have recently published updated reviews and guidelines on reflux management and monitoring[4-6]. This review highlights recent advances in GERD diagnostic testing and their utility in clinical practice. A literature search was conducted for English-language articles dealing with functional evaluation of the esophagus from 2008 to 2009. Databases included Medline and PubMed, with search

terms that included esophageal pH monitoring, GERD, and esophageal impedance.

ESOPHAGEAL pH MONITORING

Wireless capsule pH monitoring: Is it better than catheter systems?

A significant advance in pH recording has been the incorporation of an antimony electrode into a wireless capsule that transmits pH data to an external receiver *via* radiofrequency telemetry (433 MHz)[7,8]. Major advantages of the wireless system include patient tolerability and capability of performing extended recording periods of 2-4 d. Discomfort associated with conventional catheter electrodes can lead patients to minimize or avoid reflux-provoking stimuli such as meals and physical activity, thus decreasing the detection of abnormal acid exposure[9,10]. As a result of improved patient tolerability, the wireless pH system might provide a more accurate picture of an individual's acid exposure profile under more realistic conditions.

Several investigations have compared wireless to catheter-based pH monitoring. A recent study has evaluated simultaneous placement of the Bravo capsule and SlimLine catheter system in 55 patients referred with GERD symptoms and 53 healthy volunteers[11]. The Slimline system was removed after 24 h while the Bravo system recorded 48 h of data. The SlimLine catheter system recorded almost double the acid exposure time than the Bravo system in both patients and volunteers. A similar finding has been noted in previous studies[12,13]. There was correlation between pH values and a concordance of diagnostic yield of 82.1%. However, the authors argue that, due to a wide variation in repeated measurements and random variation, as measured by limits of agreement, the two methods are not interchangeable[11].

It is not clear from the study methods whether the increased acid detection by the SlimLine catheter system was due to a thermal calibration artifact intrinsic to the catheter pH recording system first reported in 2005[13]. This error has since been corrected. The SlimLine system also records a greater number of reflux events than does Bravo, which is related to a higher sampling frequency. This numerical difference has previously been shown to have a minimal effect on the overall acid exposure time[13,14]. Other potential explanations for the different measurements include lost data due to interrupted signal transmission by the wireless system, and movement of the pH sensor in the catheter system relative to the esophagogastric junction. The latter factor might be important given the axial shortening of the esophagus during swallowing, which could move the catheter electrode closer to or even transiently into the proximal stomach. The Bravo system was better tolerated and preferred by patients, although the investigators did report a failure rate of approximate 15% due to failure or premature detachment.

Prolonged monitoring: Is 4 d better than 1 d?

Extended pH monitoring using wireless technology might theoretically improve the detection of reflux and increase the sensitivity of testing. Several studies have demonstrated that increasing the recording period from 24 to 48 h increases the sensitivity of pH monitoring by 10%-26%[4,8]. Several studies have also consistently demonstrated higher acid exposure values on day 2 compared with day 1 with the wireless capsule. Although the differences are generally small, this might affect the interpretation in a subset of studies[11]. Most capsules are placed immediately after endoscopy, therefore, the observation raises concerns regarding the potential impact of conscious sedation on reflux detection in the time period immediately after endoscopy, when patients might be resting and avoiding typical activity.

Another advantage of a prolonged monitoring period is the ability to perform testing both on and off PPI therapy in a single study[15,16]. Controversy exists regarding whether pH monitoring is best done off or on PPI therapy, because there are advantages and disadvantages to each approach. Off-therapy testing evaluates the presence of abnormal acid exposure and maximizes symptom-reflux association owing to the greater number of symptom and reflux episodes. Off-therapy testing is used to document the presence of acid reflux in patients with non-erosive reflux disease, who are being considered for anti-reflux endoscopic or surgical therapy. Off-therapy testing is also employed for patients with a low index of suspicion for having reflux disease, such as those showing no symptomatic response to empiric trials of PPI therapy or those with atypical symptoms. In contrast, pH testing on PPI therapy can provide documentation of the effectiveness of PPI therapy.

The feasibility of pH monitoring for an extended duration was recently determined for 96 h (48 h off PPI therapy followed by 48 h on therapy) in 60 patients[16]. A single pH capsule was placed and calibrated to two separate receivers with the second receiver activated after 48 h upon initiation of PPI therapy. Reflux symptoms were also recorded. Complete 96-h data were available for 40 patients (67%) at completion of the study, with 20 patients having incomplete data transmission or early capsule detachment. A total of 14 patients had abnormal acid exposure in the first 48 h, and day 2 testing (off therapy) increased the detection of abnormal acid exposure by 10%. On PPI therapy, 39 out of 40 patients (97.5%) had complete normalization of acid exposure at day 4. In addition, three symptom association indices [symptom index (SI), symptom sensitivity index (SSI), and symptom association probability (SAP)] all decreased by day 4 on PPI therapy. Overall, the prolonged testing increased the detection of acid exposure and reflux events for symptom association measurements and allowed for evaluation of both acid exposure and symptom response to PPI therapy. Limitations of this approach included early capsule detachment in 15% and the need for two separate receivers. Updated models of the wireless pH capsule are expected to allow for prolonged 4-d recording with a single receiver.

pH sensor location: Is 5 cm the best site?

By convention, correct positioning of the catheter pH

electrodes is 5 cm above the proximal border of the lower esophageal sphincter (LES) and 6 cm above the squamocolumnar junction (SCJ) for the wireless pH capsule. These locations minimize potential noise from proximal stomach acid exposure, at the expense of decreased sensitivity. This is a particular concern for catheter- based systems in which esophageal shortening during deglutition results in relative movement of the pH sensor closer to the LES. Grigolon *et al*[17] recently have evaluated differences in subcardial pH measured at two different locations in GERD, as well as the role of hiatal hernia. Their study population consisted of 14 healthy volunteers and 11 and 10 GERD patients with and without a hiatal hernia, respectively. Wireless pH monitoring was performed using the Bravo capsule 2 cm below the SCJ, and all patients received a standardized lunch after placement of the capsule. The investigators confirmed that subcardial pH was highly acidic in the early stage after meals, but there was no difference between healthy subjects and GERD patients. The presence of a hiatal hernia did not affect the results. The findings build upon important observations made by this group regarding the role of the "acid pocket" in the pathogenesis of GERD. In clinical practice, substantial intrapatient variability and interpatient heterogeneity have limited the utility of intragastric pH monitoring.

Another study has evaluated 48-h pH recording, off PPI therapy, immediately above the SCJ compared to simultaneous results obtained at 6 cm above the SCJ in 62 patients with reflux symptoms and 55 controls[18]. GERD patients included those with erosive disease as well as non-erosive patients with typical reflux symptoms that are responsive to PPI therapy. Using a pre-defined specificity of 90%, monitoring immediately above the SCJ increased the sensitivity from 63% to 86% in all patients. The total percentage of time that pH was < 4 for the entire 48-h study was the parameter that best discriminated between GERD patients and controls. Patients with and without esophagitis had an increased sensitivity (78% to 97% and 47% to 73%, respectively) that indicated an increased discriminatory power for patients with more severe disease. These results were similar to another study in which pH measurements were obtained simultaneously 6 and 1 cm above the gastroesophageal junction (GEJ) in 40 GERD patients with and without erosive disease[19]. The investigators found improved diagnostic accuracy in patients with erosive disease but not non-erosive reflux disease (NERD). Although the results of these studies are encouraging for increasing the sensitivity of pH testing, especially in patients with more severe disease, more validation is needed before changing the conventional location of pH measurements.

pH-IMPEDANCE TESTING

Theoretical advantages

Intraluminal impedance monitoring detects changes in the resistance to electrical current across adjacent electrodes positioned in a serial manner along a catheter. Multiple electrodes positioned along the axial length of the impedance catheter determine the proximal extent of a reflux event. It is capable of differentiating antegrade from retrograde bolus transit, as well as liquid from gas reflux. A pH electrode incorporated into the recording assembly allows for simultaneous detection of acid content. Patient tolerability is similar to conventional pH monitoring as this is a catheter- based system. Likewise, recording has been limited to 24 h.

There is considerable debate on the current role of pH-impedance testing in clinical practice[20-22]. As PPI use for GERD has increased, patients presenting with typical or atypical reflux symptoms in spite of PPI therapy, and without erosive esophagitis, often pose a diagnostic and management challenge. The association of non-acid reflux events with symptoms has been demonstrated in several studies[23-26]. Impedance-pH monitoring is the most sensitive technique for the detection of reflux events. As a result of the ability to detect, localize and classify reflux events as acidic, weakly-acidic or alkaline, simultaneously, pH-impedance testing has been posited as the future standard for reflux detection and monitoring[27]. In addition, the more comprehensive reflux detection could guide more individualized therapy in patients based on their reflux profile as well as predict response to medical or surgical treatment[20,21].

Although theoretically superior to pH monitoring, the clinical utility of combined pH-impedance monitoring is still being investigated. Conventional pH testing has demonstrated high sensitivity and specificity in patients with GERD and erosive esophagitis. The chemical nature of non-acid reflux does not allow the presence of mucosal erosions to be used in the determination of sensitivity and specificity of impedance data. Therefore, studies that have examined the utility of impedance testing have relied upon symptom-reflux association methodology to support the clinical significance of non-acid reflux. As discussed below, substantial limitations for symptom-reflux association accuracy in the evaluation of acid reflux also apply to non-acid reflux. Furthermore, the reliance on symptom indices necessitates careful delineation of the specific symptom being evaluated. For instance, symptom association for regurgitation on PPI therapy is better detected by impedance testing than pH testing alone. However, the importance of non-acid reflux in generating symptoms of heartburn or chest pain is unclear. It has been demonstrated that the majority of persistent heartburn or chest pain events on PPI therapy are not related to either acid or non-acid reflux[26,28]. Extra-esophageal symptoms of globus, asthma and hoarseness might occur independent of individual reflux events and thus are inappropriate for reflux-symptom association analysis. GERD is often considered as a cause of chronic cough. Although studies have shown symptom correlation between cough and GERD, 50% of the cough episodes precede the individual reflux events, which demonstrates that cough-induced reflux occurs as often as reflux-induced cough[28].

Further difficulties in substantiating a role for pH-impedance monitoring arise from the absence of highly

effective, pharmacological therapies for non-acid reflux. Limited studies have used baclofen and baclofen analogs that inhibit transient LES relaxation. Surgical fundoplication is a more definitive means of arresting both acid and non-acid reflux, and ongoing studies are examining the use of pH-impedance results in predicting postoperative outcomes in refractory reflux patients. Additional limitations of impedance monitoring include low baseline impedance values generated by the mucosa of Barrett's esophagus and esophagitis, which make detection of liquid reflux problematic in such circumstances. Inaccuracies in the current versions of automated analysis software require careful and time consuming manual data correction[29].

Recent data

As a result of the ability to characterize acidity and determine number, duration, and location of reflux events, the majority of research using pH-impedance has focused on the challenges associated with diagnosing and treating NERD. A recent small study has evaluated 16 NERD patients with both pH-impedance and combined multiple pH monitoring in an effort to assess changes in reflux acidity and sensitivity to reflux events[30]. Compared to multiple site pH testing (at three locations), pH-impedance monitoring showed a small increase in sensitivity in detecting proximal reflux events. The authors reported that 30% of all distal acid reflux events became weakly acidic in the proximal esophagus, and a third of these events resulted in symptoms. Although the sample size was small, the results lend support to the concept of hypersensitivity in the proximal esophagus in a subset of NERD patients[31,32].

In a much larger study, Savarino *et al*[33] have evaluated the diagnostic utility of pH-impedance monitoring in 150 patients with NERD off PPI therapy. Among patients with normal distal esophageal acid exposure time, they found similar positive symptom associations for patients with acid reflux (15%) and non-acid reflux (12%). Twenty-six per cent of this group had a negative symptom association and were considered functional heartburn patients. The classification of patients with hypersensitive esophagus accorded by pH-impedance results (normal acid exposure time, positive symptom association) reduced the number of patients that would have been classified as having functional disease by 40%[33]. However, overall 87% of the 150 NERD patients had acid reflux identified as the etiology of their symptoms.

Impedance pH monitoring has also been used to compare reflux patterns between patients with erosive esophagitis and NERD[34,35]. In a small study of 26 patients, evenly split between NERD and erosive disease, pH-impedance monitoring did not reveal significant differences in mean reflux duration or the incidence of acid or non-acid reflux episodes. When stratified by type of reflux episode, patients with erosive disease did have slightly more liquid (mean 9 ± 2 *vs* 5 ± 1, $P = 0.07$) and acid (mean 9 ± 2 *vs* 4 ± 1, $P = 0.048$) reflux episodes in the supine position. Overall, pH-impedance could not discriminate between NERD and erosive esophagitis but this likely reflects the limited power of the sample size. In another study, Savarino *et al*[35] have compared a cohort of GERD patients with erosive and non-erosive disease with a control population and demonstrated increased acid exposure times, and frequencies of acid reflux events as well as proximal esophageal reflux extension, in both GERD subsets. Patients with erosive disease had a higher frequency and increased proximal migration of acid reflux events. Notably, the frequency of non-acid reflux events and their association with symptoms were similar in both erosive and non-erosive disease. Overall, the results of these studies lend further support to the argument for monitoring both acid and non-acid reflux episodes in further characterizing GERD and potentially directing management. However, the increased diagnostic yield of pH impedance over pH monitoring alone was limited and neither study has demonstrated that the increased detection results in improved patient therapeutic outcomes.

There has also been debate about whether pH-impedance monitoring should be performed on or off PPI therapy. This has recently been addressed in a small prospective study of patients with continued GERD symptoms on twice daily PPI therapy[36]. Using a randomized, crossover study design, combined 24-h pH-impedance monitoring was performed on (twice daily) and off PPI therapy for 7 d. Neither the number nor extent of reflux episodes was affected by PPI use. There were significantly more acidic reflux episodes off PPI therapy and more weakly acidic episodes on PPI therapy. However, there was lack of concordance between the SAP for both measurements, which was likely due to the small sample size of the study.

Ultimately, the benefit of using pH-impedance monitoring in routine clinical practice depends upon its ability to guide effective medical and surgical management. A prospective series of 12 patients in Switzerland evaluated using pH-impedance monitoring before and after anti-reflux surgery (mesh-augmented hiatoplasty)[37]. Although the sample size was small, the authors found that multichannel intraluminal pH-impedance monitoring significantly increased the number of reflux episodes detected before and after surgery compared to pH testing alone. There were also more patients identified as having a positive SI in the pH-impedance group. The study has found that pH-impedance monitoring provides increased data compared to pH testing alone, however, whether this information favorably affects management and long-term patient outcomes is yet to be determined. Future therapeutic trials using inhibitors of transient LES relaxation should provide valuable insights into the clinical significance of non-acid reflux.

SYMPTOM ASSOCIATION

Available methods

Three methods have been devised to use statistical calculations to correlate symptoms with acid reflux. Symptom correlation can be separately calculated for each symptom attributable to reflux, including heartburn, regurgitation

or chest pain. The application of symptom correlation to atypical reflux symptoms such as throat pain, hoarseness, cough and asthma is problematic given the lack of temporal association between such symptoms and individual reflux events. The first method developed was the SI[38], which involves dividing the number of symptoms associated with acid reflux events by the total number of symptoms, which yields a percentage. A second approach is the SSI[39], which divides the total number of reflux episodes associated with symptoms by the total number of reflux episodes. The third approach for symptom-reflux correlation is the SAP[40]. This involves constructing a contingency table with four fields: (1) positive symptom, positive reflux; (2) negative symptom, positive reflux; (3) positive symptom, negative reflux; and (4) negative symptom and negative reflux. Fisher's exact test is then applied to calculate the probability that the observed association between reflux and symptoms occurred by chance. An SAP value > 95% indicates that the probability that the observed association between reflux and the symptom occurred by chance is < 5%.

Both the SI and SSI do not take into account the total number of reflux and symptom events. Thus, in patients with very infrequent or frequent reflux episodes or symptoms, random, temporal associations between reflux and symptoms might produce an inaccurate result. Another important distinction between the methods is that the SAP determines the statistical validity of symptom-reflux associations, whereas the SI and SSI provide information on the strength of the association.

Does it work?

Past attempts to validate the utility of the symptom indices have shown conflicting results with some groups reporting correlation with PPI response[41,42], whereas others have shown high discordance rates of the indices and mediocre specificity and sensitivity[43]. As with any test used in clinical practice, reproducibility is paramount and this issue has been addressed recently in 21 patients with GERD symptoms[44]. The SI, SSI and SAP were determined in concert with 24-h pH-impedance monitoring. The SAP and SSI showed the highest reproducibility compared with the SI. The study was performed under "real world" conditions of ambulatory monitoring, which suggested that the symptom association indices, although far from ideal, can play a role in relating symptoms to reflux episodes. The limitations of symptom association and remaining cognizant of what the three methods do not measure should be considered before applying these in clinical practice. The symptom correlation tests should be viewed as complementary information that links symptoms with reflux events, which does not ensure response to either medical or surgical therapy.

OTHER MODALITIES AND ISSUES

Narrow-band imaging

Use of narrow-band imaging (NBI) to enhance the contrast between esophageal and gastric mucosa and improve visualization of the SCJ has been studied in GERD patients. NBI has been shown to increase reproducibility in grading esophagitis[45] and the ability to detect changes in the microvasculature at the SCJ[46]. More recently, a prospective study has evaluated the use of NBI to differentiate erosive esophagitis (EE) from NERD and controls[47]. A total of 107 patients underwent endoscopy with NBI. Compared to conventional endoscopy, NBI allowed for an increased detection of micro-erosions, vascularity, and mucosal islands ("pit patterns"). In terms of differentiating patients using these criteria, EE and NERD patients had a higher prevalence of micro-erosions and vascularity compared to controls. EE and NERD patients were only differentiated by an increased vascular surface in the absence of pit patterns (sensitivity 86.1%, specificity 83.3%). Although NBI with endoscopy is unlikely to serve as a standard for the diagnosis of GERD, it could serve as an adjunct in the classification of erosive and non-erosive disease.

Histopathology

The use of histological characteristics to help diagnose GERD, and specifically NERD, has garnered increased attention and has recently been reviewed[48]. Although there are limitations to many of the studies that have evaluated histology, dilation of the intracellular space (DIS) has emerged as a promising diagnostic marker of NERD[48,49]. There is also evidence that DIS can be affected by PPI treatment, potentially serving as a clinical endpoint in therapy. However, definitive histological parameters of DIS have yet to be defined for reflux disease. Histological parameters such as basal cell hyperplasia and papillae elongation have proven less sensitive or specific for GERD, but might ultimately play a role when used in combination with DIS[48,50]. Ultimately, histopathological characteristics will likely be used in concert with other modalities to diagnose and characterize GERD better.

Eosinophilic esophagitis as a confounder

Eosinophilic esophagitis (EoE) has been increasingly diagnosed in pediatric and adult populations over the past 15 years[51]. Patients can present with a variety of symptoms including dysphagia, food impaction, heartburn, and chest pain[52,53]. However, these symptoms are not specific for the diagnosis and it can be difficult to differentiate EoE from GERD. Presently, the diagnosis of EoE is defined by the combination of clinical symptoms and histological characteristics of mucosal eosinophilia (> 15 eosinophils/high-power field)[52]. Supportive features include the presence of mucosal rings, longitudinal furrows and exudates in the esophagus. Disorders such as hypereosinophilic syndrome, connective tissue disorders, GERD, drug hypersensitivity reactions or infectious esophagitis should either be excluded or deemed non-causal in the eosinophilia.

A recent retrospective case control study has evaluated clinical, endoscopic and histological characteristics that could differentiate GERD from EoE[54]. The combination of nine characteristics (age, dysphagia, food allergy,

esophageal rings, linear furrows, white plaques, no hiatal hernia, maximum eosinophil count, and eosinophil degranulation) differentiated GERD from EoE in their population[54]. However, as GERD is prevalent in approximate 20% of the United States population, it is inevitable that many patients will have coexisting disease[52,55]. Moreover, acid reflux itself might produce tissue eosinophilia or allow for allergen sensitization[56]. A significant proportion of suspected EoE patients respond both symptomatically and histologically to PPIs, which blurs the distinction between EoE and GERD even further[57,58].

CONCLUSION

As a result of complexities in phenotypic heterogeneity and pathophysiology, there is no single gold standard diagnostic modality for GERD. pH monitoring has the greatest accuracy in patients with typical heartburn and erosive esophagitis, but unfortunately, it suffers from significant limitations when applied to atypical manifestations in NERD patients. Advances in pH monitoring, most notably wireless pH capsule technology, have improved patient tolerability and allowed for prolonged recordings that allow for both detection of acid reflux and response to therapy. The sensitivity of pH monitoring might be enhanced by pH capsule positioning closer to the SCJ, but further validation is needed because of concerns for diminished diagnostic specificity. pH-impedance has clearly increased the understanding of acid and non-acid reflux pathophysiology. When combined with symptom indices, pH-impedance detection of weakly and non-acidic reflux has the potential to provide information that might guide management. Therapeutic trials that have demonstrated the predictive value of impedance data support this practice. Recent results using NBI and histopathology are of significance. Taken together, these methods lend themselves to a reductionist view of GERD, whereas patients are classified into better-defined sub-groups. This strategy could ultimately result in more effective, individualized management of GERD and improved outcomes.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3757

World J Gastroenterol 2010 August 14; 16(30): 3757-3761
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Gastroesophageal reflux disease and severe obesity: Fundoplication or bariatric surgery?

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Key words: Morbid obesity; Gastroesophageal reflux disease; Fundoplication; Bariatric surgery; Gastric bypass; Sleeve gastrectomy

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Prachand VN, Alverdy JC. Gastroesophageal reflux disease and severe obesity: Fundoplication or bariatric surgery? *World J Gastroenterol* 2010; 16(30): 3757-3761 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3757.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3757

Abstract

Increases in the prevalence of obesity and gastroesophageal reflux disease (GERD) have paralleled one another over the past decade, which suggests the possibility of a linkage between these two processes. In both instances, surgical therapy is recognized as the most effective treatment for severe, refractory disease. Current surgical therapies for severe obesity include (in descending frequency) Roux-en-Y gastric bypass, adjustable gastric banding, sleeve gastrectomy, and biliopancreatic diversion with duodenal switch, while fundoplication remains the mainstay for the treatment of severe GERD. In several large series, however, the outcomes and durability of fundoplication in the setting of severe obesity are not as good as those in patients who are not severely obese. As such, bariatric surgery has been suggested as a potential alternative treatment for these patients. This article reviews current concepts in the putative pathophysiological mechanisms by which obesity contributes to gastroesophageal reflux and their implications with regards to surgical therapy for GERD in the setting of severe obesity.

PARALLEL TRENDS IN GASTROESOPHAGEAL REFLUX DISEASE AND SEVERE OBESITY: CAUSALITY OR COINCIDENCE

Obesity has dramatically increased over the past few decades, with the prevalence of obesity among adults in the United States, defined as body mass index (BMI) $\geqslant$ 30 kg/m^2, increased from 13% in 1960-1962[1] to 32% in 2003-2004, with 3% of men and 7% of women classified as being severely obese (BMI $\geqslant$ 40 kg/m^2) in a recent estimate[2]. BMI itself is a strong predictor of overall mortality, with a progressive excess in mortality noted above the optimum BMI of 22.5-25 kg/m^2, due mainly to metabolic and vascular disease[3]. Indeed, the prevalence of the metabolic comorbidities that contribute to atherosclerosis appears to increase significantly with increasing BMI[4,5].

In parallel with this trend in obesity is the perception that the prevalence of gastroesophageal reflux disease (GERD) has increased as well, currently affecting between 8% and 26% of the population in the western world[6-8]. These data, however, are somewhat difficult to interpret,

as these longitudinal population-based studies rely primarily upon subjective GERD symptoms rather than physiological measures of GERD. Nonetheless, there has been a significant increase in the prevalence of serious sequelae related to GERD[9-11], including Barrett's esophagus and adenocarcinoma of the distal esophagus, which strongly suggests that the severity, if not the prevalence, of GERD is in fact increasing.

Furthermore, because the prevalence of GERD is markedly higher in overweight and obese individuals as compared to those with normal BMI[12,13], GERD itself is now recognized as obesity-related comorbidity. Indeed, the importance of the relationship between excess visceral adiposity and GERD is demonstrated by the greater correlation between GERD and waist circumference and waist-to-hip ratio (markers of central obesity) than that between GERD and BMI[14]. However, the prevalence of GERD, even in the setting of severe obesity, is < 50%[15], which suggests that severe obesity itself is not sufficient to cause GERD, and that in the majority of severely obese individuals, at least some of the physiological mechanisms that prevent GERD remain reasonably intact. As such, when managing GERD in a severely obese patient and considering surgical therapy, it is useful to review the proposed mechanisms by which obesity contributes to GERD pathophysiology.

ROLE OF SEVERE OBESITY IN GERD PATHOPHYSIOLOGY

Fundamental to the development of GERD is a failure of the anti-reflux barrier that comprises the lower esophageal sphincter (LES) and the crural portion of the hiatus. LES function is directly dependent on intrinsic LES pressure (LESP, normal, 10-24 mmHg), total LES length, intra-abdominal LES length, and the frequency and duration of transient LES relaxation (TLESR). Indirectly, LES function is affected by the pressure gradient between the intragastric and intraesophageal environment.

When compared to healthy asymptomatic control subjects, 43 consecutive severely obese patients were found to have a lower LESP (11.9 ± 5.3 mmHg *vs* 15.9 ± 2.7 mmHg), and 51% were noted to have abnormal acid exposure[16]. Similarly, in a large cohort of patients with foregut symptoms, the prevalence of a mechanically defective LES (based on hypotensive LES, total length, or abdominal length) increased as BMI increased, with 55% of obese patients demonstrating a defective LES[17]. While nearly 30% of the 1659 subjects in this study were noted to be obese, specific data regarding severely obese individuals were not described. In contrast, in another large cohort of patients with GERD, mean LESP was in fact significantly greater in subjects with severe obesity (17 ± 9.2 mmHg *vs* 14 ± 7.6 mmHg), and 62% of severely obese subjects with GERD had a normal (39%) or hypertensive (23%) LES compared to only 46% of individuals with BMI ⩽ 35 kg/m^2; 10% of whom were noted to have a hypertensive LES[18]. The authors of this study hypothesized that the mechanisms responsible for GERD might be different in the setting of severe obesity, and that the observed increased LESP could represent a compensatory mechanism against the increased pressure gradient between the stomach and esophagus, which ultimately remains inadequate to prevent GERD. This finding also has important implications with regards to surgical therapy, as conventional anti-reflux procedures (i.e. fundoplication) seek to correct the defective LES.

TLESR could be the most important reflux mechanism in the setting of a functioning LES, and it has been observed that fundoplication reduces the frequency of TLESR[19]. Based on high-resolution manometry and concurrent fluoroscopy in non-obese patients, the key events that lead to opening of the gastroesophageal junction during TLESR include LES relaxation, crural diaphragm inhibition, esophageal shortening, and a positive pressure gradient between the stomach and the gastroesophageal junction lumen[20]. Obese individuals without GERD were noted to have an increased frequency of TLESR (7.3 ± 2.0 events/2 h *vs* 2.1 ± 1.2 events/2 h) compared to normal weight individuals, whereas LESP and LES length were similar between the two groups[21]. Similar findings have been noted in the setting of severe obesity[22].

Several factors might contribute to the increased gastroesophageal gradient seen with obesity[23], including increased intra-abdominal pressure[24], increased intragastric pressure[25], increased negative inspiratory intrathoracic pressure[26], and a mechanical separation between the LES and the extrinsic compression provided by the diaphragmatic crura[23]. The latter is a key step in the development of hiatal hernia, which, based on endoscopic evidence, is more prevalent in obese individuals than normal weight individuals[27,28]. Indeed, the negative effects of the presence of hiatal hernia on LES function might in fact be greater than the effects of obesity *per se*[17].

SURGICAL TREATMENT OF GERD

There is substantial controversy regarding the long-term efficacy and durability of fundoplication in the setting of obesity, and fewer data still to inform clinicians as to its effectiveness in the setting of severe obesity. A major concern regarding the long-term durability of fundoplication in severe obesity is the presumed increased risk of hiatal hernia recurrence, projected from the well-recognized contribution of obesity to the risk of hernia recurrence following abdominal wall hernia repair[29,30]. In a study of 224 consecutive patients with 3 years follow-up who underwent laparoscopic Nissen or transthoracic Belsey Mark Ⅳ (BM-Ⅳ) fundoplication, overall symptomatic recurrence was 31.3% in obese patients (22.9% Nissen, 53.8% BM-Ⅳ), compared to 4.5% in normal-weight individuals[31]. In another cohort study, preoperative severe obesity was associated with a higher rate of fundoplication failure, defined as the need for reoperation, lack of satisfaction, or severe symptoms at follow-up[32]. This study was limited by the small number of severely obese patients (only seven out of 166) and loss of patients to follow-up.

In another study of patients who were undergoing gastric bypass after failed fundoplication, the majority of failures were found to be due to wrap disruption rather than intrathoracic wrap migration[33]; the latter being the most common anatomical failure in normal and overweight patients. In contrast, several studies have demonstrated short-term and medium-term outcomes in obese patients that are comparable to those in non-obese patients[34-37]. These data are somewhat limited in their applicability to severely obese individuals, however, due to their lack of physiological outcomes measures, small numbers of severely obese patients, and relatively short follow-up period.

BARIATRIC SURGERY AND GERD

Bariatric surgery has become a widely accepted form of treatment for severe obesity, and several studies have demonstrated a significant reduction in GERD symptoms and medication utilization, as well as weight and metabolic comorbidity, including diabetes, hypertension and dyslipidemia[38,39]. Indeed, given the frequent presence of these and other comorbidities in the setting of severe obesity, the importance of significant and sustained weight loss for the overall health of severely obese patients, and the conflicting data regarding the outcomes of fundoplication in severe obesity, bariatric surgery is increasingly being seen as a more appropriate surgical treatment for GERD in severe obesity, even though objective measures of GERD outcomes might be comparable between fundoplication and gastric bypass[40]. Earlier concerns regarding the comparative safety of bariatric surgery (gastric bypass in particular) and Nissen fundoplication have been addressed by the recent finding that the morbidity and mortality rates of the two procedures were very comparable when using the University Health System Consortium database to identify morbidly obese patients who underwent laparoscopic gastric bypass (n = 21 156) or laparoscopic Nissen fundoplication (n = 6108) at American academic medical centers between 2004 and 2007[41]. Instead, discussion today is centered around the differential effects of currently performed bariatric operations [Roux-en-Y gastric bypass (RYGB), adjustable gastric banding (LAGB), biliopancreatic diversion with duodenal switch (DS), and sleeve gastrectomy (SG)] on GERD, as well as other obesity-related comorbidity.

RYGB AND GERD

RYGB accounts for over half of the currently performed bariatric operation in the United States, and appears to have a very favorable impact on GERD[42-45]. Its recognized effectiveness has even led to its use in non-severely obese patients with GERD[46], particularly in the setting of failed fundoplication[33]. Its efficacy in treating GERD is thought to be related to the relatively low acid production of the small-volume (15-30 mL) gastric pouch[47], reduction of esophageal biliopancreatic refluxate by use of a roux limb measuring at least 100 cm in length[48,49], and weight loss. The physiological effects of the anatomic configuration of RYGB, and specifically, the configuration of the gastric pouch, might in fact be a more important contributor to reflux improvement than reducing alkaline bile reflux or weight loss. When comparing GERD remission as measured by symptom resolution and medication discontinuation, super-obese patients (BMI $\geqslant$ 50 kg/m^2) who underwent RYGB had a higher rate of GERD resolution than those who underwent DS, despite the greater weight loss seen in the latter group[15].

LAGB AND GERD

Since its FDA approval in 2001, LAGB has rapidly become a popular bariatric surgical option for patients and surgeons due to its relative technical simplicity, perceived advantageous safety profile, and lack of gastrointestinal tract division or reconstruction (and consequent malabsorption). The effects of LAGB on GERD are conflicting, however, with some studies demonstrating improvement in physiological GERD metrics[16], while others show improvement on GERD questionnaires and/or through the discontinuation of GERD medications[50,51]. In contrast, several studies have demonstrated measured exacerbation of esophageal acid exposure, GERD symptoms, and the development or worsening of esophageal dysmotility following LAGB[52-54]. The mechanism by which LAGB may improve GERD is not well characterized, but is thought to include weight loss, increase in LESP, and reconstitution of the angle of His. It has been hypothesized that the poorer GERD outcomes following LAGB might be attributable to an unrecognized hiatal hernia at the time of initial band placement, which has led some to suggest that the presence of hiatal hernia is a contraindication to LAGB[55], whereas others have suggested that aggressive identification and concomitant repair of hiatal hernia improves outcomes and reduces the need for reoperation due to band slippage or pouch dilation[56]. Given these conflicting data, most bariatric surgeons do not recommend LAGB to severely obese patients with significant GERD, particularly in the setting of hiatal hernia.

SG, DS AND GERD

SG is a restrictive procedure initially described as the first procedure of a two-staged duodenal switch operation in very-high-risk super-obese patients, and is rapidly gaining popularity as a stand-alone bariatric operation. As with LAGB, early data regarding the impact of SG on GERD are mixed[57], and very little long-term or comparative data regarding SG and GERD are available. While the resection of a substantial portion of the parietal cell mass, significant weight loss, and a possibly increased rate of gastric emptying might all contribute to improvement in GERD physiology, the relatively long and narrow anatomical configuration of the sleeve might increase resistance to esophageal emptying of physiological amounts of reflux, and the parietal cell mass remains significantly greater than that with RYGB. Furthermore, when bile reflux is

controlled as a factor (through biliopancreatic diversion in the setting of DS), symptomatic resolution of GERD is greater with RYGB[15]. As such, SG in the setting of significant GERD should be recommended with caution.

SEVERE OBESITY AND GERD: SURGICAL RECOMMENDATIONS

When surgical treatment of GERD is indicated in a severely obese patient, bariatric surgery rather than fundoplication should be strongly considered. Not only does bariatric surgery, and RYGB in particular, better address the mechanisms that lead to GERD in obese patients with the potential for greater durability, but it also addresses concomitant obesity-related comorbidity by achieving significant and sustained weight loss. Therefore, in this case, the surgeon has the opportunity to substantially improve the patient's quality of life, positively impact multiple chronic medical conditions, and possibly reduce the excess long-term mortality risk associated with severe obesity in an acceptably safe, minimally-invasive, and cost-effective manner. For many patients, this discussion might be the first in which bariatric surgery is introduced as a possible therapeutic option, and it is not uncommon for patients to express significant resistance to the idea. In other instances, patients might have been considering bariatric surgery but were hesitant to discuss the possibility with their primary care physician and are receptive to the opportunity to learn more about the procedures. Not uncommonly, this discussion might require several office visits with the surgeon, and it is important that, in addition to offering detailed information regarding the procedures, the severely obese patient with GERD undergoes multidisciplinary evaluation as do other potential bariatric surgery patients, given the need for life-long changes in eating and behavior, and the need for long-term medical follow-up and vitamin supplementation. In doing this, the surgeon can provide a therapy that goes significantly beyond treatment of GERD.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3762

World J Gastroenterol 2010 August 14; 16(30): 3762-3772
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Pathophysiology and treatment of Barrett's esophagus

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

Gastroesophageal reflux disease (GERD) affects an estimated 20% of the population in the United States. About 10%-15% of patients with GERD develop Barrett's esophagus, which can progress to adenocarcinoma, currently the most prevalent type of esophageal cancer. The esophagus is normally lined by squamous mucosa, therefore, it is clear that for adenocarcinoma to develop, there must be a sequence of events that result in transformation of the normal squamous mucosa into columnar epithelium. This sequence begins with gastroesophageal reflux, and with continued injury metaplastic columnar epithelium develops. This article reviews the pathophysiology of Barrett's esophagus and implications for its treatment. The effect of medical and surgical therapy of Barrett's esophagus is compared.

Key words: Gastroesophageal reflux disease; Barrett's esophagus; Lower esophageal sphincter; Esophageal motility; Proton pump inhibitors; Antireflux surgery

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Oh DS, DeMeester SR. Pathophysiology and treatment of Barrett's esophagus. *World J Gastroenterol* 2010; 16(30): 3762-3772 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3762.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3762

INTRODUCTION

Gastroesophageal reflux disease (GERD) affects an estimated 20% of the population, and with direct and indirect costs exceeding $10 billion annually, it is the costliest gastrointestinal disorder in the United States[1]. Much of this extraordinary sum goes to pay for increasingly more potent and widely prescribed medications to suppress gastric acid production. While these medications have been proven to relieve heartburn symptoms and heal esophagitis, they have not substantially altered the malignant complications of reflux disease. Adenocarcinoma of the esophagus, which occurs as a consequence of chronic gastroesophageal reflux, is increasing faster than any other cancer in the United States, and has surpassed squamous cell as the most prevalent type of esophageal cancer[2].

The esophagus is normally lined by squamous mucosa, therefore, it is clear that for adenocarcinoma to develop, there must be a sequence of events that results in transformation of the normal squamous mucosa into columnar epithelium. This sequence begins with gastroesophageal reflux, and with continued injury metaplastic columnar epithelium develops. Currently, in the Unites States, only an endoscopically visible segment of columnar mucosa that contains goblet cells on biopsy is considered to be premalignant, and patients with this condition are considered to have Barrett's esophagus. Barrett's esophagus is the precursor lesion for esophageal adenocarcinoma.

EPIDEMIOLOGY

The prevalence of Barrett's esophagus appears to be increasing in the Western world. It has been debated wheth-

er this represents a true rise in incidence or is secondary to a heightened awareness of the dangers of reflux disease among practitioners, and an increased use of upper endoscopy to evaluate patients with reflux symptoms[3]. The most convincing epidemiological evidence that the prevalence of Barrett's esophagus is actually increasing comes from a recent study in the Netherlands using their Integrated Primary Care Information database, which contains > 500 000 computerized patient records. In that study, there was a linear increase in the diagnosis of Barrett's esophagus that was even more pronounced if the increase was based on the number of upper endoscopies performed during the same time period (from 19.8/1000 upper endoscopies in 1997 to 40.4/1000 upper endoscopies in 2002)[4]. Epidemiological studies in England have also demonstrated an age-specific increase in the prevalence of Barrett's esophagus per 100 upper endoscopies during the years 1982-1996[5].

Thus, there is evidence that the prevalence of Barrett's esophagus is increasing, but it is clear that the true prevalence of Barrett's esophagus in the population is unknown, and likely much higher than would be expected based on clinical cases diagnosed by upper endoscopy. In one of the few autopsy studies that has evaluated the prevalence of Barrett's esophagus, Cameron *et al*[6] found 376 cases per 100 000 people in Olmsted County, MN, USA. This rate was five times higher than the clinical prevalence of Barrett's esophagus in this same area (82.6 per 100 000). Further support for the concern about a large sub-clinical population of individuals with Barrett's esophagus comes from a study done in veterans by Gerson *et al*[7]. They performed upper endoscopy in a group of patients who presented for routine sigmoidoscopy for colorectal cancer screening; none of whom had symptoms of reflux. Although there are obvious limitations to a study done primarily in older, white male military veterans, nonetheless, their finding that 25% of patients had Barrett's esophagus is concerning because, on the basis of symptoms, none of these patients would have been recommended to have upper endoscopy. These observations suggest that the majority of individuals with Barrett's esophagus go undiagnosed, either because they ignore minor reflux symptoms or, as the study in veterans suggests, they are truly asymptomatic.

PATHOPHYSIOLOGY

Overview

The development of Barrett's esophagus is likely a two-step process. The first step involves the transformation of normal esophageal squamous mucosa to a simple columnar epithelium called cardiac mucosa. This occurs in response to chronic injury produced by repetitive episodes of gastric juice refluxing onto the squamous mucosa. The change from squamous to cardiac mucosa likely occurs relatively quickly, within a few years, while the second step, the development of goblet cells indicative of intestinal metaplasia, proceeds slowly, probably over 5-10 years[8]. Once present, Barrett's esophagus can progress to low- and high-grade dysplasia, and ultimately to adenocarcinoma. This entire process is commonly described as the Barrett's metaplasia-dysplasia-carcinoma sequence.

Step one: Transition from squamous to columnar-lined esophagus

To understand what constitutes a columnar-lined esophagus an understanding of the anatomy and histology of the normal gastroesophageal junction is required. Unfortunately, the very definition of what is normal in this area remains controversial, with much debate centered on whether cardiac mucosa is normally present at the gastroesophageal junction. Although our understanding is gradually improving, Hayward's remark in 1961 that "the lower end of the esophagus is a region where the pathology, the physiology, and even the anatomy are not quite clear" remains appropriate even today[9]. In one of the first reports describing the normal gastroesophageal junction, Hayward indicated that a junctional or buffer zone of columnar mucosa is normally interposed between the acid-secreting oxyntic gastric mucosa and the acid-sensitive squamous esophageal mucosa[9]. Although an appealing concept, Hayward provided no data in support of his theory, and did not discuss the role of the lower esophageal sphincter which had been demonstrated to exist before his publication. According to Hayward, this junctional mucosa is normally found in a length of up to 2 cm at the gastroesophageal junction. He also noted the following about this junctional mucosa: (1) it was histologically distinct from normal gastric fundic and pyloric epithelium; (2) it did not secrete acid or pepsin but was resistant to both; (3) it was not congenital but acquired; (4) it was mobile and varied in length - creeping progressively higher into the esophagus with continued gastroesophageal reflux; and (5) it was potentially reversible with correction of reflux. Furthermore, he pointed out that it was located in the esophagus, and that it developed in association with gastroesophageal reflux[9].

Now, over 40 years later, there is still dispute about the histology of the normal gastroesophageal junction, but it is clear that normally there is none or at most 4 mm of cardiac mucosa in the distal esophagus at the gastroesophageal junction[10-13]. Longer lengths of cardiac mucosa are acquired secondary to chronic gastroesophageal reflux[14,15]. Supporting evidence for the concept that cardiac mucosa is acquired comes from both clinical and experimental studies. Experimental evidence comes from a 1970 study by Bremner *et al*[16] in which a series of dogs underwent stripping of the distal esophageal squamous mucosa, with or without creation of a cardioplasty to destroy the function of the lower esophageal sphincter. Squamous re-epithelialization occurred in those animals without gastroesophageal reflux, whereas in the animals with reflux after cardioplasty, the esophagus was re-epithelialized by a columnar epithelium that lacked parietal cells - the equivalent of cardiac mucosa in humans[16]. There is also clinical evidence in humans that columnar mucosa can replace normal esophageal squamous epithelium in the setting of gastroesophageal reflux. Following

an esophagectomy with gastric pull-up, reflux of gastric juice into the residual esophagus is common because there is no lower esophageal sphincter and a large hiatal hernia has been created. Postoperative endoscopy has revealed that many of these patients develop columnar epithelium that, on histology, is identical to cardiac mucosa proximal to the anastomosis in the residual esophagus, in what had pathologically been proven to be squamous mucosa at the time of the operation. Several series have revealed that this process is common, and occurs in ⩾ 50% of patients after esophagectomy with gastric pull-up, and that the length of columnar mucosa increases with longer follow-up[8,17-20]. Furthermore, the cardiac mucosa that develops in these patients proximal to the esophagogastric anastomosis has been shown to be biochemically similar to cardiac mucosa found in non-operated patients at the native gastroesophageal junction[17]. Additional support for the concept that cardiac mucosa is acquired comes from the fact that it is not found anywhere else in the gastrointestinal tract, and when present at the gastroesophageal junction, it is always inflamed and demonstrates reactive changes unrelated to either *Helicobacter pylori* infection or mucosal pathology elsewhere in the stomach[21]. This is atypical for a normal epithelium. Lastly, the presence of cardiac mucosa can be correlated with objective markers of GERD, including an incompetent lower esophageal sphincter, increased esophageal acid exposure on 24-h pH monitoring, a hiatal hernia, and erosive esophagitis[15].

The earliest manifestation of GERD might in fact be the presence of microscopic foci of cardiac mucosa at the gastroesophageal junction. This leads to the question of why the finding of a microscopic length of cardiac mucosa at the gastroesophageal junction is so common even in patients without the typical reflux symptoms of heartburn or regurgitation. This is likely to be related to the pathophysiology of early reflux disease. Evidence is accumulating that reflux disease begins with gastric distention after large and particularly fatty meals. Gastric distension leads to effacement of the lower esophageal sphincter and exposure of the squamous mucosa at the distal extent of the sphincter to gastric juice. The pathophysiology of the gastroesophageal junction has been best studied by Fletcher and McColl. They have noted that the gastric distension that occurs with eating can cause the lower esophageal sphincter to unfold by almost 2 cm in normal volunteers[22]. Moreover, they have identified an unbuffered acid pocket at the gastroesophageal junction following a meal; a phenomenon that they have attributed to gastric juice floating upon a lipid layer after ingestion of fatty food. By pulling back a pH catheter before and after a meal, they have been able to show that the pH step-up that corresponds to the functioning lower esophageal sphincter moved proximally with gastric distension, secondary to unfolding of the distal portion of the sphincter. By measuring acid exposure with a pH catheter positioned at the squamocolumnar junction, and another located 5.5 cm proximal to the squamocolumnar junction, Fletcher *et al*[23] have demonstrated significantly greater acid exposure at the squamocolumnar junction (median total percentage time pH < 4 of 11.7% *vs* 1.8% at 5.5 cm proximal to the squamocolumnar junction). This study has confirmed the presence of significant acid exposure at the most distal intrasphincteric segment of the esophagus in patients with otherwise normal acid exposure proximally at 5.5 cm above the squamocolumnar junction. These findings were subsequently extended when it was demonstrated that salivary nitrite is rapidly converted into nitric oxide when it comes in contact with gastric acid that contains physiological levels of ascorbic acid, and this reaction has been found to be maximal at the gastroesophageal junction[24]. The levels of nitric oxide generated at the gastroesophageal junction are potentially mutagenic, and might play a role in the pathophysiology of this region.

It is likely that continued injury to the distal esophagus and lower esophageal sphincter leads to progressive loss of the abdominal length of the sphincter. What started as transient sphincter unfolding with gastric distension gradually progresses to permanent sphincter destruction. With destruction of the sphincter, reflux disease is allowed to explode into the esophagus, and can lead to an increase in the length of cardiac mucosa, either as tongues or as a circumferential replacement of the distal esophageal squamous mucosa. This leads to progressive migration of the squamocolumnar junction proximally[25,26]. Confirmation of esophageal submucosal glands deep to areas lined by cardiac mucosa provides clear evidence that the development of cardiac mucosa is occurring in the esophagus in areas previously covered with squamous mucosa and not in the proximal stomach[26].

The precise details of the molecular mechanism by which squamous mucosa is transformed into cardiac mucosa remain unknown. However, there is likely to be a crucial interaction between normally sequestered esophageal stem cells and an intraluminal stimulus that drives this metaplastic process. Tobey *et al*[27] have demonstrated that exposure of esophageal squamous mucosa to gastric juice produces dilated intercellular spaces that allow molecules of up to 20 kDa to permeate down to the stem cells in the basal layer. Perhaps the sensation of heartburn occurs as a consequence of diffusion of hydrochloric acid through these intercellular spaces and stimulation of sensory afferent nerves[28]. These ultrastructural changes occur before gross or microscopic changes become apparent. Thus, one possibility is that factors present in the refluxed juice that gain access to the basal layer stem cells via these dilated intercellular spaces induce a phenotypic transformation such that cardiac columnar mucosal cells rather than squamous cells are produced.

Step two: Intestinalization of cardiac mucosa

Cardiac mucosa is thought to be an unstable epithelium, in part because of the severe inflammatory and reactive changes present on histology. It is hypothesized that cardiac mucosa progresses down one of two possible pathways, based on a combination of environmental and genetic factors. One pathway involves the expression of gastric genes and leads to the formation of parietal cells

within glands below the cardiac mucosa. Gastric differentiation leads to a mucosa called oxyntocardiac mucosa, and this is thought to represent a regressive or favorable change because oxyntocardiac mucosa is not premalignant, and appears to be protected from developing intestinal metaplasia. In the second pathway, expression of intestinal genes causes the formation of goblet cells within cardiac mucosa. In contrast to gastric differentiation, intestinal differentiation represents a progressive or unfavorable change because this mucosa is premalignant. Both oxyntocardiac mucosa and Barrett's esophagus have less inflammation than cardiac mucosa, which suggests that these mucosal types are more stable epithelia[29].

The development of goblet cells marks the transformation of cardiac mucosa into intestinal metaplasia. When an endoscopically visible length of this mucosa is present in the esophagus, the definition of Barrett's esophagus has been met. While gastroesophageal reflux is known to be the primary factor responsible for the development of Barrett's esophagus, the specific cellular events that lead to the transformation of cardiac mucosa into intestinalized cardiac mucosa are unknown. However, evidence is accumulating that intestinalization requires a specific condition or stimulus, and that Barrett's esophagus occurs in a stepwise process. The first step, from squamous to cardiac mucosa, is likely to occur in response to acid reflux. The second step, development of intestinal metaplasia, is likely to occur in response to a different type of luminal insult. Numerous studies have demonstrated that, although isolated acid reflux can cause esophagitis, Barrett's esophagus is associated with the presence of a mixture of acid and bile salts[30-32]. Furthermore, clinical experience dating back 30 years has suggested a role for refluxed bile in the development of intestinal metaplasia. In 1977, Hamilton and Yardley observed the development of columnar mucosa and intestinal metaplasia above the esophagogastric anastomosis in a group of patients after esophagectomy. They noted that "severe symptoms of gastroesophageal reflux and bile staining of the refluxed material were documented only in the group with Barrett's. In addition, pyloroplasty had been performed more commonly in this group."[33]. Recently, in two separate analyses of patients with reflux with and without Barrett's esophagus, we found that the factor most associated with the presence of Barrett's esophagus in both men and women with GERD was abnormal bilirubin reflux, as determined by Bilitec monitoring[34,35].

Fitzgerald *et al*[36] have reported several interesting observations on how the dynamics of mucosal exposure to luminal contents might affect columnar epithelial cell proliferation and differentiation. Using cultured human Barrett's esophagus biopsy specimens, they have demonstrated that continuous exposure to acidic media at pH 3.5 resulted in increased villin expression (a marker for epithelial cell differentiation) and reduced cell proliferation. Villin expression was not detected when the culture medium was made more acidic (pH < 2.5). In contrast, a dramatic increase in proliferation occurred when the Barrett's esophagus tissue was exposed to a short (1 h) pulse of acidic medium (pH 3.5) followed by a return to neutral pH. Clinically, this same group has noted that effective acid suppression results in a shift of the Barrett's epithelium away from proliferation and toward differentiation[37]. However, the cellular consequences of duodeno-gastroesophageal reflux in the setting of gastric alkalization with acid suppression medications were not addressed in that study.

It has been hypothesized that the mechanism by which acid and bile interact to cause Barrett's esophagus is related to the ionized state of bile salts[38]. It appears that in a weakly acidic environment certain bile acids are particularly toxic. At pH 3-6, these bile salts are soluble and non-ionized, and can enter mucosal cells, accumulate, and cause direct cellular injury[39]. When the luminal pH is higher than the pKa, these same bile acids are ionized and cannot cross the phospholipid membrane. Further, when the luminal pH is lower, as normally it is in the stomach, bile acids precipitate out of solution and are harmless[40]. Thus, it is only at this critical pH range of 3-5 that certain bile acids become non-ionized and able to cross the cell membrane. Once inside the cell, the pH is 7 and the bile acids become ionized and are trapped inside the cell where they have been shown to result in mitochondrial injury, cellular toxicity and mutagenesis[41-44]. Consequently, this mid-range gastric pH of 3-5 is a danger zone for patients with duodeno-gastroesophageal reflux.

It remains uncertain whether the transformation of cardiac mucosa to intestinalized cardiac mucosa represents a phenotypic change secondary to the induction of genes, or a mutational event within the columnar cells. Mendes de Almeida and colleagues have demonstrated biochemically that both cardiac mucosa and intestinal metaplasia express sucrase-isomaltase and crypt cell antigen - two small intestine marker proteins; however, in that study only three patients with cardiac mucosa were evaluated[45]. Das has developed a murine monoclonal antibody (DAS-1) that reacts specifically with normal colonic epithelial cells, and subsequently he has found that it also reacts with an unknown epitope in Barrett's mucosa[46]. Griffel *et al*[47] have reported that the DAS-1 antibody stained cardiac mucosa without intestinal metaplasia in seven patients, and that six of these patients later developed histological evidence of intestinalization on repeat biopsies. Likewise, we noted that the pattern of immunostaining with cytokeratins 7 and 20 was similar in cardiac mucosa and Barrett's esophagus[48]. These findings suggest that, biochemically, cardiac mucosa and intestinal metaplasia are similar, and that cardiac mucosa is the precursor of intestinalized columnar epithelium, or Barrett's esophagus.

Currently, the length of Barrett's esophagus is divided into short (< 3 cm) and long (≥ 3 cm) segments based on the endoscopically determined length of the columnar streak or column in the distal esophagus. Clinically, patients with long-segment Barrett's esophagus tend to have more severe reflux disease than those with short-segment disease. Patients with long-segment Barrett's esophagus have a higher prevalence of hiatal hernia, more commonly have a defective lower esophageal sphincter, and dem-

onstrate greater esophageal acid and bilirubin exposure on 24-h pH and Bilitec monitoring[30,49]. Despite the differences in length, there is evidence that short and long-segment Barrett's esophagus are biochemically similar[48,50]. This is supported by the clinical observation that the risk of malignancy is similar for both short and long segments of Barrett's esophagus[51].

The presence of goblet cells is the *sine qua non* of Barrett's esophagus. The likelihood of finding intestinalization correlates with the length of the columnar segment. Once 4 cm of cardiac mucosa are present in the distal esophagus, nearly all patients will be found to have intestinal metaplasia on biopsy[49,52]. However, the location of goblet cells in a columnar-lined segment is not uniform, and often the entire length of columnar esophagus does not demonstrate intestinal metaplasia. Goblet cell density is greatest near the squamocolumnar junction and becomes more variable distally[29]. In other words, if intestinal metaplasia is present within a columnar-lined segment of the esophagus, it will always be present proximally at the squamocolumnar junction. Goblet cells might extend throughout the entire length of the columnar segment. The length of Barrett's esophagus is determined by the endoscopic length of columnar mucosa and not by the length of mucosa showing intestinal metaplasia. In other words, a 6-cm segment of columnar mucosa with intestinal metaplasia only at the proximal 1 cm is still considered long-segment Barrett's esophagus, but the clinical behavior of this long-segment Barrett's might differ substantially from a 6-cm segment of columnar mucosa with intestinal metaplasia throughout the entire length. The current definition of Barrett's esophagus does not take this into account.

The time course to develop goblet cells is uncertain, but it appears to take a minimum of 5-10 years[38,53]. Studies involving esophagectomy patients have indicated that cardiac mucosa develops rapidly, often within 1-2 years. Intestinalization of the columnar segment in these patients occurs significantly later, typically after another 3-5 years[18-20,33,54]. These findings might reflect an accelerated course of events because these patients often have significantly greater reflux of acid and bile than the typical patient with GERD. However, this clinically relevant human model does demonstrate the two-step process of Barrett's esophagus, starting with columnarization followed by intestinalization in some patients.

The molecular mechanisms by which cardiac mucosa acquires goblet cells remain to be elucidated. However, there is increasing evidence that expression of the homeobox gene Cdx-2 plays a pivotal role. The expression of this gene increases with progression from squamous mucosa with esophagitis to cardiac mucosa, and is maximal in the setting of intestinal metaplasia[55-57]. Experimental work has suggested that Cdx-2 expression can be modulated by the pH of luminal material[58]. Furthermore, an individual's response to an inflammatory stimulus might also participate in the mucosal adaptation to reflux disease. Fitzgerald *et al*[59] have demonstrated that esophagitis and Barrett's esophagus have distinct cytokine profiles that reflect different inflammatory responses to reflux-induced injury. Moreover, even within a given Barrett's esophagus segment, the inflammatory response is more severe at the proximal end near the squamocolumnar junction, which could explain the greater tendency for intestinalization to occur at this location[60]. Furthermore, the specific cytokine polymorphism of a given individual might also influence the development of Barrett's esophagus. Preliminary work from Gough *et al*[61], for example, has demonstrated that specific polymorphisms of interleukin (IL)-1 receptor antagonist and IL-10 are more common in patients with Barrett's esophagus than those with esophagitis. Thus, a genetically determined inflammatory response to reflux might influence the pathway of disease in each individual patient.

DYSPLASIA AND MALIGNANT TRANSFORMATION

Barrett's esophagus is a premalignant mucosa, and has an increased proliferation rate, decreased apoptosis, and an increased fraction of diploid and aneuploid cells compared to normal epithelium[13,62]. The combination of increased proliferation and decreased apoptosis allows genetic abnormalities to develop and accumulate, and drives the development of dysplasia and malignant transformation in Barrett's esophagus[63]. Although non-dysplastic Barrett's esophagus is a simple columnar epithelium with homogenous nuclei arranged close to the basement membrane, dysplasia results in both cytological and architectural abnormalities, including loss of nuclear polarity, pleomorphic appearance, and the development of glandular distortion[64]. By convention, there are four broad categories used by pathologists to describe the dysplastic process: (1) no dysplasia; (2) indefinite for dysplasia; (3) low-grade dysplasia; and (4) high-grade dysplasia. This classification system has been adapted for use in Barrett's esophagus from that used in ulcerative colitis[65,66]. The most significant category, high-grade dysplasia, is characterized by carcinoma *in situ* with malignant cells that do not invade the lamina propria.

The grading of dysplasia has great clinical utility in stratifying risk of subsequent cancer in patients with Barrett's esophagus, and to date, it is the most important predictive marker for the development of invasive adenocarcinoma. However, the ability to grade dysplasia remains a subjective endeavor, particularly outside specialized centers with expert gastrointestinal pathologists[67]. Even among focused gastrointestinal pathologists there is discordance, particularly with regard to the presence of low-grade dysplasia[68]. This lack of precision inherent in histopathological grading has stimulated efforts to identify more objective molecular and biochemical indicators of an increased risk for progression in patients with Barrett's esophagus. It has been demonstrated that in medically treated patients with Barrett's esophagus and low-grade dysplasia, the risk of progression is increased in patients with aneuploidy[69]. It is hoped that other molecular mark-

ers that are better able to predict which patients with Barrett's esophagus are at increased risk for progression will be identified in the future.

NATURAL HISTORY OF BARRETT'S ESOPHAGUS

Although it is widely accepted that Barrett's esophagus is a premalignant condition, the degree of risk remains uncertain. A meta-analysis by Shaheen *et al*[70] of 25 articles published between 1984 and 1998 concluded that the incidence of adenocarcinoma in patients with Barrett's esophagus was approximately 0.5% per patient-year, with a range from 0.2% to 2.9%. However, these studies were done in patients being treated for reflux, including those that had antireflux surgery, and thus these estimates might not reflect the true natural history of Barrett's esophagus progression. Known risk factors for progression to dysplasia and cancer include hiatal hernia size, the length of Barrett's esophagus, patient age, and the presence of cellular and molecular abnormalities, including abnormal ploidy status and *p16* or *p53* gene abnormalities[69,71-74].

The natural history of dysplasia is not well characterized, but the risk of malignancy increases with the development of low- and high-grade dysplasia. The best data have come from Reid *et al*[69], and in a carefully followed group of patients. they reported that low-grade dysplasia progressed to cancer in 4% over 5 years, whereas high-grade dysplasia led to cancer in 61% at 5 years. It is also clear that progression is variable, with some patients progressing at a steady pace over several years, while others have stable non-dysplastic or low-grade dysplasia in Barrett's esophagus for many years, and then rapidly develop high-grade dysplasia and cancer. Theisen *et al*[75] conducted a review of patients who received follow-up through the entire sequence of Barrett's esophagus, low-grade dysplasia, high-grade dysplasia, and adenocarcinoma to better understand the chronology of these events. In a group of 28 patients that presented with adenocarcinoma, a median of 24 mo passed from the initial diagnosis of Barrett's esophagus. Progression from low-grade to high-grade dysplasia occurred over a median of 11 mo. Once high-grade dysplasia was diagnosed, the median time to diagnosis of cancer was 3 mo. Although this timeline was variable for each individual, in the cohort of patients that had progression of Barrett's esophagus to cancer, the process occurred within 3 years. However, because most Barrett's esophagus patients do not progress onto dysplasia and cancer, the cohort in this retrospective study might not be applicable to all patients. Furthermore, because few of these patients had been in long-term Barrett's esophagus surveillance programs, it is not possible to separate prevalent from incident cancers in this group, and the actual month and year that Barrett's esophagus developed in each patient is also unknown. Thus, information on progression of Barrett's esophagus is largely anecdotal.

IMPACT OF ANTIREFLUX THERAPY ON THE NATURAL HISTORY OF BARRETT'S ESOPHAGUS

Medical therapy of Barrett's esophagus

There are three goals for treating patients with Barrett's esophagus: (1) stop reflux; (2) promote or induce healing or regression of the metaplastic epithelium such that the high-risk mucosa (intestinal metaplasia) is eliminated; and (3) halt progression to dysplasia and cancer. Most patients with Barrett's esophagus are treated medically; however, adequate medical therapy is difficult because of the degree of impairment of the lower esophageal sphincter and the poor esophageal body motility that are frequently present. This is likely to be the reason why the least controlled symptom in patients with Barrett's esophagus receiving medical treatment is regurgitation[76]. Medical treatment options are limited to dietary and lifestyle modifications, promotility agents, and acid-suppression therapy. Sampliner and the Practice Parameters Committee of the American College of Gastroenterology have stated that "the goal of therapy of Barrett's esophagus should be the control of the symptoms of GERD", and that "symptom relief is an appropriate endpoint for the therapy of Barrett's esophagus"[77]. However, this viewpoint flies in the face of logic. Gastroesophageal reflux causes both Barrett's esophagus and esophageal cancer. Symptoms are not part of the pathophysiology of the disease. Rather, they are merely the variably expressed byproduct of reflux. Many patients with Barrett's esophagus have few or no reflux symptoms; probably as a consequence of an altered sensitivity of the metaplastic epithelium to refluxed acid. Consequently, the eradication of symptoms, if present, cannot be equated with elimination of reflux. Katzka and Castell[78] have demonstrated that standard-dose omeprazole (20 mg/d) failed to suppress acid sufficiently to keep gastric pH neutral for a full 24 h in patients with Barrett's esophagus. Furthermore, increasing the dose of the omeprazole until all symptoms were alleviated was an unreliable measure of effective therapy, since 80% of patients studied with 24-h pH still had abnormal distal esophageal acid exposure[78]. Sampliner likewise found that high-dose proton pump inhibitor administration (lansoprazole, 60 mg/d) failed to normalize the 24-h pH test in over a third of patients with Barrett's esophagus who were tested while on therapy[76]. Even if complete suppression of acid could be achieved 24 h/d, 7 d/wk, for 350 d/year, impedance studies have shown that the number of reflux events is unchanged. Acid reflux events are merely converted to non- or weak acid reflux events, because the physiological abnormalities that lead to reflux are unaddressed by medical acid suppression therapy[79,80]. The role of continued weak or non-acid reflux in the progression of Barrett's esophagus is undefined, but it may explain the paucity of evidence that acid suppression therapy alters the natural history of Barrett's esophagus.

The second and third goals of therapy in patients with Barrett's esophagus are to eliminate the high-risk mucosa,

i.e. intestinal metaplasia, and prevent progression to dysplasia and cancer. Medical therapy has not been shown to achieve either of these goals reliably. Several reports have concluded that medical therapy does not cause regression of intestinal metaplasia[81-83]. This might be different in patients with short-segment Barrett's esophagus. Weston *et al*[84] have described the loss of goblet cells from lengths of intestinal metaplasia < 2 cm in 32% of patients treated medically for 1-3 years. In contrast, only two of 29 patients (7%) with lengths of intestinal metaplasia ⩾ 3 cm had loss of goblet cells.

With respect to the efficacy of medical therapy in preventing progression of Barrett's esophagus to dysplasia and cancer, there is speculation that prolonged, and perhaps inadequate acid suppression might actually promote the development of Barrett's esophagus and its complications[32]. Lagergren *et al*[85] have recently reported that the risk of esophageal adenocarcinoma was increased nearly eightfold among persons in whom heartburn, regurgitation, or both occurred at least once weekly compared to persons without these symptoms. They noted that the risk of esophageal adenocarcinoma was three times higher among patients who used medication for symptoms of reflux compared to those who did not use any antireflux medication[85]. Others, including Ortiz *et al*[82] and Hameeteman *et al*[86] have also linked medical therapy for Barrett's esophagus with progression to dysplasia and adenocarcinoma. In the study by Hameeteman *et al*[86] from the Netherlands, 50 patients with a columnar-lined esophagus were treated medically and followed from 1.5 to 14 years (mean 5.2 years). Of these 50 patients, initially only 34 had intestinal metaplasia on biopsy of the columnar mucosa. At completion of the study, 37 patients had intestinal metaplasia, which indicated that three patients developed Barrett's esophagus during the 5-year study period. In addition, at the start of the study, six patients had low-grade dysplasia and one had high-grade dysplasia. By the end of the 5-year study, 10 patients had low-grade dysplasia, three had high-grade dysplasia, and five had adenocarcinoma[86]. Similarly, Sharma *et al*[87] followed 32 medically treated patients with short segment Barrett's esophagus (mean length: 1.5 cm) for a mean of 36.9 mo, and found a 5.7% annual incidence of progression to dysplasia. During the 98 patient-years of follow-up in their series, two patients developed high-grade dysplasia, and one of these patients progressed to cancer. Recall that the expected rate of cancer is 1 per 100 patient-years of follow-up. All patients in the study by Sharma and colleagues were treated with omeprazole, ranitidine, and/or promotility agents. They commented that most patients developed dysplasia while on acid suppression medication, and they concluded that medical treatment does not prevent the development of dysplasia. A recent retrospective observational study in patients with Barrett's esophagus suggested that proton pump inhibitor use was associated with a reduced incidence of high-grade dysplasia or adenocarcinoma compared to patients not taking such medication, but there was no difference in the incidence of dysplasia between groups[88].

Antireflux surgery for Barrett's esophagus

In contrast to the ongoing weak or non-acid reflux that occurs with acid suppression therapy, antireflux surgery restores lower esophageal sphincter function and abolishes reflux of gastric contents into the esophagus. Consequently, an antireflux operation ends the repetitive injury to both the metaplastic and normal esophageal mucosa. Randomized clinical studies have confirmed superior control of reflux following antireflux surgery compared to medical therapy, and antireflux surgery has been proven safe, effective, and durable[82,89]. In addition, many patients are candidates for a minimally invasive laparoscopic approach associated with a short hospital stay and rapid recovery. We therefore favor the performance of an antireflux procedure in patients with Barrett's esophagus.

There have been conflicting reports about whether intestinal metaplasia regresses following antireflux surgery. Brand, in 1980, described complete regression in four of 10 patients with Barrett's esophagus who underwent fundoplication[90]. Subsequently, most reports have demonstrated that while some regression of the length of Barrett's esophagus is common, complete regression occurs only rarely, particularly with long-segment disease. In contrast, intestinal metaplasia of the cardia and short segments of Barrett's esophagus much more commonly regress to no intestinal metaplasia after fundoplication[91-93]. Furthermore, during prospective follow-up of patients with a columnar-lined esophagus without intestinal metaplasia treated either medically or with antireflux surgery, Oberg *et al*[94] showed that significantly fewer patients developed intestinal metaplasia after antireflux surgery.

Perhaps of greater importance is the issue of progression of Barrett's esophagus to dysplasia or cancer after surgical treatment of reflux disease. Compared to medical therapy, antireflux surgery is associated with a reduced incidence of dysplasia and adenocarcinoma. McCallum *et al*[95] have prospectively followed 181 patients with Barrett's esophagus. Twenty-nine had antireflux surgery while the remaining 152 patients were treated medically. After a mean follow-up of 62 mo in the surgical group and 49 mo in the medical group, there was a significant difference in the incidence of dysplasia and adenocarcinoma. Dysplasia was found in 3.4% of the surgical group compared with 19.7% in the medically treated group. No patient in the surgically treated group developed adenocarcinoma of the esophagus compared with two medically treated patients. They concluded that compared with medical therapy, an antireflux operation in patients with Barrett's esophagus was significantly associated with the prevention of dysplasia and cancer. Similarly, Katz *et al*[96] have followed 102 patients with Barrett's esophagus for a mean of 4.8 years. By 3 years, approximately 8% of the medically treated patients had developed dysplasia. In contrast, patients treated by antireflux surgery had a significantly reduced risk of developing dysplasia ($P = 0.03$)[96]. In the only randomized controlled trial that has compared medical therapy with antireflux surgery for Barrett's esophagus, Parrilla *et al*[97] showed that patients with functioning fundoplication had a significantly reduced incidence of developing dysplasia

compared to patients on medical therapy. Evidence at the molecular level has shown that antireflux surgery reduces the expression of genes potentially involved in the progression of Barrett's esophagus to cancer down to the level of control subjects without reflux[98,99]. These studies provide an insight into how antireflux surgery might be protective against progression of Barrett's esophagus to cancer.

Opposing these studies are two Swedish database studies that have suggested that antireflux surgery does not protect against progression to cancer. However, the serious flaw in both these studies is that the prevalence of Barrett's esophagus was not known in either population, and it is quite likely that far more patients in the antireflux surgery group had Barrett's esophagus than the comparison groups[100,101]. The presence of Barrett's esophagus is the leading known risk factor for subsequent development of esophageal adenocarcinoma, therefore, both studies only add to the controversy rather than provide any reliable answer to this important issue. Another factor that complicates any analysis of progression of Barrett's esophagus after antireflux surgery is that the cellular and genetic alterations that lead to the development of dysplasia and adenocarcinoma might have already occurred before the antireflux procedure. It has been estimated to take up to 6 years for adenocarcinoma to develop within Barrett's esophagus with low-grade dysplasia, and thus some cancers, particularly those that present during the first few postoperative years, probably do not represent progression of disease after surgery. McDonald *et al*[102] have made this point in a study from the Mayo Clinic. They found invasive adenocarcinoma in two patients and carcinoma *in situ* in one patient during surveillance after antireflux surgery, but they noted that no patient developed carcinoma after 39 mo, despite a median follow-up of 6.5 years, and a maximum follow-up of 18.2 years.

CONCLUSION

There is increasing evidence that at the normal gastroesophageal junction, esophageal squamous mucosa abuts oxyntic fundic mucosa of the stomach. With exposure to gastric juice, the squamous mucosa is injured, and over time becomes replaced by columnar cardiac mucosa. Deterioration of the lower esophageal sphincter allows reflux to extend up into the esophagus, and the squamocolumnar junction migrates proximally. Although it is likely that acidic gastric juice drives the transformation of squamous mucosa to cardiac mucosa, there is substantial evidence that other components of gastric juice, particularly bilirubin, are essential for subsequent intestinalization of the cardiac mucosa.

Barrett's esophagus is a premalignant mucosa, and the risk of malignant transformation is approximately 0.5% per patient-year. The finding of dysplasia is currently the most commonly used indicator of increased malignant risk, but it has high inter-observer variability. It is expected that ultimately molecular markers will prove more helpful than histology in Barrett's esophagus, and there are ongoing efforts to determine biomarkers that will better delineate an individual's risk for progression to cancer. Surveillance endoscopy in patients with Barrett's esophagus has proven efficacy, but is time-consuming and haphazardly applied. Currently, screening endoscopy is not recommended for Barrett's esophagus, but given the dramatic increase in the incidence of esophageal adenocarcinoma, new technologies that permit widespread and cost-effective screening are needed. Patients with Barrett's esophagus are commonly treated with acid-suppressive medication, but there are few data that this therapy alters the natural history of the disease, and thus current medical guidelines are to treat for symptomatic relief rather than for documented pH control. Antireflux surgery abolishes reflux and has been shown to normalize gene expression in patients with Barrett's esophagus, but controversy persists regarding the impact of an antireflux procedure on the risk of Barrett's esophagus progression.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3773

World J Gastroenterol 2010 August 14; 16(30): 3773-3779
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Effect of medical and surgical treatment of Barrett's metaplasia

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

Barrett's esophagus (BE) is a change in the esophageal mucosa as a result of long-standing gastroesophageal reflux disease. The importance of BE is that it is the main risk factor for the development of esophageal adenocarcinoma, whose incidence is currently growing faster than any other cancer in the Western world. The aim of this review was to compare the common treatment modalities of BE, with the focus on proton pump inhibitors and operative fundoplication. We performed a literature search on medical and surgical treatment of BE to determine eligible studies for this review. Studies on medical and surgical treatment of BE are discussed with regard to treatment effect on progression and regression of disease. Although there is some evidence for control of reflux with either medical or surgical therapy, there is no definitive evidence that either treatment modality decreases the risk of progression to dysplasia or cancer. Even though there is a trend toward antireflux surgery being superior, there are no definitive studies to prove this.

Key words: Barrett's esophagus; Intestinal metaplasia; Proton pump inhibitors; Nissen fundoplication; Esophageal adenocarcinoma; Gastroesophageal reflux

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Wassenaar EB, Oelschlager BK. Effect of medical and surgical treatment of Barrett's metaplasia. *World J Gastroenterol* 2010; 16(30): 3773-3779 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3773.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3773

INTRODUCTION

Barrett's esophagus (BE) is defined as a change of any length in the distal esophageal epithelium, which can be recognized as columnar-type mucosa at endoscopy and confirmed as intestinal metaplasia (IM) by biopsy of the tubular esophagus[1]. BE is a complication of gastroesophageal reflux disease (GERD) through damage of the esophageal mucosa from refluxed contents[2,3]. It is thought to be present in around 10% of patients with GERD[3,4], although the exact incidence is unknown. As a result of the substantial increase of esophageal adenocarcinoma (AC) in patients with BE, it is considered the major risk factor for this form of cancer. In fact, over the past decade there has been acceleration in the incidence of AC in the Western world, presumably from a rise in GERD, its treatment, or other environmental factors. In the United States, it is estimated that 1.5-2 million people have BE[5].

It has been estimated that the risk for developing esophageal AC when IM is present is approximately 0.5% per year[6]. Although the factors that affect progression are not completely known, it is tempting to assume that the risk is increased by continued exposure of the IM to gastric contents[7].

Screening for BE in patients with chronic heartburn is not widely considered to be cost-effective, but surveillance in patients with BE is generally advised[8]. This, however, puts a heavy burden on resources for endoscopists. To prevent the development of esophageal cancer and to try and reduce the need for surveillance, the available treatment modalities for BE have been evaluated. The goal for treating patients with BE is generally directed at controlling associated symptoms of GERD, because quelling symptoms is a much more immediate endpoint for adjusting or changing therapy. Nevertheless, in this review, we discuss the possible treatment options for BE, with a focus on their effect on the Barrett's mucosa itself. The two most common treatments of GERD and associated BE are medical (proton pump inhibitors, PPIs) and surgery (fundoplication). Recently, more attention has been paid to other possible (medical) treatment options of BE that are not specifically aimed at reducing reflux. We briefly cover these treatment options as well.

LITERATURE SEARCH

A PubMed search was performed to identify publications using the following MeSH terms: "Barrett esophagus" and "proton pump inhibitors" or "surgical procedures, operative". Publications had to be published in the English language in peer-reviewed journals. Only studies published from 2000 onward with endoscopic biopsy results after treatment were deemed eligible. If publications were from the same research group, the most recent or most applicable study was chosen.

The abstracts of the results were read to determine eligibility for this review. If deemed eligible, full-text versions of the studies were acquired. From these full-text articles, references were checked to find publications that were missed using the search with MeSH terms[9-12]. Twenty studies were found to be eligible for this review. Five were on medical treatment (PPIs), 11 were on surgical treatment and four compared the two treatments.

DEFINITIONS

Progression of BE in this review is defined as a change in histological findings on biopsy from either IM to any form of dysplasia or an increase in grade of dysplasia. Development of AC is also considered progression of disease. Regression is defined as change from high-grade dysplasia (HGD) to low-grade dysplasia (LGD) or no dysplasia, change from LGD to metaplasia or loss of metaplasia, and change from IM to complete loss of metaplasia. Shortening of the segment or development of squamous cell islands, although considered by some as regression, usually is not accurately measured and reported, and is therefore, not considered regression in our report. Short-segment BE (SSBE) is defined as a length $\leqslant$ 3 cm seen at endoscopy and confirmed by biopsy. Long-segment BE (LSBE) is defined as > 3 cm.

LIMITING PROGRESSION

Ultimately, the goal of treatment for BE is to prevent cancer. Both medical and surgical treatment studies therefore have traditionally been focused on showing results of preventing progression of disease. We first discuss the results for PPI treatment, then those of operative treatment using fundoplication, and finally, studies that have compared these two treatment modalities.

Medical treatment

Three recent studies have investigated the effect of PPI treatment on the risk of progression of BE to dysplasia or AC[9,13,14]. The results of studies of PPI treatment with regard to progression and regression of disease are shown in Table 1. The results of these studies suggest a protective effect of PPIs in limiting the progression of BE.

In the study by Hillman *et al*[13], (350 patients with BE over a 20-year period), patients were stratified according to delay in starting PPI therapy after the diagnosis of BE was established. Patients who delayed PPI therapy for $\geqslant$ 2 years after being diagnosed with BE had 5.6 times higher risk of developing LGD than patients who used PPI within the first year after diagnosis. Furthermore, patients with BE had up to a 20 times higher risk of developing HGD or AC when PPI therapy was delayed for 2 years after diagnosis of BE. Although this suggests a substantial protective effect, the absolute risk of developing HGD or AC was low (n = 11; 3%) at a median follow-up of 4.7 years.

The small rate of progression of BE makes it very difficult to show a difference between treatments. In another study, the risk of developing LGD within 5 years of the diagnosis of BE was around 2.5%, and the risk of HGD/AC was around 2% while taking PPI therapy. Cooper *et al*[9] have shown this in a study of 188 patients with IM who were treated with a PPI. However, when following patients for > 5 years, Nguyen *et al*[14] recently have found a much higher risk of developing AC. They have studied 344 patients diagnosed with BE without dysplasia, with a mean follow-up of 7.6 years. They found that the chance of developing HGD or AC was 7.4%. Moreover, this risk was even higher when not taking PPIs (14.2%). Taken together, the results of these non- controlled studies suggest that PPIs have a protective effect, but they do not eliminate the risk of developing AC.

Surgical treatment

Surgical treatment of BE most often involves fundoplication for GERD. Where PPIs are only able to decrease acid content in the stomach (and thus change the pH of the refluxate), surgery has the ability to prevent any type of reflux. Therefore, many have argued that surgery is a more effective therapy for BE. All 11 publications on surgical treatment for BE that met our screening criteria included results on prevention of progression, as well as regression of metaplasia or dysplasia[15-25]. In this section, we discuss only the results of the effect of fundoplication on the rate

Table 1 Medical therapy and surgery for limiting progression and causing regression of Barrett's esophagus *n* (%)

Publication	No. of patients	Follow-up (yr)	Adenocarcinoma	Dysplasia	Regression
Medical therapy					
Hillman *et al*[13], 2004	279	4.7	7 (2.5)	5 (1.8)	NA
Cooper *et al*[9], 2006	188	5.1	3 (1.6)	6 (3.2)	NA
Nguyen *et al*[14], 2009	231	7.6	17 (7.4)	53 (23)	NA
Heath *et al*[10], 2007	82	0.9	6 (7.3)	9 (11)	34 (41)
Horwhat *et al*[11], 2007	67	3.8	2 (3.0)	21 (31)	13 (19)
Total	847	4.4	35 (4.1)	94 (11.1)	47 (31.5)
Surgery					
Hofstetter *et al*[15], 2001	79	5.0	0	4 (5)	16 (20)
Bowers *et al*[16], 2002	64	4.6	0	1 (2)	31 (48)
Mabrut *et al*[17], 2003	13	3.8	0	0	6 (46)
Oelschlager *et al*[18], 2003	90	2.6	1 (1)	3 (3)	30 (33)
Desai *et al*[19], 2003	50	3.1	0	1 (2)	9 (18)
O'Riordan *et al*[20], 2004	57	3.8	2 (4)	2 (4)	14 (25)
Abbas *et al*[21], 2004	33	1.5	1 (3)	2 (6)	13 (39)
Zaninotto *et al*[22], 2005	35	2.3	0	0	6 (17)
Ozmen *et al*[23], 2006	37	1.6	0	1 (3)	6 (16)
Biertho *et al*[24], 2007	70	4.2	0	3 (4)	23 (33)
Biertho *et al*[25], 2009	23	4.5	0	0	14 (61)
Total	551	3.4	4 (0.7)	17 (3.4)	168 (30.5)

NA: Not applicable.

of progression. The results of studies on surgical treatment for limiting progression and causing regression are summarized in Table 1.

In the reported case series, the number of patients is relatively low since a minority of patients is referred for surgery. As a result, because progression can take a long time and is still a relatively rare event (especially on medical therapy), large studies with several hundred patients would be needed to show a clinically significant benefit. Still, it is interesting to look at several trends, and as can be seen in Table 1, almost uniformly there is a low incidence of progression to dysplasia and even a lower incidence to AC.

Hofstetter *et al*[15] have published the study with the longest follow-up. They showed results for a series of 97 patients, with complete endoscopic follow-up in 79, at a median of 5 years. No patients developed HGD or AC, but four had progression of metaplasia to LGD (5%). Bowers *et al*[16], have reported a similar series with a mean follow-up of 4.6 years. Their 104 patients underwent open or laparoscopic fundoplication. Of these, 64 patients had endoscopic follow-up with biopsy. None of the patients developed HGD or AC. Only one patient had progression to LGD (1.5%).

Control of reflux

The hypothesis that surgery is superior to medical therapy comes from the assumption that surgery provides better control of GERD than do PPIs, and this should translate into lower progression rates. Indeed, there is some circumstantial evidence for this. Lagergren *et al*[26] and Csendes *et al*[27] have suggested that, when esophageal AC occurs after antireflux surgery, it is usually in the face of persistent or recurrent reflux. This observation, that control of reflux is essential in preventing progression of disease, is backed up by the fact that, in most studies, the patients with progression after surgical treatment seem to have recurrent reflux. In a series of 58 patients by O'Riordan *et al*[20] who underwent open or laparoscopic Rossetti-Nissen fundoplication, four were found to have progression of disease after a follow-up of 45 mo. All four patients were found to have abnormal postoperative acid scores[20]. In another study, Biertho *et al*[24] have published the results of 70 patients with BE who had endoscopic follow-up for 4.2 years after laparoscopic fundoplication. Three patients had progression of disease, but none developed HGD or AC. All three patients with progression had recurrence of GERD symptoms. We published our results of 106 patients with BE who underwent laparoscopic fundoplication[18]. Endoscopic follow-up with biopsies was performed in 90 patients with a median follow-up of 30 mo. One patient was found to have developed AC at 10 mo after the operation (and thus likely had at least dysplasia at the time of operation). One patient developed HGD and one LGD. The patient with HGD had LGD preoperatively and for 3 years thereafter, and then developed recurrent GERD symptoms with an abnormal 24-h pH. One year later this patient was found to have developed HGD despite being on medical therapy. Still, despite the fact that surgery is not perfect, the rate of progression to HGD or AC seems around 1.5%, which is lower than that typically seen in medical treatment.

One of the difficulties in evaluating the results of these treatments is the overall low incidence of patients with BE progressing to AC. Although decreasing the total burden of BE might actually decrease the risk of cancer, it is difficult to track. The results of the studies suggest that surveillance after medical treatment is necessary. After surgical treatment, there is also still progression of

Table 2 Medical therapy *vs* surgery for Barrett's esophagus *n* (%)

Publication	Treatments	PPI	Nissen	Progression PPI	Progression Nissen	Regression PPI	Regression Nissen	Study type
Gatenby *et al*[6], 2009	PPI *vs* Nissen	646	41	154 (24)	4 (10)	NA	NA	Cohort
Parrilla *et al*[28], 2003	H2RA/PPI *vs* Nissen	43	58	10 (23)	5 (9)	2 (5)	5 (9)	RCT
Rossi *et al*[29], 2006	PPI *vs* successful Nissen	19	16	NA	NA	12 (63)	16 (100)	Case comparison
Total		708	115	164 (23.8)	9 (9.1)	14 (22.6)	21 (28.4)	

PPI: Proton pump inhibitor; H2RA: H2 receptor antagonist; RCT: Randomized controlled trial; NA: Not applicable.

disease (particularly in patients with LSBE), although the risk seems to become very small when this treatment is successful. Patients are generally reluctant to have surveillance, as shown by the low number of patients who actually have endoscopy after fundoplication. Another difficulty in interpreting the results is the follow-up of these studies that ranges from 0.9 to 7.6 years. With a disease that, in general, progresses only slowly, studies with follow-up of 10-20 years are needed. In contrast, studies on surgical treatment with the longest follow-up have still shown very low incidence of progression. The study on medical treatment with the longest follow-up did show a higher chance of progression of disease[14], although that study was possibly confounded by selection bias.

Medical vs surgical treatment

There have been very few studies comparing medical and surgical therapy; in fact, in our review, we only found two studies on progression of disease worthy of comment. The results of these are summarized in Table 2.

In one, Gatenby *et al*[6] published the results of their review of a cohort of 738 patients with BE enrolled in a national registry. They compared patients with anti-reflux surgery (n = 41) to those treated medically with PPIs (n = 551), H2 receptor antagonists (H2RAs) (n = 42), H2RA followed by PPI (n = 95), or no treatment (n = 9). Their outcome parameters were progression of disease to LGD, HGD or AC. They could not control for many other selection factors, which might have confounded the results, such as severity of disease. After a follow-up of 5 years after medical therapy and 6 years after surgical therapy, there was however a trend toward antireflux surgery being more protective. No patients in the antireflux group developed HGD or AC as compared to 4.3% in the all-medical therapies group (P = 0.13). There were not enough patients in the surgical arm to determine if this was a significant difference.

Parrilla *et al*[28] have published the only randomized study comparing medical treatment (n = 43) and antireflux surgery (n = 58). In that study, 101 patients with BE were treated between 1982 and 2000. Medical treatment consisted of H2RA treatment initially and then omeprazole from 1992 onward. Surgery was performed through laparotomy with Nissen fundoplication in 56 patients and a Collis-Nissen procedure in the other two because of short esophagus.

All patients had annual clinical, endoscopic and histological follow-up, and patients who had an operation also had a pH study and manometry at 1 year postoperatively and every 5 years thereafter, or if they presented with recurrent GERD symptoms. Mean follow-up was 6 years for the medical therapy group and 7 years for the surgical group. Progression of BE to any dysplasia was found in eight patients (19%) in the medical treatment group and in three in the surgical group (5%). Although the P value was not specified in their paper, according to our calculations using Fisher exact test, there was a protective effect of fundoplication (P = 0.05). Two patients in each group progressed to AC, which was confirmed after esophageal resection. Although differences in progression rates between the two groups were not significant according to the authors, when a sub-analysis was performed including only patients in the surgical arm with normal pH, the progression rate dropped to 2%, which was a significantly lower chance of progression of disease than in the medical group (P < 0.05).

CAUSING REGRESSION

IM without dysplasia is a benign condition, therefore, inducing regression is not considered as important as limiting progression. Nevertheless, if IM is no longer present, then it theoretically can no longer progress to cancer, thus it has been reported as a surrogate for measuring the response of various therapies. Disappearance of IM seems to be a slightly more common occurrence after effective treatment of GERD and therefore is a more easily studied endpoint.

Medical treatment

The only two studies that we found that have published results of regression of BE following medical treatment are by Heath *et al*[10] and Horwhat *et al*[11]. The results of these studies are shown in Table 1, together with the studies on progression of disease.

The purpose of the study by Heath *et al*[10] was to investigate the effect of long-term celecoxib in patients with BE with dysplasia. The mechanism for chemoprevention of celecoxib is thought to be through inhibition of cyclooxygenase (COX)[30]. They randomized 100 patients with low or high-grade Barrett's dysplasia to treatment with either celecoxib (n = 49) or placebo (n = 51). Although this study did not focus on PPI therapy, > 90% of these patients were concomitantly on a PPI. After 48 wk of treatment, endoscopic biopsy results showed a regression of dysplasia in 41.9% of patients on celecoxib and 41%

on placebo (P = 0.89), either from LGD to no dysplasia or from HGD to LGD (although differentiation between those events in this study was not possible). In contrast, 14% (n = 6) and 15.4% (n = 6) respectively had an increase in highest grade of pathology, with three patients in each group developing AC. These mixed results might say more about the variability in interobserver reliability of dysplasia, as has been reported[31]. However, the results do suggest that patients with dysplasia can regress with medical therapy alone.

Horwhat *et al*[11] looked at LSBE and SSBE. They contacted 101 patients after a mean follow-up of 46 mo. Most patients received PPI therapy but seven underwent fundoplication. Of the 38 patients with LSBE, 23 underwent endoscopy. Six patients developed dysplasia (26%) and two cancer (9%). No patient with LSBE had regression of disease. Of the 63 patients in the SSBE group, 44 underwent endoscopy. Three patients were found to have progression of disease (7%) *vs* 13 with regression (30%). They found an almost linear relationship between BE segment length and normalization of the epithelium, that is, the chance of progression of disease is significantly higher in LSBE compared with SSBE. Unfortunately, it is unclear in this study whether the patients with regression or progression had medical or surgical treatment.

Surgical treatment

The results of regression of BE with surgical treatment are shown in Table 1, together with the results of progression. The literature suggests that regression of BE occurs with some regularity after fundoplication, even regression to completely normal squamous epithelium. Hofstetter *et al*[15] have reported that 16 of their 79 patients (20%) had regression of disease in some fashion. Of the 16 patients with LGD, seven had regression (44%), and of the 63 patients with IM, nine had complete loss of metaplasia (14%).

It is important to consider that LGD is sometimes over-reported because of inflammation from ongoing GERD, and surgery could make it easier for the pathologist to interpret the biopsies. Nevertheless, other studies have suggested regression in a substantial number of BE patients. Desai *et al*[19] have found a loss of metaplasia in seven of 50 patients (14%) postoperatively. Two out of the three patients with LGD had regression to non-dysplastic BE. In the study by Bowers *et al*[16], it has been found that 31 of 66 patients had loss of IM (47%) after antireflux surgery. Patients with regression had shorter lengths of BE preoperatively and longer follow-up after the operation.

That patients with SSBE have a higher incidence of regression than those with LSBE seems logical, and it has been consistently seen in studies where long and short-segment BE has been distinguished. In the study by O'Riordan *et al*[20], eight of 57 patients (14%) were found to have complete regression. Six of these patients had SSBE preoperatively. They have also found regression from LGD to non-dysplastic BE in six of eight patients. Biertho *et al*[24] have reported that complete regression was found in 23 of their 70 patients (33%). All patients with regression had SSBE preoperatively. Regression from LGD to non-dysplastic BE occurred in two of three patients.

Our experience mirrors that of other authors who have found that complete regression occurs only in patients with SSBE. Of the 54 patients with SSBE before surgery, 30 (54%) had no evidence of IM at last follow-up. In contrast, none of the 38 patients with LSBE before surgery had complete regression[18]. These observations suggest that the chance of accomplishing regression is especially high in patients with earlier disease. Therefore, earlier referral for surgery might increase the chance of cure from BE even further.

Medical vs surgical treatment

Only one small study comparing medical and surgical treatment directly has been published that focuses on regression of BE. The results are summarized in Table 2.

Rossi *et al*[29] prospectively studied 19 patients with high-dose PPI and 16 patients with fundoplication. All patients had LGD. After 18 mo follow-up, a high percentage of patients were found to have regressed to IM after medical (63%) as well as surgical treatment (100%). Although the rate was higher in the surgical group, the small numbers make it difficult to use the study to draw any definitive conclusions. Parrilla *et al*[28] also have reported data on regression of disease in their randomized study, although they do not comment on this, with 2/43 (4.6%) having regression from LGD to IM with medical therapy, and 5/58 (8.6%) after surgical therapy ($P > 0.05$).

When comparing both treatment modalities, antireflux surgery seems to be more successful in prevention of progression and in promoting regression than medical treatment with PPI. The number of patients studied and the quality of the studies however were low, therefore, a firm conclusion cannot be drawn. Complications from the operation are also not taken into account and these studies generally come from surgical centers of excellence. On the other hand, the patients that underwent an operation are more likely to have had more severe disease than the patients that are treated medically.

OTHER MEDICAL TREATMENT

Almost all patients with BE, because of their associated GERD, are treated with PPIs (unless they have surgery), therefore, it makes sense to evaluate the effect of acid reduction on the natural history of BE. However, there have been other medical therapies investigated for the purpose of addressing IM primarily. For example, Vaughan *et al*[32] have shown a potential role for nonsteroidal anti-inflammatory drugs (NSAIDs). The effect of NSAIDs is thought to be through their anti-inflammatory effect through inhibition of COX-2 production[33]. Ogunwobi *et al*[34] have made a theoretical argument for statins, stating that they might affect proliferation and apoptosis in esophageal cancer cells. The protective effect of these medications is further supported by a

recent study by Nguyen *et al*[35]. In this retrospective observational study using pharmacy data, they have shown a reduced risk of developing AC in patients with BE and filled NSAID prescriptions. They have also studied statins as chemopreventive medications, however, they are concerned about confounding with statin therapy because patients had short periods of use, therefore, conclusions cannot be drawn about these medications.

Other publications contradict the role of NSAIDs in preventing progression. One is the study by Heath *et al*[10] that was discussed earlier, which did not find a difference when comparing patients on or off celecoxib. Gatenby *et al*[36] have published results of a national registry in the United Kingdom of BE, where they did not find a difference in development of dysplasia or AC between patients on or off aspirin. To evaluate further the effect of aspirin treatment of BE on progression to cancer, a large randomized trial (AsPECT) is ongoing, which is comparing patients on PPI therapy with and without aspirin[37].

Many other medications, such as ursodeoxycholic acid, hormone replacement therapy and n-3 fatty acids have been studied[38-41], but all have too little information to recommend their use currently. Dietary interventions through antioxidants, fiber and vitamins have been studied for their effect on risk of cancer in general and for prevention of esophageal AC. However, mixed results have been reported[42].

Very few clinical studies have been carried out on treatment modalities other than antireflux surgery using fundoplication, or medical treatment using PPIs. Therefore more (large) studies are necessary before any firm conclusions can be drawn on the chemopreventive qualities of agents such as aspirin, selective COX inhibitors or diet modifications.

CONCLUSION

Consensus on the best treatment for BE remains elusive, because there has not been a large definitive study to date that has compared PPIs and fundoplication (nor is there likely to be one). There is, however, a trend toward lower risk of progression with anti-reflux surgery compared with anti-acid medication, especially when anti-reflux surgery is successful. In addition, there seems to be a greater chance of regression of disease with anti-reflux treatment, but the importance of this regression is unclear. Theoretically, surgery controls gastroesophageal reflux better than PPIs do (which mostly reduces the acid component), therefore, it is appealing for some to consider this a real difference, and therefore, recommend surgery for patients with BE, even though it is not definitively proven. As a result, treatment of BE has to be given based on the patient's preference and control of GERD symptoms. Just like GERD without IM, those with IM should consider fundoplication if symptomatic, despite appropriate medical therapy. The effect of fundoplication on the natural history of the epithelium should be a secondary concern. Whichever treatment is pursued, surveillance remains important, because the risk of cancer is not eliminated despite the decrease in risk through both PPIs and surgery.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3780

World J Gastroenterol 2010 August 14; 16(30): 3780-3785
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Endoscopic treatment of Barrett's esophagus: From metaplasia to intramucosal carcinoma

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

The annual incidence of adenocarcinoma arising from Barrett's esophagus (BE) is approximately 0.5%. Through a process of gradual transformation from low-grade dysplasia to high-grade dysplasia (HGD), adenocarcinoma can develop in the setting of BE. The clinical importance of appropriate identification and treatment of BE in its various stages, from intestinal metaplasia to intramucosal carcinoma (IMC) hinges on the dramatically different prognostic status between early neoplasia and more advanced stages. Once a patient has symptoms of adenocarcinoma, there is usually locally advanced disease with an approximate 5-year survival rate of about 20%. Esophagectomy has been the gold standard treatment for BE with HGD, due to the suspected risk of harboring occult invasive carcinoma, which was traditionally estimated to be as high as 40%. In recent years, the paradigm of BE early neoplasia management has recently evolved, and endoscopic therapies (endoscopic mucosal resection, radiofrequency ablation, and cryotherapy) have entered the clinical forefront as acceptable non-surgical alternatives for HGD and IMC. The goal of endoscopic therapy for HGD or IMC is to ablate all BE epithelium (both dysplastic and non-dysplastic) due to risk of synchronous/metachronous lesion development in the remaining BE segment.

Key words: Barrett's esophagus; High-grade dysplasia; Intramucosal carcinoma; Esophagectomy; Endoscopic mucosal resection; Radiofrequency ablation; Cryotherapy

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Chennat J, Waxman I. Endoscopic treatment of Barrett's esophagus: From metaplasia to intramucosal carcinoma. *World J Gastroenterol* 2010; 16(30): 3780-3785 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3780.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3780

INTRODUCTION

The annual incidence of adenocarcinoma arising from Barrett's esophagus (BE) is approximately 0.5%[1-3]. Through a process of gradual transformation from low-grade dysplasia (LGD) to high-grade dysplasia (HGD), adenocarcinoma can develop in the setting of BE[4]. The clinical importance of appropriate identification and treatment of BE in its various stages, from intestinal metaplasia (IM) to intramucosal carcinoma (IMC) hinges on the dramatically different prognostic status between early neoplasia and more advanced stages. Once a patient has symptoms from adenocarcinoma, there is usually locally advanced disease with an approximate 5-year survival rate of about 20%[5,6].

Esophagectomy has been the gold standard treatment for BE with HGD, due to the suspected risk of harboring occult invasive carcinoma, which has been

estimated to be as high as 40%[7,8]. Our previous analysis of the published literature demonstrated that the true prevalence of submucosal invasive carcinoma in the setting of HGD was actually 12%, which was much lower than the pooled reported historical rate of 40%[9]. Esophagectomy has also been routinely performed for BE with IMC, despite a low incidence of lymph node metastasis of < 1% that is associated with non-invasive T1a disease[10]. Additionally, esophagectomy is associated with significant morbidity and mortality even in high-volume centers[11,12].

With these issues in mind, the paradigm of BE early neoplasia management has recently evolved, and endoscopic therapies have entered the clinical forefront as acceptable non-surgical alternatives for HGD and IMC. The goal of endoscopic therapy for HGD or IMC is to ablate all BE epithelium (both dysplastic and non-dysplastic) due to risk of synchronous/metachronous lesion development in the remaining BE segment[10]. Endoscopic therapies can be further subdivided into tissue-acquiring and non-tissue-acquiring modalities. Tissue acquisition can be achieved through endoscopic mucosal resection (EMR), while photodynamic therapy (PDT), radiofrequency ablation (RFA), and cryotherapy all ablate tissue without the benefit of histological specimen retrieval. A brief technical review and pertinent available efficacy/safety data are summarized for these various modalities in treating stages of early BE neoplasia that ranges from IM to IMC. Modalities such as argon plasma coagulation, multipolar electrocoagulation, and laser therapies are not be discussed as current mainstay therapies due to high BE relapse rates, infrequent usage, or significant risk of buried gland development[13].

EMR

EMR can be performed through a variety of techniques: free-hand, lift-and-cut, cap-assisted, or band-assisted. Injection of saline with a sclerotherapy needle is performed to create a submucosal fluid cushion, and a snare is used to entrap directly the mucosal tissue in the free-hand method. In the lift-and-cut approach, a dual channel endoscope is used to introduce simultaneously a grasping forceps and snare for resection. The cap technique uses a clear distal attachment with an inner rim around which a crescent-shaped snare is carefully fitted. The target area is injected for submucosal lift, then suction is applied through the cap, and tissue is entrapped by the snare for subsequent mucosal excision. Band-assisted techniques are modifications of the variceal band ligation device that allows for injection and then deployment of bands for mucosal pseudopolyp creation. A snare is then introduced and the mucosa is resected either above or below the band[14].

Focal EMR can be performed for endoscopically visible lesions that are suspicious for malignancy. However, several previously published studies on focal resection have demonstrated a high rate of synchronous and recurrent lesion development, which ranged from 14% to 47%, and increased with longer observation times[15-22]. As a result of this limitation of focal EMR, complete Barrett's eradication EMR (CBE-EMR) has been advocated and performed in select centers, with the intent to remove all BE epithelium curatively, to reduce the potential risk of synchronous or metachronous lesion development. Complete responses have ranged from 76% to 100%. The complication profile of EMR includes stricture formation, with an incidence rate that approaches 50%, bleeding and perforation. Of note, most esophageal stenoses and bleeding are amenable to endoscopic treatment[23-26].

When evaluating the effect of EMR on final histopathological staging, our center long-term results with CBE-EMR have revealed that initial EMR upstaged seven of 49 (14%) and down-staged 15 of 49 (31%) final pathology results when compared to pre-EMR biopsy results. Among the upstaged group, four patients had advanced pathology that was found after index EMR (either submucosal carcinoma or IMC with lymphatic channel invasion). All four of these patients had visible lesions upon endoscopy[26]. This is the crucial point that distinguishes EMR from all other non-tissue-acquiring modalities that would have inadvertently attempted ablation of advanced pathology in the setting of presumed BE HGD treatment.

PDT

The goal of PDT is destruction of tissue through a light-sensitizing reaction sequence. A photosensitizer is first administered which accumulates in esophageal malignant and pre-malignant tissue before light activation therapy. Porfimer sodium is the most common photosensitizer, and this is delivered intravenously 72 h before the procedure. Alternatively, oral 5-aminolevulinic acid (ALA) and intravenous m-tetrahydroxyphenyl chlorine (mTHPC) can be used. Activation of the photosensitizing agent occurs upon exposure to either bare cylinder or balloon-based diffusing light fibers that are placed alongside the target tissue via an endoscopic approach. The resulting molecular excitation reacts with oxygen to create radical oxygen species that cause eventual cell apoptosis[27].

A multicenter trial by Overholt *et al*[28] randomized BE HGD patients to receive twice daily oral omeprazole (20 mg) with or without porfimer sodium PDT administration. The study found that, at 5 years, PDT was significantly more effective than proton pump inhibition (PPI) alone, in elimination of HGD (77% *vs* 39%, $P < 0.0001$). Prevention of cancer progression was a secondary outcome that also showed a significant difference, with the PDT/PPI group demonstrating half the likelihood of developing cancer and longer time to cancer progression.

Overholt *et al*[29] have conducted another porfimer PDT study of 103 patients with LGD, HGD, or IMC with a mean follow-up of 50.65 mo (SD 20.57) (range: 2-122 mo). Intention to treat success rates were 92.9%,

77.5%, and 44.4% for the respective LGD, HGD, and IMC groups. Three patients (4.6%) developed sub-squamous adenocarcinoma. Strictures occurred in 18% with one session of PDT, 50% with two treatments, and 30% in the overall group.

ALA PDT has shown 97% and 100% complete response rates for treatment of BE with HGD and IMC, respectively, in a median follow-up period of 37 mo (interquartile range: 23-55 mo). Disease-free survival of HGD patients was 89%, and 68% in patients with IMC. The calculated 5-year survival was 97% for HGD and 80% for IMC, but no deaths were related to Barrett's neoplasia[30].

In a pilot study of PDT using mTHPC for seven patients with HGD and 12 patients with IMC, Lovat and colleagues found that treatment results were variable based on red versus green light usage. Successful ablation was achieved in four out of six mucosal carcinoma and three out of four HGD patients who received red light. However green light exposure failed to achieve successful disease eradication or long-term remission[31]. Significant complications such as death occurred after premature biopsy performance after treatment. This limited sample size study demonstrated that although mTHPC can destroy BE epithelium, the optimal light and drug dosimetry are still unknown[31].

To date, no randomized, controlled prospective trials have been conducted to compare PDT and surgery for BE neoplasia management. However, a retrospective data analysis of HGD patients who received PDT (n = 129) or esophagectomy (n = 70) has revealed no statistically significant differences in mortality or long-term survival based on choice of treatment modality[32].

The major side effects of PDT include photosensitivity that requires patients to avoid post-procedure skin sunlight exposure, non-cardiac chest pain, and symptomatic stricture formation. Risk factors for post-PDT stricture development include history of prior esophageal stricture, performance of EMR before PDT, and more than one PDT treatment in a single session[33]. Another concern about PDT is development of sub-squamous BE glands that could harbor neoplastic potential. The clinical significance of this finding is still not fully understood. However, reports of adenocarcinoma arising from sub-squamous BE glands after PDT therapy have been described[29,34]. For these reasons, PDT usage has gone out of favor in recent years, with the availability of other endoscopic ablative options.

RFA

Using a either a balloon-based catheter or a focal device, RFA of BE tissue can be achieved in either a circumferential or localized fashion. After initial insertion of a sizing balloon into the esophagus, the optimal size of the circumferential balloon is selected based on various pressure measurements in different locations. The ablation process is a series of two separate applications of direct thermal energy with the electrodes embedded in either the circumferential or focal device. Scraping of treated tissue is performed between the first and second ablation to ensure adequate and uniform thermal contact. The most common complications associated with RFA include non-cardiac chest pain, non-transmural lacerations, and stricture formation (lower stricture rate when compared to EMR).

After thermal dose-escalation animal testing and pre-esophagectomy human experiments[35,36], the first larger clinical evaluation of RFA was performed on BE patients without dysplasia in the Ablation of Intestinal Metaplasia (AIM) study from 2003 to 2005. This multicenter trial demonstrated a 70% complete remission of BE in the circumferential-balloon-treated group at 1 year follow-up, without evidence of subsequent stricture formation or buried BE among 4306 biopsy fragments evaluated[37]. A subsequent AIM Ⅱ study reported 98% complete remission of IM after stepwise circumferential therapy with additional focal ablative therapy of remaining BE[38].

RFA was also studied in 142 patients with BE HGD. At 1 year follow-up, complete remission of HGD was achieved in 90.2%, complete remission of dysplasia in 80.4%, and complete remission of IM in 54.3% of patients[39]. In a recent landmark multicenter, sham-controlled trial, 127 patients with dysplastic BE were randomly assigned to receive either RFA or a sham procedure. The measured primary outcomes at 1 year included complete eradication of dysplasia and intestinal metaplasia. Based on an intention-to-treat analysis, in patients with LGD, complete eradication of dysplasia occurred in 90.5% in the ablation group, compared to only 22.7% in the control group ($P < 0.001$). In the HGD sub-group, complete eradication occurred in 81% of ablated patients as compared with 19% of the control group ($P < 0.001$). Overall, 77.4% of ablation patients demonstrated complete eradication of IM, as compared to 2.3% in the control group ($P < 0.001$). There was less disease progression in patients in the ablation group (3.6% *vs* 16.3%, $P = 0.03$) and fewer cancers developed (1.2% *vs* 9.3%, $P = 0.045$). There were more reports of chest pain after ablation than after sham procedures, and a 6% esophageal stricture rate was reported in the treated group[40]. This stricture rate is markedly lower than that commonly reported for EMR, which confers a significant advantage for RFA in treatment of BE with flat HGD.

In patients who demonstrate visible lesions in the setting of HGD, a combination of EMR and RFA has recently been studied. Pouw and colleagues have reported on performance of EMR for visible lesions with subsequent ablation of the remaining segment[41]. Complete histological eradication of all dysplasia and IM was achieved in 43 patients (98%). Post-ablation complications included mucosal laceration at prior EMR sites (n = 3) and transient dysphagia (n = 4). No dysplasia recurred after a 21-mo follow-up period[41]. A more recent multicenter European trial involved EMR of visible lesions, followed by serial RFA. Focal escape endoscopic resection was utilized in cases of BE persistence despite RFA. The study

included 24 patients, and achieved neoplasia and IM eradication in 95% and 88% of patients, respectively. These rates improved to 100% and 96%, respectively, following escape EMR in two patients. No neoplasia recurred within a median 22-mo follow-up period[42]. Neo-squamous epithelium rigorous EMR and biopsy evaluation in a group of 22 post-RFA patients with baseline BE with IMC or HGD showed no evidence of persistent genetic abnormalities or buried BE glands[43]. To date, as far as we are aware, no published studies exist on outcomes of sole RFA therapy of BE with IMC.

CRYOTHERAPY

Cryotherapy is the latest modality to arrive on the endoscopic horizon of ablative options. This technology utilizes sprayed liquid nitrogen freeze-thaw cycles that result in tissue destruction by intracellular disruption and tissue ischemia, with relative preservation of the extracellular matrix to promote less fibrosis formation[44,45]. The procedure requires placement of an orogastric decompression tube to allow for adequate excess nitrogen gas expulsion to prevent inadvertent gastrointestinal viscus perforation. Repeat treatment sessions can be conducted every 4-6 wk as needed to ensure complete remission of the target area.

In a prospective open-label trial, Dumot *et al*[46] enrolled patients with BE and HGD or IMC who were not deemed surgical candidates or who refused esophagectomy. EMR was used for pathological staging of nodular areas before cryoablation and focal residual areas during the follow-up period. Patients with prior ablation therapy were not excluded. Twenty-seven of 30 patients had pathological downgrading post-treatment. After a median follow-up of 1 year, elimination of cancer or downgrading of HGD was achieved in 80% of IMC and 68% of HGD patients. A perforation occurred in a patient with Marfan syndrome, with the prototype system. Of six patients who showed a complete response, three had recurrence of dysplasia or cancer in the gastric cardia.

The efficacy and safety of liquid nitrogen cryotherapy has been demonstrated in a four-center study of 23 patients (17 with HGD, four with IMC, and three with early-stage adenocarcinoma). Complete response to HGD was found in 94% with HGD, and 100% with IMC and cancer. Complete response to IM was noted in 53% with HGD, 75% with IMC, and 67% with cancer. No symptoms were reported in 48% of 323 procedures. Esophageal strictures developed in three patients, but all were successfully treated by dilation. Other complications included chest pain, dysphagia, sore throat, and the gastric perforation noted in the Marfan patient as above[47].

CONCLUSION

BE early neoplasia treatment has undergone transition from radical esophagectomy to endoscopic organ-preserving options. The key to successful endoscopic management hinges on appropriate selection of candidate patients and detection of visible lesions through careful white light, high-definition endoscopy and ancillary imaging techniques such as narrow-band imaging and/or endomicroscopy. All visible lesions must be removed by EMR for definitive histopathological staging and to ensure adequacy of resection margins. Total eradication of the entire BE segment must occur to protect against synchronous/metachronous lesion development.

As a result of the higher risk of stricture development associated with EMR, our center currently employs a hybrid approach to treatment of BE early neoplasia that is based on segment length. For BE segments that measure $\leqslant$ 5 cm and harbor HGD or IMC, a CBE-EMR approach is used. For patients with BE segments > 5 cm, all focal lesions are resected, and the remaining flat BE is ablated using RFA to decrease the rate of stricture formation.

The critical research issues that still remain unanswered for endoscopic BE management center on: long-term survival and remission rates of both treated neoplasia and IM; development and significance of buried BE glands; quality of life and cost assessments for the various modalities compared to surgical cohorts; the role of these therapies for LGD or non-dysplastic BE; and the clinical impact of post-endoscopic therapy surveillance.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3786

World J Gastroenterol 2010 August 14; 16(30): 3786-3792
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Esophageal resection for high-grade dysplasia and intramucosal carcinoma: When and how?

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

High-grade dysplasia (HGD) and intramucosal carcinoma (IMC) in the setting of Barrett's esophagus have traditionally been treated with esophagectomy. However, with the advent of endoscopic mucosal resection and endoscopic ablative therapies, endoscopic therapy at centers with expertise is now an established treatment of Barrett's-esophagus-related neoplasia, including HGD and IMC. Esophagectomy is today reserved for more selected cases with submucosal invasion, evidence for lymph node metastasis, or unsuccessful endoscopic therapy.

Key words: Barrett's esophagus; High-grade dysplasia; Intramucosal carcinoma; Endoscopic mucosal resection; Esophagectomy

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Konda VJA, Ferguson MK. Esophageal resection for high-grade dysplasia and intramucosal carcinoma: When and how? *World J Gastroenterol* 2010; 16(30): 3786-3792 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3786.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3786

INTRODUCTION

High-grade dysplasia (HGD) and intramucosal carcinoma (IMC) in the setting of Barrett's esophagus (BE) have traditionally been treated with esophagectomy. However, with the advent of endoscopic mucosal resection and endoscopic ablative therapies, endoscopic therapy at centers with expertise is now an established treatment of Barrett's-esophagus-related neoplasia, including HGD and IMC. Esophagectomy is today reserved for more selected cases with submucosal invasion, evidence for lymph node metastasis, or unsuccessful endoscopic therapy. This review highlights the updated role of and approaches for esophagectomy in the management of HGD and IMC in BE and discusses risk factors associated with submucosal invasion, lymph node metastasis, or unsuccessful endoscopic therapy.

TRADITIONAL APPROACH: ESOPHAGECTOMY AS THE STANDARD OF CARE FOR HGD

HGD in the setting of BE has been identified as a key risk factor in the progression to esophageal adenocarcinoma (EA). Patients with HGD are at a higher risk for progressing to EA than are patients with BE with no or low-grade dysplasia (LGD). This has given rise to performing prophylactic esophagectomy for the treatment of HGD to prevent EA. In addition to the risk of progression to EA, the surgical literature has reported a high risk of coexisting adenocarcinoma in patients with HGD that is

not diagnosed by endoscopic biopsy. The esophagectomy literature has reported varying prevalence of occult EA in patients with BE and HGD, ranging from 0% to 73%, and frequently approximates to a rate of around 40%[1-7]. Thus, the role of esophagectomy for the treatment of HGD is underlined by both prevention of cancer and cure of occult cancer.

Concerns have previously been raised as to whether esophagectomy is appropriate for most patients with HGD and IMC. Newer data have suggested that the incidence of invasive cancer is probably much lower than the 40% rate previously estimated[8]. This suggests that esophagectomy for HGD is unnecessary in more than 80% of patients in whom it is performed. At the same time, newer endoscopic techniques for evaluating and managing HGD and IMC have been developed and clinically tested. Currently, the approach to HGD and IMC is more complex and provides much more individualized care of patients than previously was available.

ENDOSCOPIC EVALUATION OF BARRETT'S-ESOPHAGUS-RELATED NEOPLASIA

The management of BE has been greatly influenced by the advent of endoscopic mucosal resection (EMR) and endoscopic ultrasound (EUS). Prior to the advent of endoscopic ablative techniques, whether intramucosal cancer was different from invasive cancer was a moot point, given that esophagectomy was indicated in either case. However, with endoscopic therapy now available for IMC, the distinction must be acknowledged. When evaluating treatment options it is crucial to understand the difference between the presence of intramucosal cancer limited to the mucosal lining, which only has a minimal nodal metastasis risk[9-11] and might be locally treatable, and the presence of cancer with invasion into the submucosa, which carries a higher nodal metastasis risk and requires surgery and/or systemic therapy[9,12-16].

Understanding pathological definitions is instrumental in managing a patient with Barrett's-related neoplasia. Dysplasia is neoplastic cytological and architectural atypia without evidence of invasion past the basement membrane. The diagnosis of LGD or HGD is based on the severity of cytological criteria that suggest neoplastic transformation of the columnar epithelium[17]. HGD and carcinoma *in situ* are regarded as equivalent. IMC is tumor that is limited to the lamina propria and is considered T1a by the American Joint Committee on Cancer staging. Submucosal carcinoma (SMC) is a tumor that invades past the muscularis mucosa into the submucosa, but not into the muscularis propria. Vessel invasion might be either venous or lymphatic channel invasion.

In a systematic review of the surgical literature that has reported the rates of cancer in patients who were undergoing esophagectomy for prophylactic treatment of HGD, the pooled average was 39.9% in the 441 patients who underwent esophagectomy for HGD among 23 studies[5]. These rates were largely based on retrospective studies with varying aims, sizes, definitions, and methodology. This average rate is consistent with previous pooled studies by Edwards *et al*[1], Ferguson *et al*[6], and Pellegrini *et al*[7] who have reported rates of 41%, 43% and 47%, respectively. However, the majority of these patients had IMC, whereas the rate of submucosal invasive cancer was decreased to 12.7% when applying both standardized criteria and strict definitions.

Prospective studies with rigorous endoscopic criteria in the EMR literature have reported lower rates of occult submucosal invasive disease. Among patients presenting with HGD and IMC who were undergoing complete BE EMR, the rate of occult submucosal invasive cancer was 4%[18]. Pech *et al*[19] have reported their long-term experience with EMR and other ablative procedures for Barrett's-esophagus-related neoplasia. They achieved a complete response in 96.6% and the 5-year survival rate was 84%. In their experience, esophagectomy was required in only 3.7% of patients initially presenting with HGD or IMC[19].

The management of HGD and IMC has now shifted from esophagectomy to endoscopic therapy to achieve total Barrett's eradication[18,20,21]. The concept of total Barrett's eradication highlights the importance of not only treating the known neoplasia, but also eradicating all of the at-risk Barrett's epithelium, to treat any synchronous lesions and hopefully prevent any metachronous lesions. Although expertise might vary from site to site and patient characteristics need to be taken into account, there is now acceptance of endoscopic therapy for HGD and IMC, and esophagectomy is no longer the standard of care[22].

Endoscopic modalities include tissue-acquiring therapies that include focal EMR, complete Barrett's EMR, and endoscopic submucosal dissection. Tissue-acquiring modalities are important to stage a visible lesion in the setting of HGD or for the treatment of IMC. HGD might also be treated with ablative therapies, such as photodynamic therapy, which has the longest experience of the ablative therapies[23], radiofrequency ablation, which has demonstrated initial success[24], and cryotherapy, which is a newer modality[25]. Chennat and Waxman have described these endoscopic therapies in further detail in their article in this issue.

HIGH-RISK CHARACTERISTICS OF BARRETT'S NEOPLASIA

Endoscopic therapy has advantages in that it is organ-preserving and does not have the same morbidity and mortality as surgery. However, not all cases are successful or appropriate for endoscopic therapy. Indications for esophagectomy include lymph node metastasis and failure of endoscopic therapy. Risk factors for submucosal invasion, lymph node metastasis, and failure of endoscopic treatment need to be incorporated into the management strategy of a patient with HGD and IMC. These risk factors are evident in endoscopic appearance, pathological characteristics, and results of endoscopic treatment (Table 1).

Table 1 High-risk characteristics associated with submucosal invasion, lymph node metastasis, or unsuccessful endoscopic therapy

Endoscopic characteristics
Long-segment Barrett's esophagus
Visible lesions with high risk endoscopic characteristics
Polypoid mass
Excavated lesions or ulcers
Evidence of lymph node involvement by EUS + FNA
Pathological characteristics
Multifocal HGD
Evidence of submucosal invasion
Deeper two thirds of the submucosa carries high risk of lymph node metastasis
Moderately or poorly differentiated tumor
Evidence of lymphatic channel invasion
Evidence of vascular invasion
Evidence of neural invasion
Treatment characteristics
Failure of ablation of remainder for Barrett's epithelium
Piecemeal endoscopic resection (as opposed to *en bloc* resection)
Longer time to achieve eradication

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; HGD: High-grade dysplasia.

Endoscopic characteristics

Long-segment BE has been identified as a risk factor for cancer[26] and for recurrence of neoplasia with endoscopic therapy[19]. Furthermore, visible lesions in the setting of HGD are more at risk for harboring occult cancer than flat dysplasia[5,27,28].

Careful white light examination is essential for targeting biopsies and resection of visible lesions because visible lesions in the setting of dysplasia have a high risk of occult cancer. Furthermore, the type of lesion is correlated with risk of submucosal invasion. Standardization of endoscopic appearance of visible lesions is now developing, and more attention is being given to non-protruding lesions. The updated Paris classification is based on the Japanese classification of gastric lesions. In the esophagus, superficial lesions based on endoscopic appearance include the following classifications: protruding pedunculated (type 0- Ⅰ p), protruding sessile (0- Ⅰ s), slightly elevated (0- Ⅱ a), completely flat (0- Ⅱ b), slightly depressed (0- Ⅱ c), excavated (0-Ⅲ), or a mixed pattern[29]. Type 0-Ⅲ is suspicious for submucosal invasion. Type 0- Ⅰ and type 0- Ⅱ c lesions are also associated with increased risk of submucosal penetration[30]. Thus, protruding or depressed lesions are at higher risk than those slightly raised or flat areas. EMR provides an opportunity to stage the depth of a lesion in areas of question.

Endoscopic ultrasound in BE demonstrates a thickened mucosal lining. It is not optimal for differentiation between a T1a tumor (IMC) and a T1b (SMC) tumor, and EMR is better suited for depth staging at this range[31]. However, given the risk of lymph node metastasis in patients with IMC, EUS with fine needle aspiration (FNA) might identify patients not eligible for endoscopic therapy[32]. EUS with or without FNA is a reasonable procedure in all patients with IMC and patients with visible lesions, who have a higher risk of occult cancer. Any patient found with lymph node involvement should be referred for esophagectomy. The utility of EUS in flat HGD might be questioned[33].

Pathological characteristics

The diagnosis of HGD, IMC, and invasive cancer represents a biological and histological continuum. Although pathological assessment is the gold standard, interpretation is subject to a great deal of variability among pathologists. There is high inter-interpreter variability in diagnosing HGD as reported in the literature[34-38]. Due to limited sample size and depth, as well as potential crush artifacts, pathologists might not reliably be able to distinguish between HGD, IMC and SMC on a single biopsy specimen. One of the advantages of EMR specimens is that pathologists are better able to stage lesions because they provide large and intact pathological specimens.

In evaluations of specimens from EMR for Barrett's neoplasia, moderately or poorly differentiated cancers are more likely to invade the submucosa[30,39]. HGD obtained from multiple levels throughout a BE segment has a higher risk of being associated with occult cancer[28]. Furthermore, in a risk analysis performed on patients with either HGD or IMC, multifocal neoplasia has been cited as a risk factor for recurrence after endoscopic therapy[19]. Risk factors for lymph node metastasis in EA are vascular invasion, lymphatic channel permutation, neural invasion, and grade of the tumor[40,41]. In EA, submucosal invasion of the most superficial third does not carry the same lymph node metastasis risk as the deeper two thirds[40]. Manner *et al*[42] have reported favorable outcomes with endoscopic resection of low-risk SMC in their long-term experience of endoscopic resection. However, larger trials are needed before adopting endoscopic therapy as standard practice for these superficial submucosal invading tumors.

Treatment characteristics

Endoscopic resection specimens not only provide a histological specimen that is important for accurate pathological diagnosis, but also provide a means for assessing treatment adequacy. Lateral margins might indicate that further endoscopic treatment is necessary, whereas positive deep margins indicate that surgery is appropriate. The following are associated with a higher risk of recurrence: length of time to complete eradication of neoplasia with multiple endoscopic treatment sessions; piecemeal resection; and no ablative therapy to target the remainder of the at-risk Barrett's epithelium[19].

Although there is ongoing interest and early investigations for genetic or molecular markers to predict endoscopic response[43], none of these markers has been validated for clinical use.

ADVANTAGES OF ESOPHAGECTOMY

The strategy of performing esophagectomy for HGD or IMC not only cures the index condition, but also addresses occult cancer and prevents cancer death[44]. Although endoscopic treatment is an appropriate and cost-effec-

tive[45] approach for the treatment of many patients with HGD and IMC, patients who are appropriate surgical candidates can benefit from esophagectomy. The surgical specimen enables accurate staging of disease to diagnose areas of occult cancer, and confirms treatment adequacy with negative margins and lymph nodes. Conventional approaches are transhiatal esophagectomy and transthoracic esophagectomy. Minimally invasive esophagectomy (MIE) techniques are growing in popularity because of their perceived benefits of reduced pain, lower incidence of postoperative complications, and faster recovery. These MIE techniques include video-assisted thoracoscopy surgery with laparotomy or laparoscopy, laparoscopy with a right thoracotomy, or laparoscopic transhiatal resections. These procedures have been studied in mostly retrospective studies and conclusions are limited in terms of direct comparisons to open surgery due to lack of prospective randomized trials[46,47].

The issue of the morbidity and mortality of esophagectomy is the major concern for either open esophagectomy or MIE. Adverse outcomes include pulmonary complications, hemorrhage, anastomotic leakage, infections, and recurrent nerve palsy. Although one study based on a national Veteran's Affairs database has reported morbidity of almost 50% and mortality of 10%[48], the expertise and volume of the center, the experience of the surgeon, the patient risk factors, and the indications for esophagectomy should be taken into account[49-51]. In institutions with expertise and high volumes, the mortality rate is 2%-3%[52]. It is also important to note that esophagectomy specifically for HGD has a different risk profile than that of esophagectomy for cancer. Comorbid diseases, debilitation from cancer and/or neoadjuvant therapy, and issues with locally advanced disease are not as predominant in patients with HGD. A pooled mortality rate of 1% was calculated among six studies that involved esophagectomy for HGD[49]. Quality of life indicators for patients who underwent esophagectomy for HGD and IMC are equivalent to those of the general population[53].

INDICATIONS FOR ESOPHAGECTOMY FOR BARRETT'S HGD OR IMC

Strong indications for esophagectomy include lymph node metastasis and failure of endoscopic therapy. Invasion of tumor into the submucosa is still considered a strong indication for esophagectomy, although invasion into the superficial third of the submucosa does not carry the same lymph node metastasis risk as the deeper two thirds, and potentially could be treated endoscopically[29,42]. Factors to consider in the management strategy for HGD and IMC include characteristics that are associated with lymph node metastasis, submucosal invasion, and failure of endoscopic therapy, as listed in Table 1, and may serve as milder indications for esophagectomy. Excavated lesions (Paris classification 0-Ⅲ) are not typically considered to be amenable to endoscopic therapy due to high suspicion of submucosal invasion, whereas protruding lesions (0-Ⅰ) and depressed lesions (0-Ⅱc) are a concern for submucosal invasion and should be approached with caution endoscopically (Table 2). These circumstances allow for endoscopic resection to serve as a diagnostic tool to stage the lesion accurately to determine if the lesion is amenable to endoscopic therapy. Multifocal high grade is a milder indication for esophagectomy than previously considered, due to the evolving options of ablative therapy. These risk factors, as listed in Table 1, need to be weighed with patient characteristics, patient preferences, available surgical expertise, available endoscopic expertise, and surgical approach options to decide if esophagectomy or endoscopic therapy is appropriate for each case.

Table 2 Relative risk of submucosal invasion associated with endoscopic appearance of lesions

Endoscopic appearance	Paris classification	Relative risk of submucosal invasion
Polypoid	0-Ⅰp	Higher
Sessile	0-Ⅰs	Higher
Slightly raised	0-Ⅰa	Low
Flat	0-Ⅰb	Low
Slightly depressed	0-Ⅰc	Higher
Excavated	0-Ⅲ	Very high

WHICH OPERATION FOR BARRETT'S HGD OR IMC?

Selection of the appropriate approach to esophagectomy for HGD or IMC is based on a number of factors (Table 3). Prior surgery in the chest or abdomen might require an open rather than a minimally invasive approach, and prior esophageal surgery such as fundoplication might limit consideration of a vagal-sparing approach. Comorbidity such as severe pulmonary disease, or advanced age might encourage some surgeons to pursue an approach associated with less postoperative pulmonary morbidity, such as transhiatal esophagectomy[54]. Whether minimally invasive approaches offer a lower risk of postoperative pulmonary morbidity compared to open transthoracic approaches has not yet been adequately determined[47,55-57].

The appropriate extent of operation for HGD or IMC is somewhat complex and controversial, and is related to the length of esophagus that must be resected, the extent of soft tissue resection around the esophagus, and the regions for lymph node dissection. It is appropriate to examine the surgical specimen at the time of resection, and usually to perform a frozen section analysis of the proximal margin, to ensure that all the Barrett's mucosa has been removed. Limiting the resection to encompass just the Barrett's segment is probably not a good long-term strategy, because most reconstructive techniques using a gastric tube create a model of frequent reflux, thus exposing patients to the possibility of developing Barrett's mucosa in the remaining esophagus[58]. Indeed, this phenomenon has been well documented in the esophageal remnant after standard subtotal esophagectomy, and theoretically, the risk would be increased if more esophagus were left in place[58-64]. Some cases of adenocarci-

Table 3 Selecting an appropriate surgical approach

Patient characteristics
Prior surgery (thoracic, abdominal, esophageal)
Obesity
Age
Pulmonary function
Other comorbid factors
Surgical options
Standard open resection
Transhiatal esophagectomy (2 or 3 holes)
Minimally invasive esophagectomy
Vagus sparing esophagectomy
Mucosal stripping esophagectomy?
Extent of operation
Extent of esophageal resection
Limited resection of Barrett's segment
Near-total esophagectomy
Extent of soft tissue resection
Minimal
Standard
Extended
Extent of nodal dissection
Minimal
Standard
Extended 3-field
Surgical results
Accuracy of staging
Number of lymph nodes
Effects on long-term survival
Effects on perioperative outcomes

noma arising in such metaplastic epithelium have been described[65,66]. Therefore, a near total esophagectomy is recommended for patients who are undergoing esophagectomy for HGD or IMC.

The lateral extent of soft tissue resection for HGD or IMC is a more controversial problem, with the possible range extending from a vagal-sparing esophagectomy, in which no additional soft tissues are removed, to an extended *en bloc* esophagectomy, which sometimes includes the azygos vein, thoracic duct, contralateral pleura, a rim of diaphragm, and in some cases, even the posterior pericardium. With the increasing accuracy of EUS in assessing the depth of penetration of the primary tumor, anything more than removing a standard amount of soft tissue representing the lateral margins is not likely to provide the patient with benefits regarding local recurrence, but might add to postoperative morbidity. Whether a vagal-sparing operation offers the same freedom from local recurrence has not been sufficiently studied to date[67].

The appropriate extent of nodal dissection for HGD or IMC is also controversial. In order to stage esophageal cancer accurately it has been suggested that a minimum of 10 lymph nodes be resected for early-stage cancers[68]. The use of more extensive nodal dissections, especially three-field lymphadenectomy, are controversial for regionally advanced cancers and are likely inappropriate for HGD and IMC, although this question has not been formally studied.

The best surgical option for HGD or IMC is the one that produces the least morbidity, balanced against the best long-term survival. As present, any standard resection technique including open transthoracic, minimally invasive, and transhiatal approaches provide similar long-term outcomes, and transhiatal esophagectomy might have an advantage in reducing postoperative morbidity. The more extensive resections (open transthoracic, and minimally invasive) are likely to improve staging accuracy, particularly with regards to nodal status. Long-term functional status is similar regardless of the surgical approach. The use of vagal-sparing techniques, especially for HGD, has potentially interesting advantages with regard to quality of life, but has not been adequately evaluated in terms of staging accuracy and long-term outcomes. In the end, it is the surgeon's training and experience, in combination with the individual patient's needs that determines the most appropriate approach to esophagectomy for HGD or IMC.

CONCLUSION

Barrett's HGD or IMC can be primarily treated endoscopically with endoscopic resection and endoscopic ablation with the goal of total Barrett's eradication. Evidence of submucosal invasion, lymph node metastasis or failure of endoscopic therapy or their risk factors, which can be ascertained by endoscopic appearance, pathological characteristics, and treatment course, need to be incorporated into the decision-making process for endoscopic versus surgical treatment. Longer-term studies with additional risk analysis need to be carried out to be able to predict reliably which patients are amendable to endoscopic therapy and who may benefit from esophagectomy.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3793

World J Gastroenterol 2010 August 14; 16(30): 3793-3803
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Neoadjuvant treatment of esophageal cancer

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

The management of esophageal cancer has been evolving over the past 30 years. In the United States, multimodality treatment combining chemotherapy and radiotherapy (RT) prior to surgical resection has come to be accepted by many as the standard of care, although debate about its overall effect on survival still exists, and rightfully so. Despite recent improvements in detection and treatment, the overall survival of patients with esophageal cancer remains lower than most solid tumors, which highlights why further advances are so desperately needed. The aim of this article is to provide a complete review of the history of esophageal cancer treatment with the addition of chemotherapy, RT, and more recently, targeted agents to the surgical management of resectable disease.

Key words: Esophageal cancer; Multimodality therapy; Neoadjuvant therapy; Chemotherapy; Radiotherapy; Targeted agents; Disease management

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Campbell NP, Villaflor VM. Neoadjuvant treatment of esophageal cancer. *World J Gastroenterol* 2010; 16(30): 3793-3803 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3793.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3793

INTRODUCTION

Esophageal cancer is the most rapidly increasing tumor type in the Western world[1,2]. Globally, esophageal cancer is the eighth most common malignancy and sixth most fatal, with approximately 460 000 new diagnoses and > 380 000 deaths annually[3]. The lifetime risk, as well as histology of esophageal cancer varies worldwide from 1 in 200 in the United States, with more than half of new cases being adenocarcinoma (AC) to more than 10 times that risk in Iran, Northern China, India, and Southern Africa, where the histology is > 90% squamous cell carcinoma (SCC), and mirrors the growing epidemic of tobacco abuse[3-5].

Although there are multiple, rare esophageal cancer histologies (e.g. gastrointestinal stromal tumors, leiomyosarcoma, and liposarcoma), AC and SCC are the two principle variants and account for > 98% of esophageal cancer diagnoses[6]. Historically, AC and SCC have been treated as a single disease entity with many older clinical trials not differentiating between the two histologies, even in study populations[7]. Over the years, however, a great deal of evidence has been compiled to support the notion that AC and SCC represent two separate diseases based on their differing etiology, epidemiology, prognosis, and response to treatment[8-11].

AC is highly associated with obesity and gastroesophageal reflux disease (GERD). Obesity increases the risk of developing GERD by approximately twofold due to elevated intra-abdominal pressure and a resultant laxity in the lower esophageal sphincter[12]. GERD leads to chronic

irritation of the distal esophagus and can eventually cause metaplasia by the replacement of normal, squamous epithelium by columnar epithelium and the formation of what is referred to as Barrett's esophagus. The new, secretory columnar cells are thought to be better-suited to withstand the erosive contents that spill over from the gastroesophageal junction (GEJ), but unfortunately, this change also increases the risk for dysplasia by sevenfold, with Barrett's esophagus evolving to AC at a rate of approximately 1% per year[13,14].

SCC, on the other hand, is almost always linked to tobacco and alcohol abuse. Current smokers have a ninefold increased risk of developing SCC of the esophagus, while heavy drinkers of alcohol have an increased OR of 5[15]. Combined, however, the synergistic effects of tobacco and alcohol abuse lead to a 20-fold increased risk of developing esophageal cancer[16], although more extreme abusers of the two have been reported to have an increased OR as high as 50 and even 107 in studies from Italy and South America, respectively[17,18].

Epidemiologically, there has been a dramatic shift in the two histologies[5]. In the United States between 1974 and 1994, there has been a staggering 350% increase in the number of patients with esophageal AC, which now represents 60% of all new esophageal cancer diagnoses. Prior to 1974, SCC constituted 90% of esophageal cancer in the United States, which was likely secondary to increased rates of tobacco abuse[5,19]. The median age of diagnosis for SCC is approximately one decade prior to that of AC, yet surprisingly, patients with SCC have been documented in more recent studies to fair worse[7-9,20,21]. This difference is likely to be secondary to the increased comorbidity of patients with SCC but, even more importantly, the location of the primary tumor. Compared to age and lung function, the adjusted OR for postoperative death for a tumor located in the upper third of the esophagus is 4[7,22]. SCC is usually a proximal lesion, with 75% of these cancers found to have contact with the tracheobronchial tree, while 94% of ACs are below the tracheal bifurcation[7].

With regard to location, it should be noted that the pathology, treatment and prognosis of SCC of the cervical esophagus are more closely related to that of SCC of the head and neck[23]. As such, this review instead focuses on the multimodality treatment of localized and locoregional cancer involving the thoracic esophagus and GEJ. The definition of what constitutes the GEJ is debatable in itself. Siewert and Stein have described the most accepted classification scheme for AC at the GEJ: type Ⅰ, AC arising from an area of intestinal metaplasia of the esophagus, which can infiltrate the GEJ from above; type Ⅱ, AC arising from the cardia of the stomach; type Ⅲ, subcardial gastric carcinoma that infiltrates the GEJ from below[24]. With the exception of overexpression of *COX-2* with type I GEJ AC, no known significant gene expression profile changes have been noted that differentiate the three sub-types consistently[25]. Type I GEJ tumors tend to have lymphatic drainage toward lower mediastinal and upper gastric lymph nodes, whereas type Ⅱ and Ⅲ GEJ tumors are more likely to drain to celiac axis nodes. As such, type Ⅰ GEJ tumors are generally treated as distal esophageal cancer, whereas type Ⅱ and Ⅲ GEJ tumors are viewed by many as gastric carcinomas[24,25].

TREATMENT

Surgery alone

Debate regarding the current standard of care for the management of esophageal cancer is ongoing[26-28]. Surgical resection alone has been the mainstay of treatment for decades[29], although its necessity has been called into question more recently for patients with SCC[30,31]. Although surgery is considered to offer the best chance of prolonged survival, alone it will only cure 15%-20% of patients with localized disease[32-35], and unfortunately, 50%-60% of patients with esophageal cancer have tumors that are considered inoperable, secondary to either tumor extension or medical comorbidity[29]. Contemporary outcome data for treatment with surgery alone report a median survival of 16 mo with a 1-, 2- and 3-year survival rate of 60%, 37% and 26%, respectively[32]. Local disease-failure rates with surgery alone are quite high at 58%, with two-thirds of those failures from lack of complete (R0) resection and one-third recurring locally despite an R0 resection[36]. Surgical approaches and techniques - transthoracic *vs* transhiatal resection with limited *vs* extended-field lymphadenectomy - are highly debated[34,35], and are beyond the scope of this review. What is clear, however, is that postoperative morbidity and mortality are decreased while overall survival (OS) is significantly improved in high-volume, expert academic centers[37,38]. Currently, National Comprehensive Cancer Network guidelines suggest surgery as a single-modality treatment option only for non-cervical T1 lesions without lymph node involvement[39].

Radiotherapy

Radiotherapy alone has been the historical treatment of choice for patients with esophageal cancer who are not surgical candidates. Radiotherapy delivered at 60-66 Gy over 6-6.5 wk has been associated with a 5-year OS ranging from 5% to 20% depending on tumor extent[40-42]. In a review by Earlam and colleagues, 49 earlier series that involved 8489 patients with SCC treated with radiotherapy alone have been reported to yield a 1-, 2- and 5-year survival rate of 18%, 8% and 6%, respectively[43]. Adding radiotherapy to the surgical management of esophageal cancer has the advantage of increasing local control of disease. In the adjuvant setting, radiotherapy can treat microscopic disease left behind after an incomplete surgery. In the neoadjuvant setting, radiotherapy can theoretically decrease the size of a lesion prior to surgery and potentially make that lesion more resectable. The obvious trade-off of increased local control with radiotherapy is poor wound healing in both settings and an increasingly difficult resection of previously irradiated tissue in the neoadjuvant setting.

As it stands, there have been five separate phase Ⅲ trials that have compared adjuvant radiotherapy with sur-

Table 1 Randomized controlled trials of adjuvant radiotherapy *vs* surgery alone for esophageal cancer

Studies	Histology	Treatment	*n*	MS (mo)	5-yr OS (%)	*P*	RT dose (Gy)
Kunath *et al*[44], 1984	SCC	ART	23	9		NS	50-55
		Surgery	21	6			
Ténière *et al*[45], 1991	SCC	ART	102	18	19	NS	45-55
		Surgery	119	18	19		
Fok *et al*[36], 1993	SCC	ART	42	11	10	NS	43-53
		Surgery	39	22	16		
Zieren *et al*[46], 1995	SCC	ART	33		23[1]	NS	56
		Surgery	35		22[1]		
Xiao *et al*[47], 2003	AC/SCC	ART	220		41	NS	50-60
		Surgery	275		32		

[1]3-year OS. MS: Median survival; RT: Radiotherapy; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; ART: Adjuvant radiotherapy; NS: Not significant; OS: Overall survival.

Table 2 Randomized controlled trials of neoadjuvant radiotherapy *vs* surgery alone for esophageal cancer

Studies	Histology	Treatment	*n*	MS (mo)	5-yr OS (%)	*P*	RT dose (Gy)
Launois *et al*[48], 1981	SCC	NART	77	10	10	NS	40
		Surgery	57	12	12		
Gignoux *et al*[49], 1987	SCC	NART	106	11	11	NS	33
		Surgery	102	11	10		
Arnott *et al*[50], 1992	AC/SCC	NART	90	8	9	NS	20
		Surgery	86	8	17		
Nygaard *et al*[51], 1992	SCC	NART	48[1]		21[3]	NS	35
		Surgery	41[2]		9[3]		
Wang *et al*[52], 1989	SCC	NART	104		35	NS	40
		Surgery	102		30		

[1]Group 3: NART; [2]Group 1: Surgery alone; [3]3-year OS. MS: Median survival; RT: Radiotherapy; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; NART: Neoadjuvant radiotherapy; NS: Not significant; OS: Overall survival.

gery alone[36,44-47] (Table 1), and another five phase Ⅲ trials that have compared neoadjuvant radiotherapy to surgery alone[48-52] (Table 2). Although local control of disease was improved in each of the adjuvant radiation arms, there were increased complications secondary to adhesions, scarring and fistulas, and none reported an OS advantage in their entire study population as a whole. Among these trials, however, Xiao and colleagues randomized 495 patients with SCC to surgery followed by adjuvant radiotherapy or to surgery alone. Although the 5-year OS was not statistically different for all-comers (41% *vs* 32%, $P = 0.45$), a 5-year OS advantage was noted in a subgroup analysis of patients with stage Ⅲ disease (35% *vs* 13%, $P < 0.003$), which favored the arm that received adjuvant radiotherapy[47].

Of the five phase Ⅲ trials that have evaluated neoadjuvant radiotherapy in esophageal cancer, none has demonstrated an increase in resectability or OS in those treated with preoperative radiotherapy alone[48-52]. Although Nygaard and colleagues have reported a 3-year OS benefit, this was only after pooling patients who had received neoadjuvant radiotherapy with those who had also received neoadjuvant chemoradiotherapy, as there was no significant difference in survival found otherwise[51]. A meta-analysis of trials that have used neoadjuvant radiotherapy with a median follow-up of 9 years, and including data from 1147 patients who almost exclusively had SCC, has revealed a trend toward improved 5-year OS (OR: 0.89, 95% CI: 0.78-1.01, $P = 0.062$), but ultimately has failed to show a statistically significant survival advantage[53].

Chemotherapy

The theoretical advantages of adding chemotherapy to the treatment of esophageal cancer are for potential tumor down-staging prior to surgery, as well as targeting micrometastatic disease, and thus decreasing the risk of distant spread. Adjuvant chemotherapy with cisplatin-based regimens compared to surgery alone has been examined in three separate phase Ⅲ trials[54-56] (Table 3), with none of them reporting a statistically significant difference in OS, although Ando and colleagues have reported a 5-year disease-free survival (DFS) advantage (55% *vs* 45%, $P = 0.037$)[56]. In the neoadjuvant setting, there have been multiple randomized trials that have compared varying chemotherapeutic regimens to surgery alone[32,51,57-63] (Table 4). Clinical complete responses based on direct visualization and an assortment of imaging modalities have ranged from 19% to 58%, but the rate of pathological complete response (pCR) at the time of surgery was a disappointing 2.5%-13%. This is an unsurprising trend considering the relative ineffectiveness of chemotherapy alone in the treatment of esophageal cancer[32,51,57-63].

The UK Medical Research Council (MRC) trial included 802 patients of all histologies, and randomized patients

Table 3 Randomized controlled trials of adjuvant chemotherapy *vs* surgery alone for esophageal cancer

Studies	Histology	Treatment	*n*	MS (mo)	5-yr OS (%)	*P*
Pouliquen *et al*[54], 1996	SCC	CF	52	13		NS
		Surgery	68	14		
Ando *et al*[55], 1997	SCC	CV	100		45	NS
		Surgery	105		48	
Ando *et al*[56], 2003	SCC	CF	120		61	NS
		Surgery	122		52	

MS: Median survival; SCC: Squamous cell carcinoma; C: Cisplatin; F: Fluorouracil; V: Vindesine; NS: Not significant; OS: Overall survival.

Table 4 Randomized controlled trials of neoadjuvant chemotherapy *vs* surgery alone for esophageal cancer

Studies	Histology	Treatment	*n*	MS (mo)	3-yr OS (%)	*P*
Schlag *et al*[57], 1992	SCC	CF	22	7		NS
		Surgery	24	6		
Nygaard *et al*[51], 1992	SCC	BC	44	7	3	NS
		Surgery	41	7	9	
Maipang *et al*[58], 1994	SCC	BVC	24	17	31	NS
		Surgery	22	17	36	
Law *et al*[59], 1997	SCC	CF	74	17	40	NS
		Surgery	73	13	13	
Kelsen *et al*[32], 1998	AC/SCC	CF	213	15	19[1]	NS
		Surgery	227	16	20[1]	
Ancona *et al*[60], 2001	SCC	CF	47	25	34[1]	NS
		Surgery	47	24	22[1]	
MRC[61], 2002	AC/SCC	CF	400	17	43	< 0.01
		Surgery	402	13	34	

[1]5-year OS. MS: Median survival; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; C: Cisplatin; F: Fluorouracil; B: Bleomycin; V: Vindesine; NS: Not significant; OS: Overall survival.

to two cycles of neoadjuvant cisplatin 80 mg/m^2 and infusional fluorouracil 1000 mg/m^2 per d for 4 d *vs* surgery alone. A rather striking distinction of this trial compared to others was that clinicians could give their patients neoadjuvant radiotherapy (25-32.5 Gy) irrespective of randomization, and 9% of patients on each arm received radiotherapy. R0 resections were reported in 60% of assessable patients that were treated with neoadjuvant chemotherapy *vs* 54% of patients treated with surgery alone ($P < 0.0001$). OS was also improved in the neoadjuvant group (HR: 0.79, 95% CI: 0.67-0.93, $P = 0.004$), with a median OS of 16.8 mo *vs* 13.3 mo, respectively[61]. Another large trial by Kelsen *et al*[32] has evaluated neoadjuvant chemotherapy in the Intergroup (INT) 0113 study with 440 patients, however, reported no difference in OS was reported. Two large meta-analyses also have failed to demonstrate a survival advantage with neoadjuvant chemotherapy[64,65], although another meta-analysis by Gebski *et al*[66] has reported a statistically significant OS benefit with neoadjuvant chemotherapy (HR: 0.90, 95% CI: 0.81-1.00, $P = 0.05$), which corresponds to a 2-year absolute survival benefit of 7%. Caveats to this meta-analysis are that no statistically significant benefit was seen for patients with SCC treated with neoadjuvant chemotherapy (HR: 0.88, 95% CI: 0.75-1.03, $P = 0.12$) and that, although there was a benefit seen with AC (HR: 0.78, 95% CI: 0.64-0.95, $P = 0.014$), these results were based solely on the single trial whose data were available for review - the MRC trial[61,66].

At least four separate trials have compared cisplatin-based perioperative regimens (neoadjuvant and adjuvant chemotherapy) to surgery alone in esophageal cancer[32,67-69] (Table 5). Those that focused solely on esophageal cancer did not reveal survival benefits[32,67], whereas the two that included patients with AC of the stomach and GEJ did show such a benefit[68,69]. The largest of these, published by Cunningham and colleagues, randomized 503 patients with AC to three preoperative and three postoperative courses of epirubicin 50 mg/m^2 and cisplatin 60 mg/m^2 with infusional fluorouracil 200 mg/m^2 per day for 21 d *vs* surgery alone. Although the majority of patients had gastric AC, approximately 26% of the patients enrolled had AC of the GEJ or distal esophagus. Despite the fact that 58% of patients were unable to tolerate all six cycles of chemotherapy, the perioperative chemotherapy group had a statistically significant higher likelihood of OS compared to those treated with surgery alone (HR: 0.75, 95% CI: 0.60-0.93, $P = 0.009$), with an improved median OS (24 mo *vs* 20 mo) and 5-year OS (36% *vs* 23%). Although postoperative complications were not increased (46% *vs* 45%), there was also no difference in the rate of R0 resection (69% *vs* 66%) or pCR (both 0%). Importantly, there was no evidence of heterogeneity of treatment effect based on the location of the primary tumor[68].

Chemoradiotherapy

Chemotherapy in conjunction with radiotherapy was

Table 5 Randomized controlled trials of perioperative chemotherapy *vs* surgery alone for esophageal cancer

Studies	Histology	Treatment	*n*	MS (mo)	5-yr OS (%)	*P*
Roth *et al*[67], 1988	AC/SCC	BVC	19	9	25	NS
		Surgery	20	9	5	
Kelsen *et al*[32], 1998	AC/SCC	CF	213[1]	15	19	NS
		Surgery	227	16	20	
Cunningham *et al*[68], 2006	AC[2]	ECF	250	24	36	NS
		Surgery	253	20	23	
Boige *et al*[69], 2007	AC[3]	CF	113[4]		38	< 0.05
		Surgery	111		24	

[1]Of 213 patients in the perioperative arm, only 66 later received adjuvant chemotherapy; [2]26% had AC of the GEJ and lower esophagus; [3]11% had esophageal AC; [4]Of 113 patients in the perioperative arm, only 54 later received adjuvant chemotherapy. MS: Median survival; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; B: Bleomycin; C: Cisplatin; V: Vindesine; F: Fluorouracil; E: Epirubicin; NS: Not significant; OS: Overall survival.

initially evaluated as a definitive treatment for patients deemed unable to proceed with surgery[70]. In combination, chemotherapy not only compliments but augments the effect of radiotherapy in a process known as radiation sensitization, secondary to synergistic DNA damage, cell cycle synchronization, and inhibition of repair and resistance pathways[71,72]. In addition to increasing the efficacy of radiotherapy and thus controlling local tumor growth, as mentioned earlier, chemotherapy theoretically also offers the ability to eradicate micrometastatic disease and decrease the risk of distant recurrence[73].

The seminal Radiation Therapy Oncology Group (RTOG) 85-01 trial has compared radiotherapy (50.4 Gy over 5 wk) with concurrent cisplatin 75 mg/m^2 and infusional fluorouracil 1000 mg/m^2 per day for 4 d to radiotherapy alone (64 Gy over 6.4 wk). The chemotherapy arm consisted of four cycles delivered every 4 wk during radiotherapy (cycles 1 and 2) and every 3 wk for the remainder (cycles 3 and 4). The study included 134 patients with 90% having SCC and all with T1-3 N0-1 M0 disease. The trial was closed early once an interim analysis revealed that there was a statistically significant survival advantage that favored concurrent chemoradiotherapy that later amounted to a 5-year OS of 27% *vs* 0%. There was no statistically significant difference in OS based on histology[70].

Although those who received concurrent chemoradiotherapy had a decreased risk of persistent disease or local recurrence compared to those who received radiotherapy alone in the RTOG 85-01 trial, the incidence of locoregional failure was still 47%[70], and the INT 0123 trial was launched in an effort to improve upon this, with the theory that higher doses of radiotherapy would be beneficial. A total of 236 patients with T1-3 N0-1 M0 disease were enrolled (85% with SCC) and randomized to high-dose radiotherapy (64.8 Gy) *vs* low-dose radiotherapy (50.4 Gy), with both arms receiving four cycles of concurrent chemotherapy (cisplatin 75 mg/m^2 and infusional fluorouracil 1000 mg/m^2 per day for 4 d every 4 wk). The INT 0123 trial was also stopped early after an interim analysis failed to reveal a significant difference in median OS (13 mo *vs* 18.1 mo), 2-year survival (31% *vs* 40%), or locoregional persistence/recurrence of disease (56% *vs* 52%) between the high-dose and low-dose radiotherapy arms, respectively[74]. With such unacceptably high locoregional failure rates with definitive chemoradiotherapy, in addition to the dismal prognosis of patients treated with surgical resection alone[32-35], numerous trials were begun to evaluate multimodality treatments that combine chemotherapy, radiotherapy, and surgical resection.

To date, at least nine randomized phase Ⅲ clinical trials have compared neoadjuvant chemoradiotherapy with surgery alone[33,51,75-82] (Table 6). These trials incorporated multiple chemotherapy regimens, doses of radiotherapy used (20-50.4 Gy), and timing of radiotherapy with regard to chemotherapy (sequential *vs* concurrent), in addition to differing by surgical procedures performed and histological types of esophageal cancer enrolled (AC, SCC, or both). Only two of these trials have revealed a significant survival benefit that favored multimodality treatment, and neither was without its imperfections[77,81]. Walsh and colleagues randomized 113 patients with AC to two courses of neoadjuvant cisplatin 75 mg/m^2 and fluorouracil 15 mg/kg per day for 5 d with concurrent radiotherapy (40 Gy over 3 wk) or to surgery alone. The median OS was 16 mo *vs* 11 mo (P = 0.01) with a 3-year OS of 32% *vs* 6% (P = 0.01), which favored the multimodality treatment arm[77]. This single-institution-based trial, however, has been heavily criticized for an OS of patients with localized esophageal cancer treated with surgery alone (6%) that was far inferior to historical controls[32].

The second study, the Cancer and Leukemia Group B 9781 trial, was closed early with only 56 of an expected 500 patients enrolled, secondary to poor accrual that was reportedly due to the unwillingness of many patients and physicians to enroll in the control surgery-alone arm. Patients were randomly assigned to two cycles of cisplatin 100 mg/m^2 and fluorouracil 1000 mg/m^2 per day for 4 d with concurrent radiotherapy (50.4 Gy over 5.5 wk) prior to surgery, or to surgery alone. An impressive 5-year OS of 39% *vs* 16% was reported with a median OS of 4.48 years *vs* 1.79 years (P = 0.002), respectively. Although the obvious clinical significance of these findings is hard to dispute, a trial with more robust participation would have gone a long way to alleviate any uncertainties regarding the best treatment strategy for resectable esophageal cancer[81].

Table 6 Randomized controlled trials of neoadjuvant and adjuvant chemoradiotherapy *vs* surgery alone for esophageal cancer

Studies (yr)	Histology	Treatment	*n*	MS (mo)	5-yr OS (%)	*P*
Nygaard *et al*[51], 1992[1]	SCC	BC + 35 Gy	47	8	17[3]	NS
		Surgery	41	7	9[3]	
Apinop *et al*[75], 1994[1]	SCC	CF + 20 Gy	35	10	24	NS
		Surgery	34	7	10	
Le Prise *et al*[76], 1994[1]	SCC	CF + 20 Gy	41	10	19[3]	NS
		Surgery	45	11	14[3]	
Walsh *et al*[77], 1996[1]	AC	CF + 40 Gy	58	16	32[3]	< 0.05
		Surgery	55	11	6[3]	
Bosset *et al*[33], 1997[1]	SCC	C + 37 Gy	143	19	7	NS
		Surgery	139	19	9	
Urba *et al*[78], 2001[1]	AC/SCC	CFV + 45 Gy	50	17	20	NS
		Surgery	50	18	10	
Lee *et al*[79], 2004[1]	SCC	CF + 45 Gy	51	28	49[3]	NS
		Surgery	50	27	41[3]	
Burmeister *et al*[80], 2005[1]	AC/SCC	CF + 35 Gy	128	22	17	NS
		Surgery	128	19	13	
Tepper *et al*[81], 2008[1]	AC/SCC	CF + 50.4 Gy	30	54	39	< 0.01
		Surgery	26	21	16	
Macdonald *et al*[82], 2001[2]	AC[4]	F + 45 Gy	281	36	50[3]	< 0.01
		Surgery	275	27	41[3]	

[1]Neoadjuvant chemoradiotherapy; [2]Adjuvant chemoradiotherapy; [3]3-year OS; [4]20% of patients enrolled had AC of the gastroesophageal junction (GEJ). MS: Median survival; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; B: Bleomycin; C: Cisplatin; F: Fluorouracil; V: Vindesine; NS: Not significant; OS: Overall survival.

With such inconclusive and often contradictory results in trials that have evaluated neoadjuvant multimodality treatment based on disparate study populations, a myriad of regimen protocols, and more importantly, small numbers of patients, numerous meta-analyses have subsequently been performed in an effort to synthesize these data into larger pools and discover if a survival benefit exists[66,83-87]. One of the first, published by Urshel and Vasan, included nine randomized controlled trials with 1116 patients and reported a 3-year survival benefit that favored neoadjuvant chemoradiotherapy (OR: 0.66, 95% CI: 0.47-0.92, $P = 0.016$), which was most pronounced when the chemotherapy and radiotherapy were given concurrently (OR: 0.45, 95% CI: 0.26-0.79, $P = 0.005$) instead of sequentially (OR: 0.82, 95% CI: 0.54-1.25, $P = 0.36$). Although patients who received neoadjuvant chemoradiotherapy were less likely to proceed to surgery (OR: 2.50, 95% CI: 1.05-5.96, $P = 0.038$), they were still more likely to have an R0 resection (OR: 0.53, 95% CI: 0.33-0.84, $P = 0.007$) with 21% having a pCR. Although there was a decreased risk of local-regional recurrence for those who received multimodality treatment compared to those who received surgery alone (OR: 0.38, 95% CI: 0.23-0.63, $P = 0.0002$), there was no difference in risk for distant recurrence. There was a statistically insignificant but nonetheless concerning trend toward increased treatment mortality (OR: 1.63, 95% CI: 0.99-2.68, $P = 0.053$)[84]. The most recent meta-analysis published by Gebski and colleagues has evaluated 1209 patients in 10 trials, and likewise found a statistically significant benefit with neoadjuvant chemoradiotherapy compared to surgery alone, with a 19% decreased risk of death (HR: 0.81, 95% CI: 0.70-0.93, $P = 0.002$) for both AC and SCC, which corresponded to a 13% absolute difference in survival at 2 years[66].

As noted earlier, Gebski *et al*[66] also have evaluated neoadjuvant chemotherapy compared to surgery alone in a meta-analysis. These separate meta-analyses have been published at the same time in conjunction with each other. Although the two neoadjuvant chemotherapy and chemoradiotherapy data pools are not directly comparable, the absolute survival benefit of chemotherapy appears to be less than that of chemoradiotherapy (7% *vs* 13% at 2 years). This point was further supported although not confirmed by Stahl *et al*[88] who randomized 126 patients with AC of the GEJ (55% were type I GEJ tumors) to 16 wk neoadjuvant chemotherapy using cisplatin and leucovorin-modulated fluorouracil, or 12 wk of the same regimen followed by 3 wk of cisplatin and etoposide with concurrent radiotherapy (30 Gy) prior to surgical resection. Those treated with multimodality neaodjuvant chemoradiotherapy did not have a significant increase in R0 resection (72% *vs* 70%), but did have an increased probability of achieving a pCR (15.6% *vs* 2%, $P = 0.03$) and having tumor-free lymph nodes at the time of resection (64% *vs* 38%, $P = 0.01$) compared to those treated with neoadjuvant chemotherapy. There was a trend toward improved 3-year OS (47% *vs* 28%, $P = 0.07$), which favored neoadjuvant chemoradiotherapy, but with just a third of the expected 354 patients enrolled in the trial prior to its closure due to poor accrual, there was no statistically significant difference noted.

Anecdotally, patients with esophageal cancer often lack the strength to complete adjuvant chemoradiotherapy, although there are data to support its use and tolerability in patients with tumors of the GEJ[82]. The U.S. INT 0116 trial enrolled 556 patients with resected AC of the stomach and GEJ; approximately 20% of those participating had GEJ tumors. Patients were randomized to either sur-

gery alone or surgery followed by four cycles of adjuvant leucovorin-modulated fluorouracil, with the second cycle concurrent with radiotherapy (45 Gy). The median OS was 27 mo *vs* 36 mo (HR: 1.35, 95% CI: 1.09-1.66, P = 0.005), which favored the adjuvant chemoradiotherapy arm. Although 17% of patients were unable to finish the protocol because of treatment-related toxicity, an impressive 64% of patients were able to finish the protocol completely. There was no difference in survival based on the location of the primary tumor[82].

Targeted therapy

Despite improvements seen with the multimodality treatment of esophageal cancer, cure rates remain disappointingly low[66]. As such, targeted agents that have been found to benefit patients with head and neck, breast, lung, colon, and pancreatic cancers have generated intense interest in esophageal cancer[89-91]. Multiple pathways have been evaluated at the molecular level with potential targets in esophageal cancer including cyclin-dependent kinases, nuclear factor κB, matrix metallopreoteinases, and the inhibition of *COX-2*. The most promising targets at present, however, appear to be the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF)[89].

There are at least four types of EGFR: EGFR (human EGFR-1, HER-1), HER-2, HER-3, and HER-4. EGFR signaling plays a crucial role in modulating cell proliferation, invasion, metastasis, and resistance to cell death[89]. Overexpression of EGFR proteins has been reported in 30%-70% of AC and SCC of the esophagus, with such overexpression correlating with more aggressive disease and worse outcome[92-94]. Multiple clinical trials have been launched in an effort to target EGFR in esophageal cancer, with the most common drug used being the IgG1 monoclonal antibody cetuximab[95-99]. A trial by Gold *et al*[95] using cetuximab as a second-line monotherapy in the metastatic setting was discouraging, although regimens using cetuximab in combination with FOLFIRI[96], cisplatin and docetaxel[97], and cisplatin and fluorouracil[98] have revealed that the drug shows promise in the treatment of esophageal cancer. A phase Ⅱ trial by Safran *et al*[99] has evaluated 57 patients with esophageal cancer that were treated with weekly carboplatin, paclitaxel and cetuximab with concurrent radiotherapy (50.4 Gy). Seventy percent of patients achieved a complete clinical response and, of the 49 patients who went on to surgery, 27% had a pCR. The RTOG 0436 trial - a phase Ⅲ trial that is evaluating carboplatin, paclitaxel, and concurrent radiotherapy with or without cetuximab - is currently ongoing.

Another EGFR that is more famously associated with breast cancer, HER-2, is also overexpressed in 19%-43% of patients with esophageal cancer, and can be targeted by trastuzumab - a humanized IgG1 monoclonal antibody against the same receptor[100]. The phase Ⅲ ToGA trial randomized 594 patients with locally advanced, recurrent, or metastatic gastroesophageal cancer with HER-2 overexpression to treatment with cisplatin and fluorouracil or capecitabine, with or without trastuzumab. The median OS was significantly improved and favored the arm that received trastuzumab (13.5 mo *vs* 11.1 mo, HR: 0.74, 95% CI: 0.60-0.91, P = 0.0048)[101]. How these results will affect future multimodality neoadjuvant treatment is unknown, especially considering the potential for cardiotoxicity in a patient population that is already at risk. Although there were no differences in symptomatic congestive heart failure between the two arms, the patients who received trastuzumab were more likely to experience asymptomatic decreases in their left ventricular ejection fraction (4.6% *vs* 1.1%)[101].

VEGF is a regulator of angiogenesis and is yet another potential target. Similar to EGFR, VEGF is also overexpressed in 30%-60% of esophageal cancer patients and is likewise associated with poor outcome[102]. There is even evidence to suggest that the level of VEGF expression increases during treatment with chemotherapy and radiotherapy, which makes it a particularly attractive target for multimodality neoadjuvant treatment[103,104]. Promising phase Ⅱ data with surgically unresectable AC of the GEJ combining bevacizumab - a humanized monoclonal antibody against VEGF - with cisplatin and irinotecan[105], as well as docetaxel, cisplatin and fluorouracil[106] are available, while trials that are incorporating neoadjuvant chemoradiotherapy with the addition of bevacizumab are currently ongoing[91]. As with trastuzumab, it is unknown how the potential toxicities inherent to bevacizumab - hypertension, thromboembolism, poor wound healing, bowel perforation, worsening arterial disease, and an increased risk of bleeding - will affect the treatment of esophageal cancer patients who often present with multiple comorbidities[107].

CONCLUSION

The optimal treatment strategy for resectable esophageal cancer is still a controversial topic. Multimodality neoadjuvant chemotherapy with concurrent radiotherapy has been accepted by many - although not all - as the standard of care, because such a regimen increases rates of pCR, R0 resection, and local tumor control, which all correlate with improved OS[33,66,77,78,81,84-86]. If one accepts the most recent meta-analysis, an absolute OS benefit exists but is likely to be just 13% at 2 years[66]. With such a small benefit, it is no wonder that the multiple underpowered clinical trials that have compared neoadjuvant chemoradiotherapy with surgery alone have found it difficult to demonstrate a survival difference.

Although such a survival benefit might seem small, it should be noted that it is in line with accepted treatment algorithms of other lethal malignancies, such as the addition of adjuvant chemotherapy in completely resected non-small cell lung cancer[108]. The need to treat approximately eight patients with a difficult-to-tolerate regimen to cure just one additional person is hardly ideal, yet these odds are not inconsequential when discussing them face-to-face with a patient who is at least felt to be sufficiently medically fit enough to withstand an esophagectomy.

Although neoadjuvant and perioperative chemother-

apy have also been found to be effective approaches for treating esophageal cancer, there is a reasonable amount of evidence to support the notion that such treatments are inferior to neoadjuvant chemoradiotherapy[66,88], while the data supporting adjuvant chemoradiotherapy can only be applied to patients with GEJ tumors at the present time[82]. How targeted therapy will affect our approach to resectable esophageal cancer is currently unknown as many of the trials to determine this are ongoing[91,99]. By participating in clinical trials and enrolling as many appropriate patients as we possibly can, these questions will hopefully be answered in a more timely and conclusive manner than previously seen in the history of esophageal cancer treatment.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3804

World J Gastroenterol 2010 August 14; 16(30): 3804-3810
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Transhiatal *versus* transthoracic esophagectomy for esophageal cancer

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

Esophageal cancer continues to represent a formidable challenge for both patients and clinicians. Relative 5-year survival rates for patients have improved over the past three decades, probably linked to a combination of improved surgical outcomes, progress in systemic chemotherapy and radiotherapy, and the increasing acceptance of multimodality treatment. Surgical treatment remains a fundamental component of the treatment of localized esophageal adenocarcinoma. Multiple approaches have been described for esophagectomy, which can be thematically grouped under two major categories: either transthoracic or transhiatal. The main controversy rests on whether a more extended resection through thoracotomy provides superior oncological outcomes as opposed to resection with relatively limited morbidity and mortality through a transhiatal approach. After numerous trials have addressed these issues, neither approach has consistently proven to be superior to the other one, and both can provide excellent short-term results in the hands of experienced surgeons. Moreover, the available literature suggests that experience of the surgeon and hospital in the surgical management of esophageal cancer is an important factor for operative morbidity and mortality rates, which could supersede the type of approach selected. Oncological outcomes appear to be similar after both procedures.

Key words: Esophageal cancer; Transhiatal esophageal resection; Transthoracic esophageal resection

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Barreto JC, Posner MC. Transhiatal *versus* transthoracic esophagectomy for esophageal cancer. *World J Gastroenterol* 2010; 16(30): 3804-3810 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3804.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3804

INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide, with a wide variation in its frequency between high- and low-incidence regions. There are two main histopathological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma. SCC is the most common subtype in several endemic regions of the world[1], with a high correlation to smoking and alcohol abuse, as well as chronic inflammation[2]. On the other hand, adenocarcinoma is commonly associated with Barrett's metaplasia, gastroesophageal reflux disease (GERD), and obesity[3]. It has become the most common subtype in the western hemisphere, and frequently involves the gastroesophageal junction (GEJ) and proximal stomach. SCC and adenocarcinoma of the esophagus are distinct entities and should be considered as such when defining optimal therapy. As

a result of its increasing incidence[4] and relationship with GERD, the following review focuses on adenocarcinoma of the esophagus.

Despite improvements in systemic chemotherapy and radiotherapy, and the increasing acceptance of multimodality treatment that have resulted in enhanced survival rates over the past three successive decades[5], surgical resection continues to be the mainstay of care for treatment of localized esophageal adenocarcinoma. Multiple approaches have been described for esophagectomy, and they can be thematically categorized under two major headings: transthoracic or transhiatal. The transthoracic procedure is performed more commonly by means of combined laparotomy and right thoracotomy (Ivor Lewis procedure). Other options include left thoracotomy with or without cervical incision, a single left thoracoabdominal incision, or a three-incision resection with a cervical anastomosis (McKeown procedure). The transhiatal approach is performed through midline laparotomy and left cervical incision. There has been considerable controversy about which procedure provides the best short- and long-term outcomes. The discussion centers around whether more extended resection through thoracotomy provides superior oncological outcomes than resection with relatively limited morbidity and mortality through a transhiatal approach. Decisions regarding surgical technique are frequently based on personal bias, surgeons' experience and comfort with a procedure. The issue of the extent of surgical resection is addressed first, with a brief description of each approach. The relevance of surgeon/hospital volume and its relationship with adequate outcomes after esophagectomy, and the role of surgery in the context of multimodality treatment are discussed separately.

TRANSTHORACIC ESOPHAGECTOMY

Transthoracic esophagectomy is most commonly performed *via* laparotomy followed by right thoracotomy and intrathoracic anastomosis (Ivor Lewis procedure). It was originally described in 1946 in two stages[6], and historically, it is the standard procedure against which all other techniques are measured. Left thoracotomy or thoracoabdominal incision provides adequate exposure to the distal esophagus, but presents greater difficulty to access the upper and middle thirds and to perform an anastomosis high in the chest.

Ivor Lewis esophagectomy starts through a midline incision in the abdomen. The left lobe of the liver is mobilized and retracted laterally, and the stomach is fully mobilized and freed from its vascular attachments, including an upper abdominal lymphadenectomy, while preserving the right gastroepiploic and right gastric vessels on whose pedicle the reconstructive conduit is based. The duodenum is mobilized completely *via* a Kocher maneuver and a pyloric drainage procedure is performed, to diminish gastric stasis and minimize aspiration[7,8]. The right diaphragmatic crus is divided with electrocautery to allow access to the mediastinum and to avoid constricting the transposed stomach. Placement of a feeding jejunostomy is commonly performed before abdominal closure and repositioning for the thoracic component of the procedure. Muscle-sparing right lateral thoracotomy is then performed through the fifth intercostal space. The mediastinal pleura that overlies the esophagus is incised, the azygos vein is divided, the intrathoracic esophagus is mobilized, and *en bloc* resection of the surrounding periesophageal tissue is performed, including mediastinal lymph node dissection.

After division of the proximal esophagus in the chest to ensure an adequate margin, the GEJ and stomach are transposed into the thoracic cavity. A gastric conduit is now created, with a linear stapler parallel to the greater curve, and the fundus is removed with a portion of the lesser curvature. The specimen is removed, and an esophagogastric anastomosis is performed. The McKeown procedure is an alternative three-incision approach, in which right thoracotomy is the initial stage of the procedure, followed by repositioning of the patient in the supine position for abdominal and left cervical incision, to achieve a cervical esophagogastric anastomosis.

The theoretical advantage of the transthoracic approach is a more thorough oncological operation as a result of direct visualization and exposure of the thoracic esophagus, which allows a wider radial margin around the tumor and more extensive lymph node dissection. However, the combined effects of an abdominal and thoracic incision might compromise cardiorespiratory function, especially in patients with coexisting lung or heart disease. The other disadvantage is that an intrathoracic anastomotic leak can lead to catastrophic consequences including mediastinitis, sepsis, and death. The three-incision modification of the procedure effectively eliminates the potential for complications associated with an intrathoracic esophagogastric anastomosis.

The perioperative mortality of transthoracic esophagectomy in experienced centers ranges from 9% to as low as 1.4%[9-15]. Five-year survival in approximately 25% of patients who undergo transthoracic esophageal resection has been reported. These reports include heterogeneous populations of patients with esophageal cancer that underwent a variety of surgical approaches, the use of adjuvant treatment in some but not all patients, and combined histologies (SCC and adenocarcinoma).

TRANSHIATAL ESOPHAGECTOMY

Transhiatal esophagectomy was first performed by Turner in 1933 for esophageal carcinoma[16]. During subsequent decades, it was not routinely performed since the transthoracic approach was preferred after general anesthesia became available. In 1978, Orringer described his initial series of blunt transhiatal esophagectomy, which kindled new interest in this procedure[17]. It has gained favor among surgeons concurrent with the rising incidence of adenocarcinoma of the distal esophagus, which is readily approachable through the diaphragmatic hiatus.

The abdominal portion of the procedure duplicates that of the previously described transthoracic approach

and includes mobilization of the stomach, pyloromyotomy and placement of a feeding jejunostomy. Again, cautery division of the right crus allows access to the mediastinum and dissection under direct vision of the distal and middle third of the esophagus. A left cervical incision along the anterior border of the sternocleidomastoid muscle provides exposure to the cervical esophagus. Circumferential dissection of the cervical esophagus is carried down to below the thoracic inlet, and blunt dissection is continued into the superior mediastinum to mobilize the upper thoracic esophagus, with care to avoid injury to the recurrent laryngeal nerve in the tracheoesophageal groove. The remainder of the dissection at the level of and superior to the carina is completed by blunt dissection through the esophageal hiatus. The cervical esophagus is then divided, the stomach and attached intrathoracic esophagus are delivered through the abdominal wound, and a gastric conduit is fashioned using a linear stapling device in the same manner as described above. The gastric tube is delivered through the posterior mediastinum to the cervical wound, where a cervical esophagogastric anastomosis is performed. The stomach is considered by most surgeons as the ideal replacement for the resected esophagus, although a segment of colon or a free flap of small bowel can be used as alternative conduits[18,19].

The postulated advantages of the transhiatal approach to esophagectomy are avoidance of a thoracotomy incision, which thereby minimizes pain and subsequent postoperative pulmonary complications; elimination of potentially life threatening mediastinitis as a result of an intrathoracic anastomotic leak; and a shorter duration of operation, which potentially results in decreased morbidity and mortality[17]. Leak of a cervical esophagogastric anastomosis can be handled in the vast majority of patients with opening of the cervical wound, followed by local wound care. Compared to transthoracic esophagectomy, transhiatal esophagectomy is associated with poor visualization of upper and middle thoracic esophageal tumors (potentially compromising the oncological integrity of the operation), increased anastomotic leak rate with subsequent stricture formation, and a higher risk of recurrent laryngeal nerve injury[20,21].

The reported postoperative mortality after transhiatal esophagectomy in individual series tends to be slightly lower than that of the transthoracic approach, between 1% and 7.5%[22-26], and 5-year survival rate is approximately 25%, which is not substantially different from that accomplished after the transthoracic approach. Orringer *et al*[26] have reported the most extensive experience with transhiatal esophagectomy. Their latest report involved 2007 patients, of which 1525 had a diagnosis of cancer. Seventy-two percent had adenocarcinoma, and 38% received neoadjuvant chemoradiation, with a 5-year survival rate of 29%. Among this series, their most recent group of 944 patients had a hospital mortality of 1%. The anastomotic leak rate was 9% in this same group, and recurrent laryngeal nerve injury occurred in 2% of cases. These results reflect those reported from other surgical series of transhiatal esophagectomy.

Table 1 Meta-analysis comparing transthoracic and transhiatal esophagectomy

Meta-analysis	Rindani *et al*[20]	Hulscher *et al*[21]
No. of patients	5483	7527
Postoperative mortality (%)		
TT	9.5	9.2
TH	6.3	5.7
Intraoperative blood loss (mL)		
TT	1171	1001
TH	1311	728
Hospital stay (d)		
TT	19.8	21
TH	19.5	17.8
Pulmonary complications (%)		
TT	25	18.7
TH	24	12.7
Cardiac complications (%)		
TT	10.5	6.6
TH	12.4	19.5
Anastomotic leakage (%)		
TT	10	7.2
TH	16	13.6
Vocal cord paralysis (%)		
TT	4.8	3.5
TH	11.2	9.5
5-yr OS (%)		
TT	26	23
TH	24	21.7

TT: Transthoracic esophagectomy; TH: Transhiatal esophagectomy; OS: Overall survival.

STUDIES COMPARING TRANSTHORACIC *VS* TRANSHIATAL ESOPHAGECTOMY

The question of one approach being superior to the other continues to generate considerable controversy among surgeons. No definitive advantage in oncological outcome or postoperative morbidity and mortality can be concluded from the non-comparative case series mentioned above.

Two large meta-analyses have addressed these issues by utilizing collective reviews of numerous individual studies that have compared transhiatal esophagectomy to transthoracic esophagectomy[20,21]. Most of the studies included in these meta-analyses were retrospective in nature and were not consistent with respect to the surgical technique utilized and which therapy in addition to surgery was delivered. Nevertheless, the results of both were very similar.

The meta-analysis by Rindani *et al*[20] included almost 5500 patients from 44 series published between 1986 and 1996 (Table 1). The statistical analysis was descriptive rather than comparative due to the diverse nature of the series, and there was only one prospective randomized trial included, with a small sample and short follow-up. Postoperative respiratory and cardiovascular complications were almost identical between the two groups. The transhiatal group had a higher incidence of anastomotic leaks and recurrent laryngeal nerve injuries. Thirty-day mortality was 6.3% after transhiatal and 9.5% after transthoracic resection, but survival at 5 years was equivalent between the two procedures.

The second meta-analysis, by Hulscher *et al*[21], involved

Table 2 Randomized trials comparing transthoracic and transhiatal esophagectomy

Meta-analysis	Goldminc *et al*[27]	Chu *et al*[28]	Jacobi *et al*[29]	Hulscher *et al*[30,31]
No. of patients	67	39	32	220
Postoperative mortality (%)				
TT	8.6	0	6	4
TH	6.2	0	6	2
Intraoperative blood loss (mL)				
TT	[1](2.3 units transfused)	671	2270	1900
TH	[1](2.3 units transfused)	724	1000	1000
Hospital stay (d)				
TT	18	27	21	19
TH	20.5	18	23	15
Postoperative pneumonia (%)				
TT	20	0	31	57 (atelectasis included)
TH	19	10	19	27 (atelectasis included)
Cardiac complications (%)				
TT	[1]	15.8	19	26
TH	[1]	15	31	16
Anastomotic leakage (%)				
TT	9	0	12.5	16 (subclinical included)
TH	6	0	12.5	14 (subclinical included)
Vocal cord paralysis (%)				
TT	3	[1]	[1]	21 (transient)
TH	3	[1]	[1]	13 (transient)
Reported survival (%)				
TT	22 at 3 yr	Median survival 13.5 mo	77 at 1 yr	36 at 5 yr
TH	30 at 3 yr	Median survival 16 mo	70 at 1 yr	34 at 5 yr

[1]Data not reported or did not occur. TT: Transthoracic esophagectomy; TH: Transhiatal esophagectomy.

over 7527 patients derived from 50 studies from 1990 to 1999 (Table 1). Six were prospective comparative studies, three of which were randomized, all with a relatively small sample size. None of these three studies could demonstrate a significant difference in morbidity, mortality, or long-term survival[27-29]. When all 50 studies were analyzed, no significant differences were demonstrated in the overall morbidity rate. Blood loss was higher after transthoracic esophagectomy, and it had a higher risk of pulmonary complications, chylous leakage (2.4% *vs* 1.4%) and wound infection (7.7% *vs* 4.3%). Similar to the previous meta-analysis, transhiatal esophagectomy had a higher incidence of anastomotic leakage and recurrent laryngeal nerve injury. Length of stay in the intensive care unit (ICU) and hospital were longer in the transthoracic group, and in-hospital mortality was significantly higher as well. Again, there was no difference in 5-year survival rates.

There have been a total of four randomized trials that have compared both techniques (Table 2). Three of them, included in the previous meta-analyses described above, could not provide definitive conclusions and each was hampered by an extremely small sample size, with non-significant differences reported between the two arms[27-29].

The fourth randomized trial, published in 2002 by Hulscher *et al*[30], has provided level I evidence regarding this controversial issue. Two hundred and twenty patients were assigned to either transhiatal or transthoracic esophagectomy with cervical anastomosis. The transthoracic esophagectomy procedure included *en bloc* resection of the thoracic duct, azygos vein, ipsilateral pleura, and all peri-esophageal tissue in the mediastinum, including a formal lymphadenectomy. Transhiatal esophagectomy had a shorter operative duration than transthoracic esophagectomy (3.5 h *vs* 6 h), with lower blood loss (1 L *vs* 1.9 L). Perioperative morbidity rate was also lower in the transhiatal group (pulmonary complications, 57% *vs* 27%; chylous leakage, 10% *vs* 2%). Duration of mechanical ventilation, ICU stay and hospital stay were all shorter in the transhiatal group. However, there was no significant difference in hospital mortality (transthoracic: 4%; transhiatal: 2%). Although initially a trend toward a survival benefit with transthoracic approach was seen, after longer follow-up, no difference in 5-year overall survival was found (transthoracic: 36%; transhiatal 34%). Notably, the transthoracic approach was of benefit in some subgroups; patients with 1-8 positive lymph nodes had better disease-free survival rate (64% *vs* 23%), and patients with tumors arising from the distal esophagus (rather than gastric cardia) tended towards a survival benefit (51% *vs* 37%, not statistically significant)[31]. However, this phase Ⅲ study was not adequately powered to address these subgroup analyses.

A large population-based study has been published recently, which has evaluated the results of both approaches through the Surveillance, Epidemiology and End Results (SEER) - Medicare linked database from 1992 to 2002[32]. A lower operative mortality was found after transhiatal esophagectomy (6.7% *vs* 13.1%). Although observed 5-year survival was higher after transhiatal esophagectomy, after adjusting for stage, patient and provider factors, no significant 5-year survival difference was found.

These data suggest that perioperative and oncological outcomes are not substantially influenced by the surgical approach to esophagectomy, and that either procedure is associated with acceptable results in the hands of expe-

rienced surgeons. Ideally, surgeons and hospitals treating patients with esophageal carcinoma should have expertise in both techniques. Some patients might even benefit from an individualized approach. For an older or higher-risk surgical patient, for whom perioperative recovery is an even greater concern than usual, a transhiatal approach could confer an advantage. In a fit patient with evidence of a limited number of involved lymph nodes, there is some evidence (although not level I evidence) that suggests a benefit in survival with the transthoracic approach. Still, available literature suggests that experience of the surgeon and hospital is likely to be a more important factor than is the type of approach selected.

SURGEON/HOSPITAL VOLUME AND ESOPHAGECTOMY

There is increasing evidence that confirms that patients who undergo complex oncological resections, such as esophagectomy, at high-volume hospitals by experienced surgeons have significantly lower rates of perioperative morbidity and mortality[33-35]. This association has been shown for several surgical procedures in studies that have used health-services-linked databases. However, the association between volume and outcome for esophagectomy is one of the strongest among all complex cancer operations[33-35]. Furthermore, a recent analysis of the SEER - Medicare linked data base[36] suggests that long-term survival, and therefore oncological outcome, is also volume dependent. The probability of surviving 5 years following esophagectomy in high-volume hospitals was 34%, whereas 5-year survival probability in low-volume hospitals was only 17%. This 17% absolute difference in 5-year survival following esophagectomy between high-volume and low-volume hospitals was the highest amongst all cancer resections surveyed. Volume-dependent discrepancy in 5-year survival could not be attributed to differences in the delivery of adjuvant therapy. Therefore, not only are short term procedure-related outcomes associated with surgical experience but long-term oncological outcomes might also be affected by surgeon and center volume/experience with esophageal resection. The basis for this improved survival has not been defined and requires further investigation.

ROLE OF SURGERY IN THE MULTIMODALITY THERAPY ERA

Relative 5-year survival rates for patients with esophageal cancer have improved over the past three successive decades[5,37]. The reasons for this trend are surely multifactorial and could include the widespread acceptance and use of a multimodality treatment approach, improved surgical outcomes, and progress made in systemic chemotherapy and radiotherapy.

Based on the current level I evidence, it can be reasonably argued that the addition of surgery to an effective regimen of chemoradiotherapy in patients with SCC of the esophagus might not improve outcome. Two randomized trials have addressed the role of chemoradiotherapy alone *vs* chemoradiotherapy followed by surgery in patients with SCC. The German Esophageal Cancer Study Group[38] has demonstrated better 2-year local, progression-free survival in the surgical group (64.3% *vs* 40.7%), although with increased treatment-related mortality (12.8% *vs* 3.5%), and equivalent overall survival between the two treatment groups. The FFCD 9102 trial[39], in which 90% of the patients had a diagnosis of SCC, found a higher frequency of locoregional relapse in the chemoradiotherapy alone group, but with a lower 3 mo mortality rate. As in the German study, survival rates were similar in both groups.

In contrast to SCC, the controversy regarding patients with esophageal adenocarcinoma has been centered on the added value of preoperative combined modality therapy, and not the necessity of surgical resection. Despite the fact that numerous phase Ⅲ trials[14,40-43] have compared preoperative chemoradiotherapy followed by surgery to surgery alone, it is not clear that preoperative chemoradiotherapy can be declared as a standard of care.

One randomized trial from Ireland[41] has shown a benefit in patients with adenocarcinoma, but definitive conclusions are hampered by the small sample size, unusually poor results with surgery alone, and short follow-up. More recently, the Cancer and Leukemia Group B initiated a trial that was closed prematurely due to poor accrual[43]. The most common histological tumor subtype in this study was adenocarcinoma. Reported median survival (4.48 years *vs* 1.79 years) and 5-year survival (39% *vs* 15%) favored trimodality therapy. Its major limitation was the incredibly small patient sample size due to poor accrual, although the findings had statistical significance.

Although the survival benefits have not been consistent, the majority of patients are down-staged with preoperative chemoradiotherapy, and for those patients who have a substantial response (complete pathological or major partial response defined by residual microscopic disease in the resected specimen) to preoperative chemoradiotherapy, there is a survival advantage. Surgery appears to be a crucial component of combined modality therapy to eliminate residual disease following chemoradiotherapy that leads to improved locoregional control and improved long-term survival. However, failure at a distant site is common and is the most frequent cause of death.

Even though the evidence for the benefit of preoperative chemoradiotherapy in the treatment of patients with esophageal cancer is not compelling, the combined modality approach has gained acceptance in most centers in the United States, and is by far the most frequent therapeutic option offered to patients with cancer of the esophagus. A meta-analysis has reported that preoperative chemoradiotherapy improved 3-year survival by 13% over surgery alone with similar improvement identified in patients with either SCC or adenocarcinoma histology[44]. Although the role of surgery has been questioned, especially for SCC, it can be reasonably concluded that esophageal resection remains an important, if not the most impor-

tant, therapeutic component of a combined modality approach to esophageal cancer. However further refinement of our treatment of patients with esophageal cancer is warranted. Patients who achieve a complete pathological response to combined chemoradiotherapy probably will obtain no advantage from undergoing esophagectomy, considering the substantial morbidity and mortality associated with the procedure. Unfortunately, current diagnostic methods are not reliable to identify this group of patients preoperatively. In contrast, it is reasonable to expect that patients with residual disease, either apparent or occult, following preoperative combined modality treatment will benefit from eradicating that residual disease with resection to give them the best opportunity for a long-term disease-free state. Surgeons interested in this lethal disease should direct their efforts to more accurate identification of those patients that will likely benefit from different single or combination treatment modalities, and tailor their therapeutic interventions accordingly.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3811

World J Gastroenterol 2010 August 14; 16(30): 3811-3815
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco G Patti, MD, Professor, Director, *Series Editor*

Minimally invasive esophagectomy

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Received: April 24, 2010 Revised: June 7, 2010
Accepted: June 14, 2010
Published online: August 14, 2010

Abstract

Esophageal resection is associated with a high morbidity and mortality rate. Minimally invasive esophagectomy (MIE) might theoretically decrease this rate. We reviewed the current literature on MIE, with a focus on the available techniques, outcomes and comparison with open surgery. This review shows that the available literature on MIE is still crowded with heterogeneous studies with different techniques. There are no controlled and randomized trials, and the few retrospective comparative cohort studies are limited by small numbers of patients and biased by historical controls of open surgery. Based on the available literature, there is no evidence that MIE brings clear benefits compared to conventional esophagectomy. Increasing experience and the report of larger series might change this scenario.

Key words: Esophageal resection; Transhiatal esophagectomy; Transthoracic esophagectomy; Esophageal cancer; Minimally invasive esophagectomy; Laparoscopy; Thoracoscopy

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Herbella FA, Patti MG. Minimally invasive esophagectomy. *World J Gastroenterol* 2010; 16(30): 3811-3815 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3811.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3811

INTRODUCTION

Esophageal cancer is a devastating disease. It was estimated in 2002 that 462 117 individuals developed the disease and 385 892 died worldwide[1], which corresponds to a mortality rate of 83.5%. Surgery has been considered an essential part of the treatment of patients with esophageal adenocarcinoma. However, surgery has been traditionally associated with a high morbidity and mortality rate. A lot of progress has been made since Earlam and Cunha-Melo in 1980 reviewed the literature and reported 29% mortality for esophagectomy[2]. Recent series have shown much improved rates, but they are still far from ideal. For these reasons, minimally invasive esophagectomy (MIE) brought high hopes to this field.

This final paper from a seminar on heartburn and adenocarcinoma focuses on the minimally invasive approach to esophagectomy; a treatment that is suitable for Barrett's esophagus and high-grade dysplasia and for esophageal adenocarcinoma.

TECHNIQUE

The techniques for esophagectomy can be simplistically described as those that include thoracotomy (transthoracic) and those without thoracotomy (transhiatal). The same classification can be used for MIE. According to the preferred approach, thoracotomy can be replaced by thoracoscopy and/or laparotomy can be replaced by laparoscopy. Thus, the following different combinations can be found in the literature: (1) transhiatal esophagectomy -

laparoscopy and cervicotomy[3,4]; (2) transthoracic esophagectomy (three-field) - laparoscopy, thoracoscopy and cervicotomy[5,6]; (3) transthoracic esophagectomy (three-field) - laparotomy, thoracoscopy and cervicotomy[6]; (4) transthoracic esophagectomy (three-field) - laparoscopy, thoracotomy and cervicotomy[7]; (5) transthoracic esophagectomy (Ivor Lewis) - laparoscopy and thoracoscopy[5]; (6) transthoracic esophagectomy (Ivor Lewis) - laparotomy and thoracoscopy[8]; and (7) transthoracic esophagectomy (Ivor Lewis) - laparoscopy and thoracotomy[9].

Laparoscopy

The laparoscopic approach to esophagectomy has the purpose of: (1) dissection of the abdominal esophagus and esophageal hiatus; (2) abdominal lymphadenectomy; (3) preparation of the stomach to replace the esophagus; (4) pyloroplasty or pyloromyotomy; and (5) placement of a feeding jejunostomy.

Dissection of the abdominal esophagus and esophageal hiatus follows the same principles of laparoscopic antireflux surgery. In summary, five abdominal ports are usually used. The abdominal esophagus and esophageal hiatus are dissected. The gastro-hepatic ligament is open, which preserves the right gastric artery. The greater curvature of the stomach is mobilized, which preserves the right gastroepiploic artery. The left gastric artery and coronary vein are isolated and divided with an endo-GIA stapler. A gastric conduit is constructed by dividing the stomach, starting on the lesser curvature and finishing at the angle of His. Pyloroplasty or pyloromyotomy is usually performed. The tip of the gastric conduit is sutured to the esophageal specimen that is retrieved through the neck or through the thorax if the anastomosis is performed in the chest[5,9,10]. Alternatively, the gastric conduit might be created through a mini-laparotomy[11]. The colon is rarely used for esophageal replacement during MIE.

Extended abdominal lymphadenectomy might be added to the procedure based on the philosophy adopted for the treatment of esophageal cancer by the surgeon. It is safe and feasible with a laparoscopic approach, after the lessons learned with laparoscopic treatment of gastric cancer[12].

Thoracoscopy

The thoracoscopic approach to esophagectomy has the purpose of: (1) dissection of the thoracic esophagus; (2) thoracic lymphadenectomy; and (3) esophageal anastomosis.

Dissection of the esophagus is performed using four ports in the right chest. Carbon dioxide insufflation is not considered necessary by most surgeons. The deflated lung is retracted anteriorly and the mediastinal pleura overlying the esophagus is divided. The azygos vein is then divided using an endo-GIA stapler with a vascular cartridge. A Penrose drain is placed around the esophagus to facilitate retraction. The esophagus is circumferentially mobilized from the esophageal hiatus up to the thoracic inlet. An esophageal anastomosis might be performed above the level of the azygos vein with the aid of a linear stapler. Otherwise, once the thoracoscopic dissection is completed, the operation can continue with cervicotomy, and the continuity of the digestive tract is restored with transposition of the stomach to the neck[5,9,10].

Similarly to the laparoscopic approach, extended mediastinal lymphadenectomy might be performed.

TECHNICAL VARIATIONS

Hand-assisted esophagectomy

Some surgeons perform transhiatal MIE using a laparoscopic approach to the abdomen but include a subxiphoid midline incision for manual mobilization of the mediastinal esophagus through a hand-port[5].

Prone position

Some surgeons have proposed a prone position for thoracoscopy instead of a left lateral decubitus approach[13,14]. This approach is used in order to improve ergonomics, operative time and pulmonary complications. The patient is placed in the prone position and the esophagus is approached through the right chest. The right lung is kept ventilated but it is collapsed due to the action of gravity and an 8-mmHg CO_2 pneumothorax[13,14].

Palanivelu *et al*[13] have reported an incidence of 2% for pleural and pulmonary complications in 130 patients. Fabian *et al*[15] have shown no differences in blood loss, number of lymph nodes dissected, and complications in two small cohorts of patients operated in left lateral decubitus *vs* prone position. However, operation time was significant shorter. Although good results have been reported, this technique is not widely accepted.

Robotic surgery

Robotic surgery claims to have the advantages of: (1) eliminating the counter-intuitive motion of standard laparoscopy; (2) aligning the eyes and hands over the area of interest with improved ergonomics; (3) increasing freedom of instrument movement by allowing wrist and finger movements that standard laparoscopic instruments do not have; (4) minimizing instrument tremor; and (5) 3D stereoscopic vision with dual camera technology[16]. Different types of esophageal operations have been performed with the aid of a robotic platform. Cases of robotic esophagectomy have been shown to be safe and feasible, either through thoracoscopy[17] or laparoscopy[4].

Early results have shown a conversion rate ranging from 0% to 15%[4,18]. Operating time is still high for transthoracic robotic esophagectomy, at an average of 7.5 h, which leads to a high incidence of pulmonary complications that decreases with experience[18]. Long-term outcomes are still elusive.

Vagal-sparing esophagectomy

Vagal-sparing esophagectomy is an attractive alternative to conventional procedures to avoid postoperative complications associated with vagotomy. Vagal-sparing

MIE has been described and popularized by the Portland Group[19]. The technique follows the same principles as open surgery: the vagal nerves are mobilized off the distal esophagus and stomach to the level of the pylorus; two nasogastric tubes are passed distally through the cervical esophagus and into the gastric remnant; the gastric remnant is divided and the nasogastric tubes are incorporated into the staple line; and finally, the esophagus is inverted, stripped out and removed through the cervicotomy[19].

OUTCOMES

Intraoperative complications are still frequent and they are the main cause for conversion to open surgery. During laparoscopy, bleeding is the main complication, either at the splenic hilum or parenchyma (often requiring splenectomy) or during division of gastric vessels at the time of the preparation of the gastric conduit[5]. Liver injury has also been reported[6,20]. During thoracoscopy, bleeding is reported as well[6]; however, the presence of pleural adhesions is the main cause for conversion[5,6]. Overall, the conversion rate ranges from 3% to 18%[5,6] with an average of 5%-7% depending on the technique[21,22].

Postoperative complications average 40%-50%, but can reach 80%[6,21,22]. Pleural and pulmonary complications still account for a significant proportion of morbidity; an average of 22%[22]. Nguyen *et al*[5] have reported, in a large series of 104 patients, that postoperative major morbidity occurred in 12.5%, especially anastomotic complications, staple line leaks and pulmonary complications. Minor complications occurred in an additional 15% of cases[5].

Review papers show a median length of intensive care unit stay of 2-5 d, and a median length of hospital stay of 9-18 d after MIE[21,22]. Mortality rate ranges between 0% and 4%[5,6,20-22].

COMPARISON WITH OPEN SURGERY

As far as we are aware, no randomized controlled trials have compared MIE and open esophagectomy to date. Available data suggests that MIE is similar but not superior to conventional esophagectomy.

Morbidity and mortality

MIE was expected to reduce the morbidity and mortality rate of esophageal resection when compared to conventional surgery. However, a recent meta-analysis[23] has shown similar results for major morbidity, pulmonary complications and mortality when MIE and open surgery are compared either to transhiatal or transthoracic esophagectomy. Nguyen *et al*[5] also have shown similar pulmonary complications when MIE and open cohorts were compared. Perry *et al*[24] have compared the outcomes of open and laparoscopic transhiatal esophagectomy in two sets of patients from different periods of time. They have found that lower intraoperative blood loss and overall length of hospital stay favor MIE. Complication rates were no different.

Cost

As far as we are aware, no studies have compared cost for MIE and open surgery. It is intuitive, however, that direct operative costs are higher for MIE, especially with the use of endoscopic staplers. Moreover, the clinical benefits of MIE are not yet proven to be greatly superior to open surgery in order to decrease indirect costs.

Oncological radicality

Advantages of minimally invasive techniques include a magnified view of the operative field. This advantage theoretically enhances the ability to perform more radical lymphadenectomy. In contrast, surgeons might be less confident to work close to important vascular structures without a tactile feeling and the possibility to use their hands to control bleeding. Reported experiences with different types of cancer, such as colon[25] and stomach[26], have shown a comparable number of lymph nodes retrieved when open or minimally invasive surgery are compared. MIE shows similar results. Decker *et al*[22] have shown a mean 10-27 lymph nodes were dissected in MIE, depending on the technique adopted, and these numbers are comparable to open surgery and considered adequate[27].

Survival is expectedly similar to open surgery with an average of 40% at 5 years[22].

Learning curve

It has been shown that esophagectomy outcomes are highly linked to the experience and volume of the centers performing the operation[28]. The same seems to be true for MIE[22]. To the best of our knowledge, no studies have defined the number of procedures necessary for these techniques to become safe and effective. Advanced laparoscopic skills and experience with major foregut surgery (open and laparoscopic) are clearly necessary.

CONCLUSION

Minimally invasive surgery has the advantages of better cosmetic results, reduced operative stress, postoperative immobility, and pain. These advantages are obtained by minimizing the incisions to obtain access to natural cavities, i.e. decreasing the external surgical stress. Minimally invasive surgery does not change, however, the internal part of the operation and the surgical stress determined by it. The minimally invasive approach has gained rapid acceptance and has become the gold-standard operation where external stress is higher than internal stress, such as for cholecystectomy and hiatal hernia repair[29,30]. In operations in which internal surgical stress is intensive, such as a Whipple procedure, the minimally invasive approach is questionable[31]. This is also true for MIE. This review shows that, even with a minimally invasive approach, patients are not discharged earlier and the clinical consequences of intense internal aggression, such as systemic inflammatory response syndrome[32], are still noticed after MIE. For these reasons and for the

technical skills necessary to perform a MIE, it is not a disseminated and widely used approach for esophageal resection. Boone *et al*[33] have surveyed 269 surgeons, members of the International Society for Diseases of the Esophagus, the European Society of Esophagology Group, and the World Organization for Specialized Studies on Diseases of the Esophagus. They have found that MIE was the operation of choice for only 14% of the responders, while 60% of them never used the MIE approach. Similar results have been presented by Enestvedt *et al*[34]. Not surprisingly, they also have shown that MIE is performed more frequently by high-volume surgeons compared to those from low-volume centers.

The available literature on MIE is still crowded with heterogeneous studies with different techniques. As far as we are aware, there have been no controlled comparative trials, and the few retrospective comparative cohort studies have been limited by small numbers of patients and biased by historical controls of open surgery[22]. Moreover, few studies have included > 100 patients. Based on the available literature, there is no current evidence that MIE brings clear benefits compared to conventional esophagectomy. Growing experience and studies with larger numbers of patients could change this situation.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3816

World J Gastroenterol 2010 August 14; 16(30): 3816-3826
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Portal vein ligation accelerates tumor growth in ligated, but not contralateral lobes

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Received: March 3, 2010 Revised: April 7, 2010
Accepted: April 14, 2010
Published online: August 14, 2010

Abstract

AIM: To investigate the mechanisms of liver growth and atrophy after portal vein ligation (PVL) and its effects on tumor growth.

METHODS: Mice were subjected to PVL, partial hepatectomy, or sham surgery. The morphological alterations, activation of transcription factors, and expression of cytokines and growth factors involved in liver regeneration were evaluated. In a separate set of experiments, murine colorectal carcinoma cells were injected *via* the portal vein and the effect of each operation on liver tumor growth was studied.

RESULTS: Liver regeneration after PVL and partial hepatectomy were very similar. In ligated lobes, various cytokines, transcription factors and regulatory factors were significantly upregulated compared to non-ligated lobes after PVL. Atrophy in ligated lobes was a result of early necrosis followed by later apoptosis. Tumor growth was significantly accelerated in ligated compared to non-ligated lobes.

CONCLUSION: Tumor growth was accelerated in ligated liver lobes and appeared to be a result of increased growth factor expression.

Key words: Portal vein ligation; Tumor growth; Growth factor; Atrophy; Apoptosis

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Sakai N, Clarke CN, Schuster R, Blanchard J, Tevar AD, Edwards MJ, Lentsch AB. Portal vein ligation accelerates tumor growth in ligated, but not contralateral lobes. *World J Gastroenterol* 2010; 16(30): 3816-3826 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3816.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3816

INTRODUCTION

Liver resection is the standard treatment for patients with primary or secondary liver malignancies, and offers the only chance of long-term survival[1,2]. With advances in surgical techniques, perioperative management, and anatomical knowledge of the liver, major hepatectomy usually does not carry a high operative mortality in patients with normal hepatic function, or in those with large tumors, unless accompanied by technical failure. However, the morbidity and mortality after extensive hepatectomy, or major hepatectomy in patients with obstructive jaundice, hepatic dysfunction, or tumors increase due to postop-

erative liver failure caused by excessive loss of functional residual liver mass[3,4]. It has been reported that there is a strong correlation between the expected remnant liver volume and postoperative liver failure in patients who undergo liver resection[5]. Surgical resection of liver tumors requires a sufficient surgical margin that can lead to substantial loss of residual mass. However, it is essential to secure sufficient functional liver mass to prevent postoperative liver failure.

In 1920, Rous and Larimore showed that selective portal occlusion can produce atrophy of the occluded lobe and compensatory hypertrophy of the contralateral lobe in rabbits[6]. In the clinical setting, Makuuchi *et al*[7] first proposed portal vein embolization as a preoperative treatment to avoid postoperative liver failure due to insufficient remnant liver mass. Portal vein embolization is now widely accepted as a useful procedure to extend eligibility of patients with liver cancer for liver resection. However, one study has shown that some patients can become ineligible for scheduled surgery due to tumor progression after portal vein embolization[8], whereas another study has indicated that portal vein embolization neither prevents nor accelerates tumor growth[9]. Thus, the effect of portal vein embolization on tumor growth prior to resection is not well understood.

In the present study, we used a murine model of portal vein ligation (PVL) to determine the effects of ligation on the mechanisms of liver growth and regeneration. In addition, we evaluated how these mechanisms influence the growth of colorectal carcinoma tumors in ligated and contralateral lobes after PVL.

MATERIALS AND METHODS

Animal model

Male C57BL/6J and BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) weighing 20-26 g were used in all experiments. This project was approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. The C57BL/6J mice were randomly separated into a PVL group, partial hepatectomy group, and sham operation group. All mice were anesthetized with sodium pentobarbital (60 mg/kg, ip) and a midline laparotomy was performed. For PVL, the branch of the portal vein that fed the left and median hepatic lobes, which corresponded to 70% of the whole liver, was dissected under an operative microscope and ligated with an 8-0 PROLENE suture (Ethicon, Inc., Somerville, NJ, USA). Partial hepatectomy was performed according to the method of Higgins and Anderson[10], with slight modification. 7-0 PRONOVA sutures (Ethicon) were secured around the base of the left and median hepatic lobes, and the lobes were resected. Mice were sacrificed at the indicated time points after operation, and blood and liver samples were taken for analysis. The liver lobes to body weight ratio was determined.

Blood and tissue analysis

Blood was obtained by cardiac puncture for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnosis kit by bioassay (Wiener Laboratories, Rosario, Argentina). Liver tissues were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin for light microscopy. Sections were stained with hematoxylin and eosin (HE) for histological examination. Liver content of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor β1 (TGFβ1) was assessed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Liver samples were weighed and immediately placed in 10 volumes (wt/vol) of a protease inhibitor cocktail that contained 10 nmol/L EDTA, 2mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL soybean trypsin inhibitor, 1.0 mg/mL bovine serum albumin, and 0.002% sodium azide in isotonic PBS, pH 7.0. Tissues were disrupted with a tissue homogenizer, and lysates were incubated at 4℃ for 2 h. Samples were clarified by two rounds of centrifugation at 12 500 *g* for 10 min at 4℃.

Liver neutrophil accumulation

Liver myeloperoxidase (MPO) content was assessed by methods described elsewhere[11]. Liver tissue (100 mg) was homogenized in 2 mL buffer A (3.4 mmol/L KH_2HPO_4, 16 mmol/L Na_2HPO_4, pH 7.4). After being centrifuged for 20 min at 10 000 *g*, the pellet was resuspended in 10 volumes of buffer B (43.2 mmol/L KH_2HPO_4, 6.5 mmol/L Na_2HPO_4, 10 mmol/L EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 s. After being heated for 2 h at 60℃, the supernatant was reacted with 3,3',3,5'-tetramethylbenzidine, and the optical density was read at 655 nm.

Proliferating cell nuclear antigen staining

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed on paraffin-embedded liver tissue with anti-PCNA antibody using DakoCytomation ARK kit (Dako, Copenhagen, Denmark). A three-step peroxidase method was performed according to the manufacturer's instructions. PC-10 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:50, for 15 min at room temperature. The sections were counterstained with hematoxylin. Evaluation of PC-10 immunostaining was performed based on the percentage of positive nuclei of 400-600 hepatocytes from the 4-6 highest positive fields at high power (400 ×), and was expressed as PCNA labeling index.

Western blotting

Liver samples were homogenized in lysis buffer (10 mmol/L HEPES, pH 7.9, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.6% NP-40, 0.5 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, and 1 μg/mL pepstatin). Samples were then sonicated and incubated for 30 min on ice. Cellular debris was removed by centrifugation at 10 000 r/min. Protein concentrations

of each sample were determined. Samples that contained equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylamide gel and transferred to a 0.1-μm pore nitrocellulose membrane. Nonspecific binding sites were blocked with Tris-buffered saline (TBS; 40 mmol/L Tris, pH 7.6, 300 mmol/L NaCl) that contained 5% non-fat dry milk for 1 h at room temperature. Membranes were then incubated with antibodies to cyclin D1 (Santa Cruz Biotechnology), signal transducer and activator of transcription 3 (STAT3) (Cell Signaling Technology, Boston, MA, USA), and phosphorylated STAT3 (Cell Signaling Technology) in TBS with 0.1% Tween 20. Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence.

Electrophoretic mobility shift assay

Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon[12], and analyzed by electrophoretic mobility shift assay. Double-stranded consensus oligonucleotides to nuclear factor (NF)-κB and activator protein (AP)-1 (Promega, Madison, WI, USA) were end-labeled with γ[^{32}P]-ATP (3000 Ci/mmol at 10 mCi/mL; Perkin Elmer, Waltham, MA, USA). Binding reactions (total volume 15 μL) that contained equal amounts of nuclear protein extract (20 μg) and 35 fmol (approximate 50 000 cpm, Cherenkov counting) of oligonucleotide were incubated at room temperature for 30 min. Binding reaction products were separated on a 4% polyacrylamide gel and analyzed by autoradiography.

Liver tumor model

The CT26 cell line is from an undifferentiated colon adenocarcinoma induced by N-nitroso-N-methylurethane injection in BALB/c mice. The CT26.WT cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). CT26.WT cells were maintained in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cells were incubated at 37℃ in a humidified atmosphere that contained 5% CO_2 in air. The cells were harvested from subconfluent cultures by 0.05% trypsinization and washed twice in PBS on the day of implantation. For the portal vein injection model, a midline incision was made and the portal vein was exposed by removing the intestine. A suspension of 2×10^5 CT26.WT cells was injected into the portal vein using a 31 G needle. After injection, a small piece of Gelfoam (Pharmacia Co., Kalamazoo, MI, USA) was pressed over the injection site for 2-3 min to obtain hemostasis. One week after tumor cell implantation, mice were subjected to portal vein ligation or sham surgery. One week after the operation, all mice were sacrificed and blood and liver samples were collected. The tumor growth was evaluated on HE slides and tumor area was determined by morphometry. Morphometric analysis was performed by image analysis software in five representative fields at low power (10 ×).

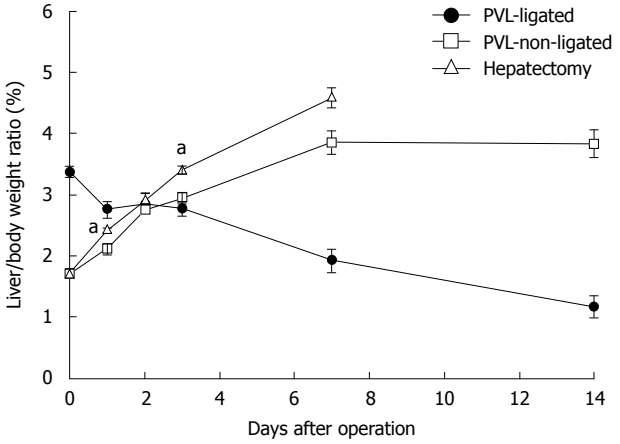

Figure 1 Changes in liver lobe to body weight ratio after portal vein ligation or partial hepatectomy. To evaluate liver regeneration after portal vein ligation (PVL) or partial hepatectomy, liver lobe to body weight ratio was determined. Data are mean ± SE with n = 4-6 per group. $^aP < 0.05$ *vs* PVL-non-ligated.

Statistical analysis

All data are expressed as mean ± SE. Data were analyzed with one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

RESULTS

Liver growth and regeneration after PVL vs partial hepatectomy

To evaluate liver growth and regeneration after PVL or partial hepatectomy, we measured liver/body weight ratios. After partial hepatectomy, liver regenerated at the expected rate and returned to normal liver mass within 7 d (Figure 1). After PVL, non-ligated lobes grew at a rate similar to liver after partial hepatectomy, but reached a plateau of mass below that after partial hepatectomy (Figure 1). The ligated lobes atrophied at a constant rate and after 14 d, the mass of the ligated lobes was approximately one third of the starting mass (Figure 1).

In accordance with the changes in liver growth and regeneration, similar patterns were found when we examined hepatocyte proliferation by staining for PCNA. PCNA-positive hepatocytes increased in a similar fashion after partial hepatectomy and in non-ligated lobes after PVL (Figure 2). However, there were subtle differences noted. While the number of PCNA-positive hepatocytes was maximal in both partial hepatectomy and non-ligated lobes after PVL at 2 d after surgery, there were significantly more PCNA-positive hepatocytes in the partial hepatectomy group (Figure 2). Furthermore, the number of PCNA-positive hepatocytes dropped dramatically by day 3 after partial hepatectomy, whereas after PVL, the number of PCNA-positive hepatocytes in non-ligated lobes had a more gradual decrease and was significantly higher compared to that after partial hepatectomy (Figure 2). Despite these minor differences, our data confirm previous studies that the mechanisms of liver growth and regeneration are

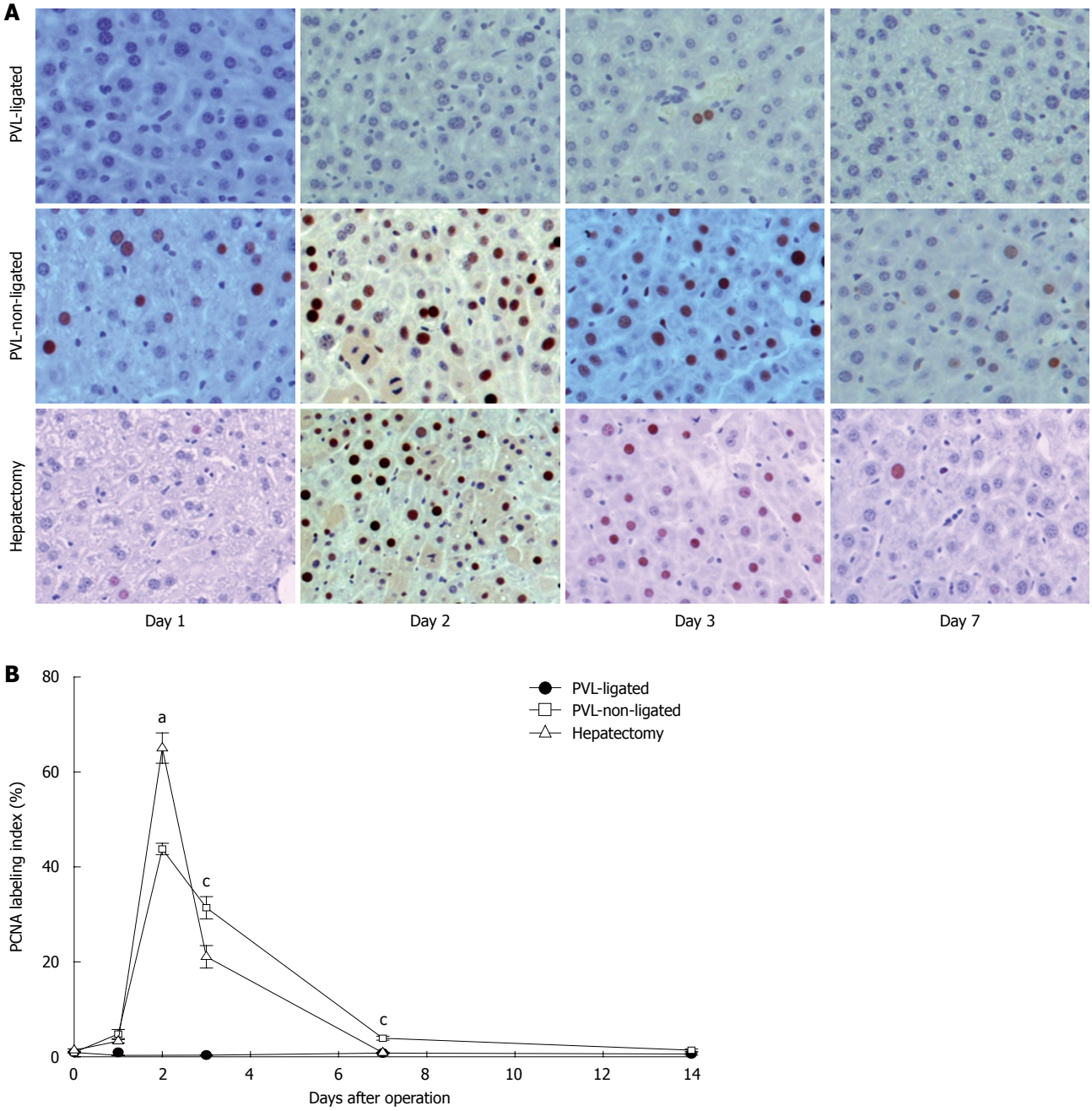

Figure 2 Hepatocyte proliferation after portal vein ligation. A: Hepatocyte proliferation was determined by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Original magnification was 200 ×; B: Quantitative analysis of PCNA labeling. PCNA labeling index was expressed as percentage of positive nuclei of 400-600 hepatocytes from the 4-6 highest positive fields at high power (400 ×). Data are mean ± SE with n = 4-6 per group. $^{a}P < 0.05$ *vs* PVL-non-ligated; $^{c}P < 0.05$ *vs* partial hepatectomy.

similar between that occurring in non-ligated lobes after PVL and that occurring after partial hepatectomy[13].

We next examined the mode of cell death after PVL. Serum levels of ALT were assessed as a measure of hepatocyte necrosis and TUNEL staining was performed to determine the amount of hepatocyte apoptosis. Serum levels of ALT peaked 1 d after PVL, but remained elevated for 14 d (Figure 3A). Corresponding with the ALT data, ligated lobes showed areas of necrosis within 1 d after PVL (Figure 3B, upper panels). These regions persisted for up to 7 d after PVL and were undetectable by day 14. In contrast, significant hepatocyte apoptosis was detected in ligated lobes, beginning at 7 d after PVL and persisting until 14 d after PVL (Figure 3B, lower panels). In non-ligated lobes, no evidence of hepatocyte necrosis or apoptosis was observed (data not shown).

Lobar differences in cytokine and growth factor expression after PVL

A variety of cytokines and growth factors are known to modulate liver growth and regeneration. To evaluate whether expression of relevant cytokines and growth factors is related to the growth of non-ligated lobes and/or the atrophy of ligated lobes, we measured the protein

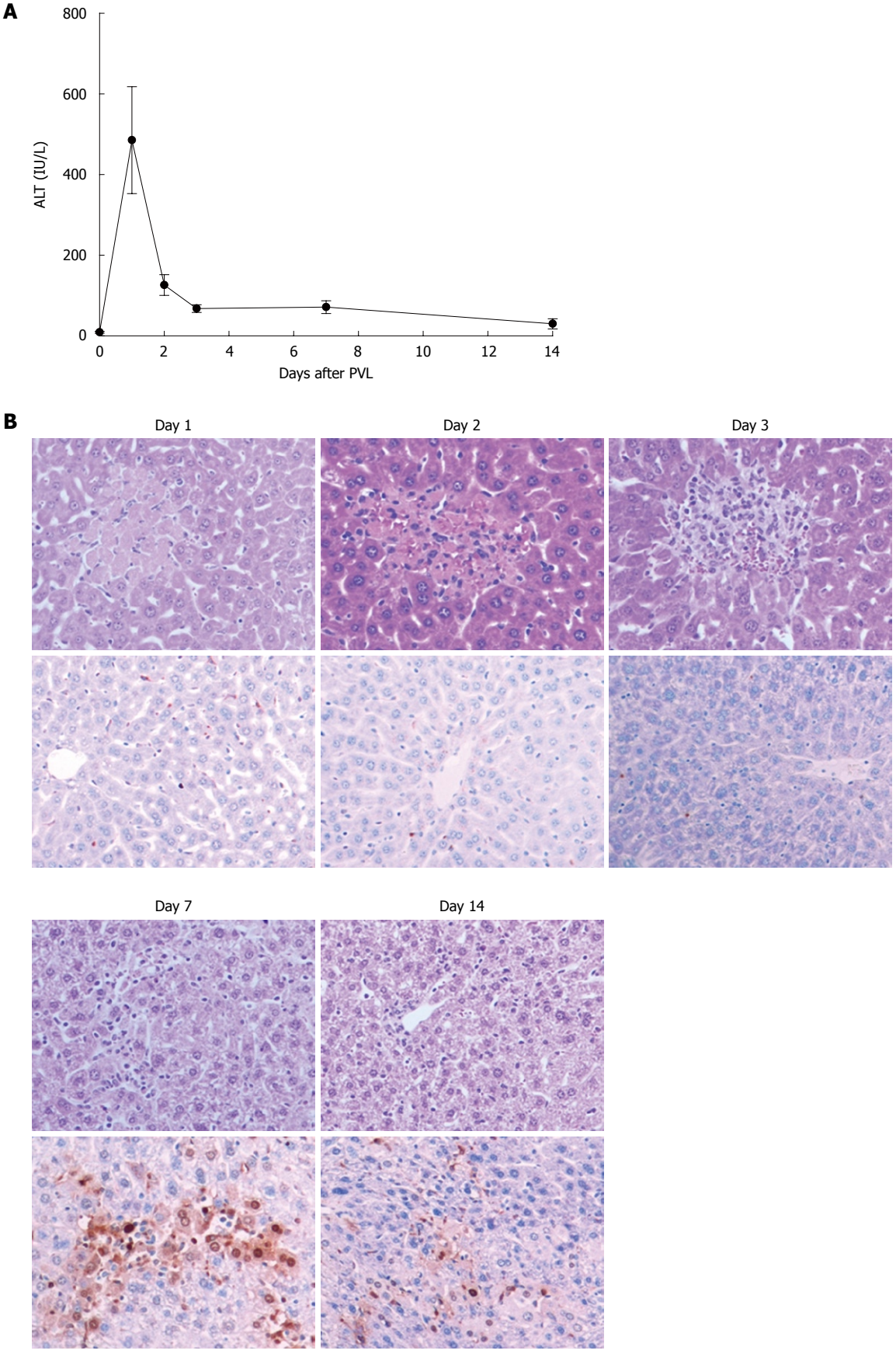

Figure 3 Effects of portal vein ligation on liver necrosis and apoptosis. A: Liver injury was measured by serum levels of alanine aminotransferase (ALT). Data are mean ± SE with n = 4-6 per group; B: Representative pictures of HE staining (upper) and TUNEL staining (bottom). TUNEL staining was performed to determine the amount of hepatocyte apoptosis. Original magnification was 200 ×. PVL: Portal vein ligation.

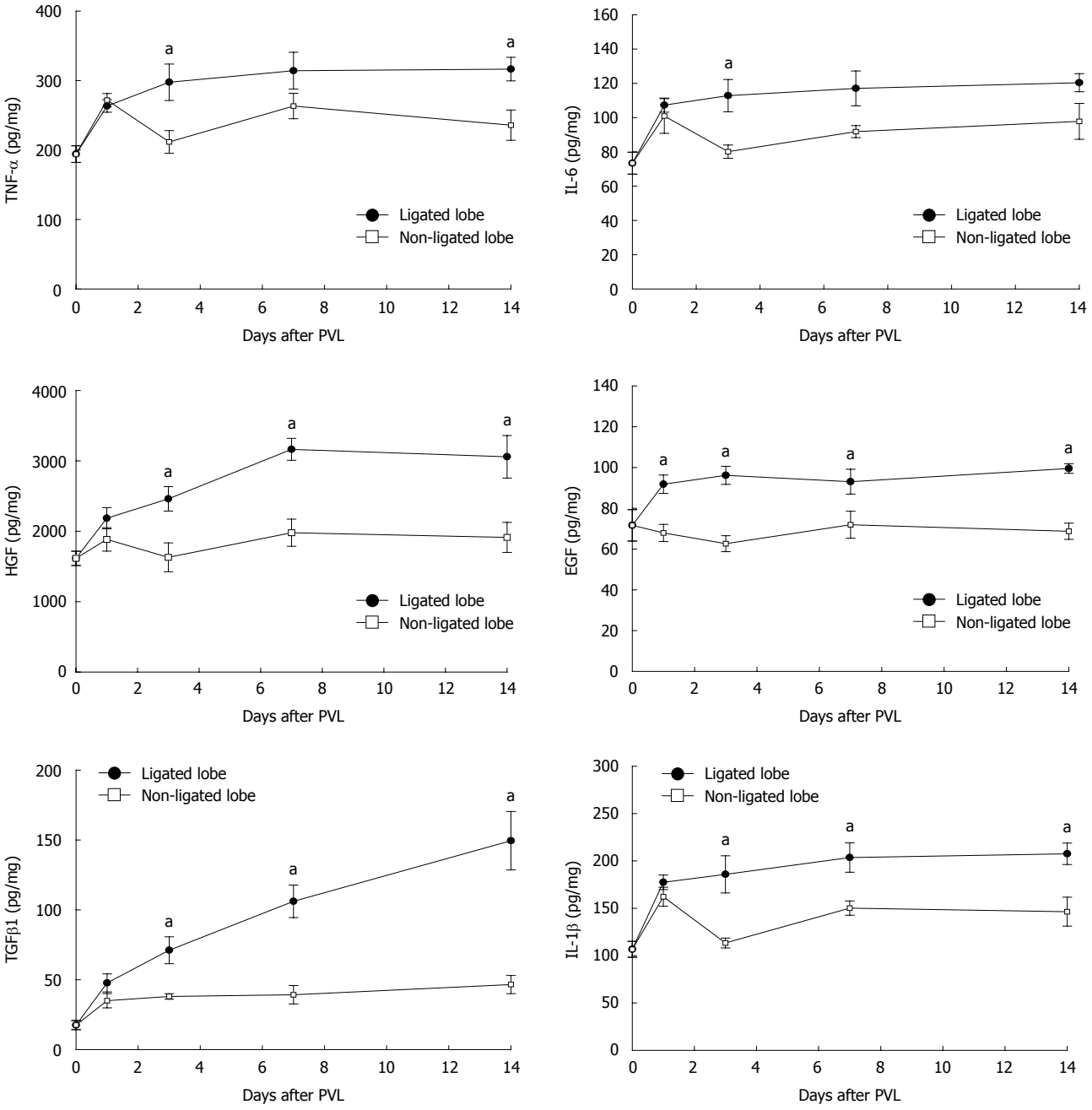

Figure 4 Effect of portal vein ligation on liver cytokines, growth factors, and chemokines. To evaluate whether expression of relevant cytokines and growth factors are related to the growth of non-ligated lobes and/or the atrophy of ligated lobes, liver levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor β1 (TGFβ1), and IL-1β were analyzed by enzyme-linked immunosorbent assay (ELISA). Liver lysates were processed for ELISA. Data are mean ± SE with n = 4-15 per group. $^{a}P < 0.05$ *vs* portal vein ligation (PVL)-non-ligated.

levels of HGF, EGF, TNF-α, IL-6, TGFβ1, and IL-1β in liver tissues. HGF and EGF are direct mitogens for hepatocytes and are crucial inducers of liver regeneration[14-16]. Expression of HGF and EGF were increased in both ligated and non-ligated lobes after PVL (Figure 4). However, expression of these mediators was much higher in ligated lobes compared to non-ligated lobes. TNF-α and IL-6 have been implicated as important contributors to liver growth and regeneration[14-16]. Expression of TNF-α and IL-6 increased similarly at 1 d after PVL in ligated and non-ligated lobes (Figure 4). By day 3, expression of TNF-α and IL-6 was significantly higher in ligated lobes compared to non-ligated lobes. TGFβ1 and IL-1β are known as suppressors of cell proliferation and might be involved in termination of liver regeneration[15,17]. We found that expression of TGFβ1 and IL-1β was increased 1 d after PVL in both ligated and non-ligated lobes (Figure 4). However, by day 3, expression of TGFβ1 had reached a plateau and IL-1β decreased in non-ligated lobes, whereas their expression had increased further in ligated lobes.

Divergent signaling mechanisms in ligated and non-ligated lobes after PVL

NF-κB, AP-1 and STAT3 are known to be important mediators of liver growth and regeneration[18,19], therefore,

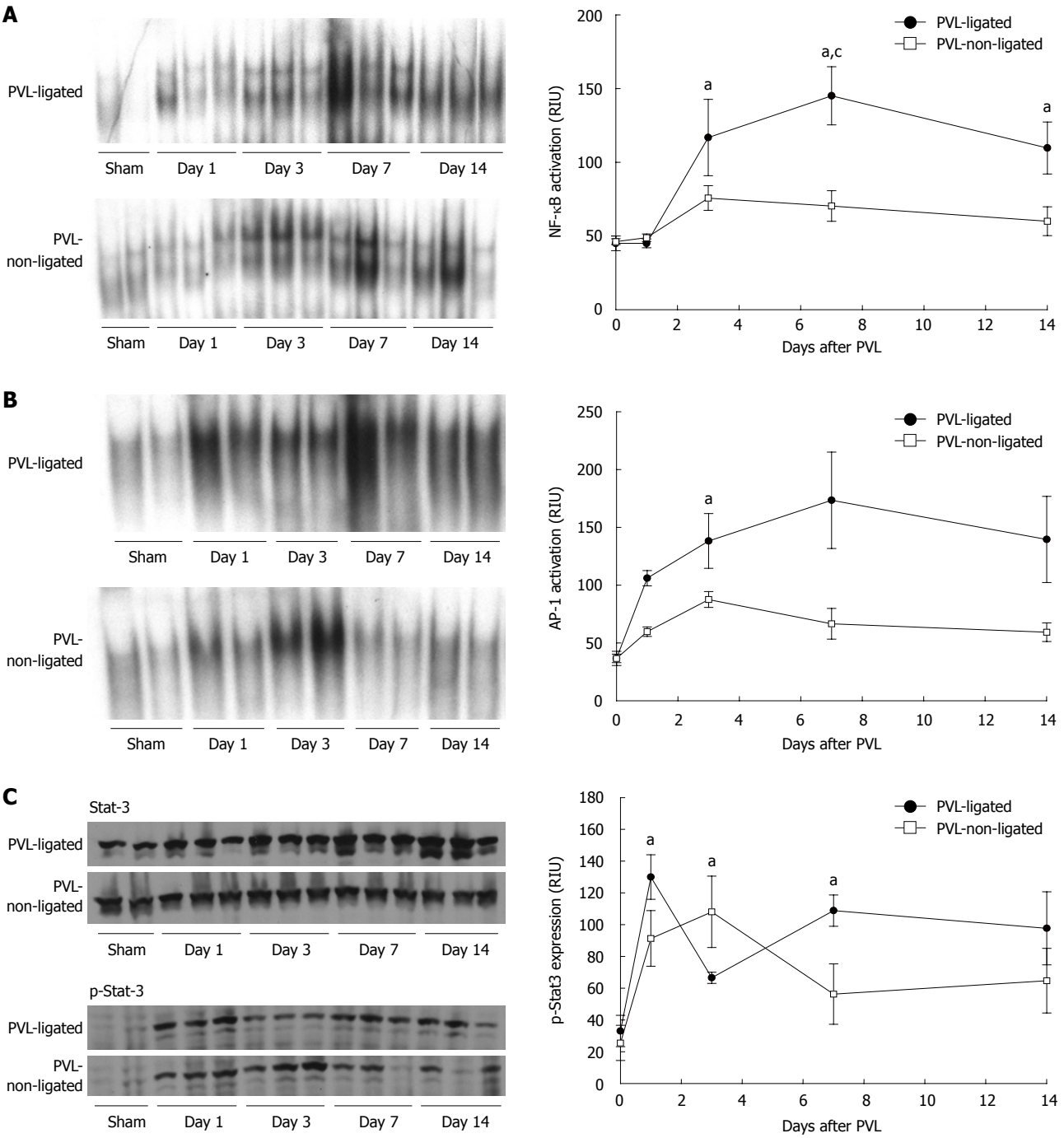

Figure 5 Transcription factor activation after portal vein ligation. Nuclear factor (NF)-κB (A), activator protein (AP)-1 (B) and signal transducer and activator of transcription 3 (STAT3) (C) were examined in liver extracts. For NF-κB and AP-1, liver nuclear extracts were analyzed by electrophoretic mobility shift assay. For STAT3, liver lysates were assessed by Western blotting. Results were quantitated by image analysis of autoradiograms and chemiluminescence films. Data are mean ± SE with $n = 4$ per group. A: $^{a}P < 0.05$ *vs* sham-operated group; $^{c}P < 0.05$ *vs* portal vein ligation (PVL)-non-ligated group; B: $^{a}P < 0.05$ *vs* PVL-non-ligated group; C: $^{a}P < 0.05$ *vs* sham-operated group.

we assessed the activation of these transcription factors in ligated and non-ligated lobes after PVL. NF-κB activation increased in both ligated and non-ligated lobes by day 3 after PVL; however, it was much greater in ligated lobes (Figure 5A). In non-ligated lobes, NF-κB activation remained elevated, albeit modestly, throughout the 14-d experimental period (Figure 5A). In contrast, activation of NF-κB in ligated lobes increased further, and remained significantly higher than in non-ligated lobes (Figure 5A). Supershift assays of NF-κB from each lobe indicated that the composition was composed primarily of p50/p65 heterodimers (data not shown).

In contrast to NF-κB, which did not become activated until 3 d after surgery, AP-1 activation occurred rapidly after PVL in both ligated and non-ligated lobes (Figure 5B). In non-ligated lobes, activation of AP-1 was increased modestly throughout the 14-d experiment. Similar to NF-κB, activation of AP-1 was much greater in the ligated

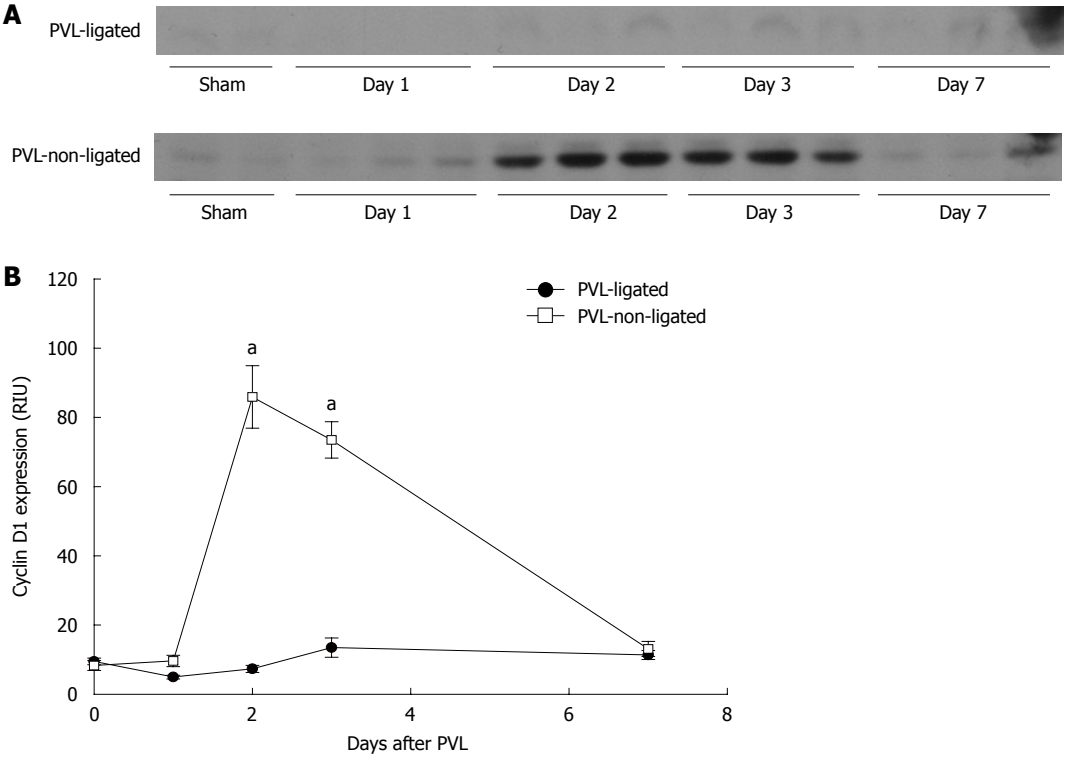

Figure 6 Liver cyclin D1 expression after portal vein ligation. A: Liver lysates were assessed for cyclin D1 protein expression by Western blotting; B: Chemiluminescence films were quantitated by image analysis. Data are mean ± SE with $n = 4$ per group. $^{a}P < 0.05$ *vs* portal vein ligation (PVL)-ligated group.

lobes compared to non-ligated lobes at every time point (Figure 5B). Supershift assays determined that the composition of AP-1 was similar in each lobe, primarily c-Fos, JunB, and JunD (data not shown).

STAT3 activation, as determined by STAT3 phosphorylation, was rapidly increased in both ligated and non-ligated lobes after PVL (Figure 5C). Interestingly, STAT3 activation decreased in ligated lobes at day 3 and then increased at days 7 and 14. In contrast, STAT3 activation in non-ligated lobes peaked at day 3 and then decreased at days 7 and 14.

Cyclin D1 is known to play a crucial role in the control of hepatocyte proliferation from G1- to S-phase[16,20,21]. Expression of cyclin D1 in non-ligated lobes was significantly increased after PVL, whereas there was no induction of cyclin D1 expression in ligated lobes (Figure 6).

PVL accelerates tumor growth in ligated, but not in non-ligated lobes

To investigate how the different milieus in ligated *vs* non-ligated lobes might alter the growth of liver tumors, mice were injected *via* the portal vein with murine colorectal carcinoma cells 7 d prior to PVL or sham surgery. We used murine colorectal carcinoma cells, CT26.WT, to reproduce the nature of colorectal liver metastases by injecting the cells into the portal vein. In sham-operated mice, there were similar amounts of small tumors in lobes that corresponded to ligated and non-ligated lobes (Figure 7A and B). In mice undergoing PVL, ligated lobes had large tumor nodules that were clearly visible on gross examination as well as histologically (Figure 7A and B). Quantitation of tumor area in liver sections demonstrated a four-fold increase in relative tumor size in ligated lobes *vs* non-ligated lobes (Figure 7C).

DISCUSSION

In the current study, we evaluated the effects of PVL on expression of cytokines and growth factors and signaling pathways that are known to contribute to liver growth and regeneration. Although the trigger for growth of contralateral lobes after PVL has not been fully elucidated, hemodynamic changes after PVL have been proposed as an initial event that contributes to this process[22]. Following PVL, arterial blood flow to the ligated lobe roughly doubles, while arterial blood flow to the non-ligated lobes is roughly 60% of normal[22]. Portal flow to the non-ligated lobes, however, more than doubles[22] and this increase in supply helps trigger growth mechanisms in the non-ligated lobes[23]. Hemodynamic changes are more drastic after partial hepatectomy because both arterial and portal flows to the remnant liver are increased. The difference in hemodynamic changes between PVL and partial hepatectomy could affect the degree and/or timing of expression of some proteins involved in regeneration[13,24] and cause a slight delay of regeneration in the non-ligated lobes. However, the gross regenerative responses are similar, as shown by our data.

Some studies have shown that the early growth/regenerative response, including activation of NF-κB, STAT3, IL-6, c-fos, c-myc, and c-jun, are similarly induced in both ligated and non-ligated lobes[18]. Other studies have shown

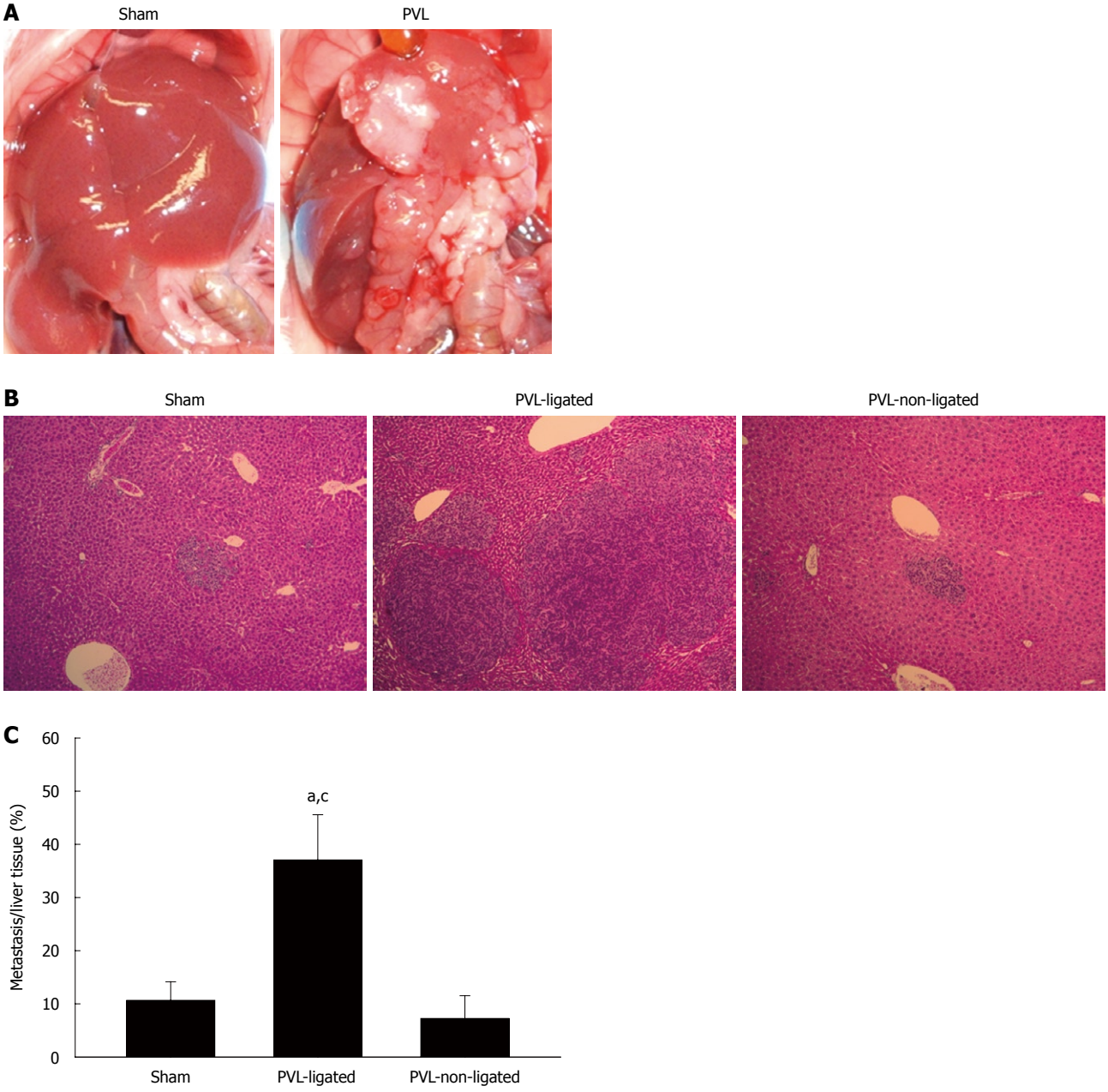

Figure 7 Effect of portal vein ligation on tumor growth. A: CT26.WT cells were injected into portal the vein. Mice were sacrificed at 14 d after injection following sham operation or portal vein ligation (PVL) performed on day 7 after injection; B: Representative pictures of liver histology after PVL. Small metastatic foci were observed in sham-operated and non-ligated lobes. Large metastatic foci were observed in ligated lobes. Original magnification was 10 ×; C: The ratio of metastases to normal liver was measured by morphometry. Data are mean ± SE with $n = 4$ per group. $^{a}P < 0.05$ *vs* sham-operated group; $^{c}P < 0.05$ *vs* PVL-non-ligated group.

differences in growth factor mRNA expression between ligated and non-ligated lobes[25]. Our data demonstrate that these growth and regenerative mechanisms are greatly increased in both ligated and non-ligated lobes, but are significantly greater in the ligated compared to non-ligated lobes. Cyclin D1 is the sole exception, being induced only in the non-ligated lobes. Cyclin D1 is known to play a crucial role in the control of hepatocyte proliferation from G1- to S-phase[20,21], and appears to be the determinant of proliferation or atrophy in non-ligated and ligated lobes, respectively.

The milieu in the ligated lobe, with greatly increased expression of cytokines and growth factors and increased activation of NF-κB and AP-1, is rather chaotic and not indicative of either a "survival" or "death" mode. TNF-α can function to promote hepatocyte proliferation or death, depending on the co-stimuli present[26-28]. NF-κB activation in hepatocytes is pro-survival and anti-apoptotic[29,30], whereas activation of AP-1 promotes hepatocellular injury and apoptosis[31]. The fate of the ligated lobe might be less dependent upon the changes in these factors, and more on the lack of nutrient and oxygen delivery. As is clear, the end result is atrophy of the ligated lobe through necrotic and apoptotic mechanisms. Despite this atrophy and the pro-hepatocyte death milieu, colorectal carcinoma metastases grew much faster in the ligated lobes compared to the non-ligated lobes after PVL. These findings are consistent with other studies

that have shown increased tumor growth in the ligated lobes after PVL[32,33]. However, our study offers more insight into the potential mechanisms that contribute to the increased tumor growth, as our data provide important information about the expression of growth factors and signaling pathways. HGF and EGF have a stimulatory effect on tumor cells[34,35], and therefore, the increased HGF and EGF observed in the ligated lobe after PVL could explain the accelerated tumor growth. TGFβ1 was also increased in the ligated lobe. Although TGFβ1 is generally known as a negative regulator in liver regeneration[15], some recent studies have reported a tumor promoter role for TGFβ1 in hepatocellular carcinoma and liver metastasis[36-38]. It has been shown that TGFβ1 is highly proliferative in CT26 cells, the colorectal carcinoma cell line used in our studies[39]. Furthermore, TGFβ1 is known to contribute to hepatocyte apoptosis, and colorectal carcinoma cells secrete significant amounts of TGFβ1, which might contribute to tumor growth[39,40]. Therefore, it is plausible that increased TGFβ1 expression in the ligated lobes significantly contributed to the accelerated growth of colorectal carcinoma tumors after PVL.

In summary, the present study demonstrated the signaling pathways that were activated in ligated and non-ligated lobes after PVL. Both lobes had increased expression of pro-proliferative cytokines and growth factors, as well as activation of pro-regenerative transcription factors, which help to define the molecular events that contribute to growth of contralateral lobes. Ligated liver lobes had significant increases in proliferative cytokines, growth factors and transcription factors compared to non-ligated lobes. While this response might constitute a survival mode for the hepatic parenchyma, it appears to provide an environment that facilitates tumor growth. PVL is a proven modality for increasing the functional liver remnant and extending the indications for surgery for metastatic liver disease. Future studies are needed to assess the effects of adjuvant or neoadjuvant chemotherapy on the hepatic expression of growth factors and tumor growth rate in this model.

COMMENTS

Background

Liver resection is the standard treatment for patients with primary or secondary liver malignancies, and offers the only chance of long-term survival. Although, the outcome of hepatic resection is improving, postoperative liver failure that results from insufficient functional liver volume after surgery could be lethal. Portal vein embolization is now widely accepted as a useful procedure to increase remnant liver volume and extend eligibility of patients with liver cancer for liver resection.

Research frontiers

Portal vein embolization is well known to induce hypertrophy of contralateral lobes. However, the manner in which portal vein embolization alters growth of the contralateral lobes and atrophy of the embolized lobe(s) is incompletely understood. Moreover, the effect of portal vein embolization on tumor growth is controversial.

Innovations and breakthroughs

In the current study, the authors demonstrated that various cytokines, transcription factors and regulatory factors were significantly upregulated in ligated lobes compared to non-ligated lobes after portal vein ligation. Tumor growth was accelerated in the ligated compared to non-ligated lobes and appeared to be a result of increased growth factor expression.

Applications

The results provide strong evidence of accelerated tumor growth in ligated lobes. This should be taken into account for the treatment strategy when patients undergo portal vein embolization.

Peer review

The experiments were well designed and well conducted. The topic relates to the advantages and/or disadvantages of the surgical procedure of portal vein embolization prior to major liver resections for hepatocellular carcinoma or other liver cancers.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3827

World J Gastroenterol 2010 August 14; 16(30): 3827-3833
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Decreased IgA+ plasma cells and IgA expression in acute liver necrosis mice

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Author contributions: Fu JL and Liu P contributed equally to this work; Liu P and Fu JL designed the research; Fu JL and Wang ZH performed the research; Li GZ and Fu JL analyzed the data; Fu JL and Wang YR wrote the paper.

Supported by National Science Foundation of China Grant No. 30670947; and National Ministry of Health, China, No. 97100252

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Received: March 4, 2010 Revised: April 23, 2010
Accepted: April 30, 2010
Published online: August 14, 2010

Abstract

AIM: To investigate the number of intestinal immunoglobulin A (IgA+) plasma cells and expression of intestinal IgA in mice with acute liver necrosis.

METHODS: A model of acute liver necrosis was established by intraperitoneal injection of galactosamine (GalN) and lipopolysaccharide (LPS). Sixty mice were randomly divided into one of 4 equal groups: normal control, acute liver necrosis, LPS, or GalN. Hematoxylin and eosin staining, immunohistochemistry, and an enzyme-linked immunosorbent assay were employed to assess liver and intestinal injury, count intestinal IgA+ plasma cells, and measure the expression level of IgA and interferon γ (IFN-γ) in the small intestinal mucosa of mice.

RESULTS: Injured intestinal mucosa was observed in the acute liver necrosis group but not in the normal, LPS or GalN groups. Compared with the normal group, intestinal IgA+ plasma cells were slightly decreased in the LPS and GalN groups [429 ± 20 per high power field (HPF), 406 ± 18/HPF, respectively], whereas they were markedly decreased in the acute liver necrosis group (282 ± 17/HPF *vs* 495 ± 26/HPF in normal group, $P < 0.05$). The expression of intestinal IgA was also slightly decreased in LPS and GalN groups, but was markedly reduced in the acute liver necrosis group as determined by enzyme-linked immunosorbent assay ($P < 0.05$). In contrast, the level of IFN-γ was slightly increased in LPS, GalN and acute liver necrosis groups, but with no statistical significance ($P > 0.05$).

CONCLUSION: Intestinal IgA+ plasma cells and IgA expression levels indicating that mucosal immune barrier dysfunction, does exist in acute liver necrosis.

Key words: Acute liver necrosis; Intestinal mucosa; Immunity; Immunoglobulin A

Peer reviewers: Damiao Carlos Moraes Santos, DCM, PhD, Department of Quality Control (deque), Bio-Manguinhos - FIOCRUZ, Avenida Brazil, 4365, Manguinhos - Rio de Janeiro, 21045-900, Brazil; Mara Massimi, PhD, Department of Basic and Applied Biology, University of L'Aquila, Via Vetoio, Coppito, 67010, Italy

Fu JL, Wang ZH, Li GZ, Wang YR, Liu P. Decreased IgA+ plasma cells and IgA expression in acute liver necrosis mice. *World J Gastroenterol* 2010; 16(30): 3827-3833 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3827.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3827

INTRODUCTION

Patients with acute liver necrosis are at high risk for enterogenic infections. Enterogenic infections are an important cause of death in patients with acute liver

necrosis associated with intestinal barrier injury, including immunological barrier injury[1,2]. Immunoglobulin A (IgA) is an important component of the intestinal immunological barrier and is the most abundant immunoglobulin at mucosal surfaces where it plays crucial roles in mucosal protection[3]. The protective barrier of the gastrointestinal system is impaired in IgA deficiencies, and IgA-deficient individuals have a tendency to develop gastrointestinal infections[4]. Previous studies have shown decreased levels of secretory IgA and decreased numbers of IgA+ plasma cells in the intestinal tract during stress and thermal injury suggesting that the humoral immune function was dramatically inhibited in these situations[5,6]. Intestinal IgA was also decreased in endotoxemia and intra-abdominal sepsis models[7,8].

Previous studies have primarily focused on mechanical barrier interruption in acute liver necrosis models[9]. So far, no studies have shown a role for the intestinal immunological barrier in acute liver necrosis. It has been reported that an increase in levels of interferon γ (IFN-γ), a TH1 cytokine, was related to tissue injury[10] and led to a decreased expression of IgA[11].

This study set out to determine whether the number of intestinal IgA+ plasma cells and the expression of IgA were modified in mice with acute liver necrosis, in an attempt to establish whether dysfunction of the intestinal immunological barrier occurs during acute liver necrosis. In addition, IFN-γ levels in the intestinal mucosa were also evaluated.

MATERIALS AND METHODS

Animals

Sixty male BALB/c mice 6-8 wk of age (provided by the Laboratory Animal Center of the China Medical University) were housed under constant room temperature and humidity, and subjected to a 12 h light/dark cycle. Experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Mice were equally and randomly divided into one of 4 groups: normal control, acute liver necrosis, lipopolysaccharide (LPS), or galactosamine (GalN). GalN (800 mg/kg body weight, Sigma, USA) and LPS (10 μg/kg body weight, Sigma, USA) were injected intraperitoneally to induce acute liver necrosis as previously described[12,13]. Serum, liver and proximal small intestinal tissues samples were obtained 9 h after GalN/LPS injection.

Blood biochemistry assay

Serum alanine transaminase (ALT) levels were determined using an automatic analyzer (Hitachi 7250; Hitachi, Japan).

Histological testing

The liver and proximal small intestinal tissue were separately stored in formalin, and embedded by paraffin. The liver and intestinal sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (HE) to explore the histopathological changes in the liver and intestinal mucosa.

Immunohistochemistry for intestinal IgA+ plasma cells

Intestinal IgA+ plasma cells were investigated by immunohistochemistry (IHC). Sections of proximal small intestine were deparaffined, and antigen retrieval was performed by pressure cooker boiling for 2 min in 10 mmol/L citrate buffer (pH 6.0). IHC analysis was performed using goat anti-mouse IgA (Zymed, USA, diluted 1:50) for 12 h at 4℃, and the secondary antibody (rabbit anti-goat IgG) was applied for 2 h at 37℃. Fresh peroxidase reaction mixture containing equal amounts of 0.02% hydrogen peroxide in H_2O and 0.1% diaminobenzidine in H_2O were prepared. Sections were mounted on Uvinert mountant (BDH, UK). Five fields of small intestinal mucosa lamina propria were examined in each section at high magnification (200 ×), and the number of IgA+ cells were counted (i.e. lymphocytes that stained a brownish-yellow color). The average number of IgA+ was calculated.

Enzyme-linked immunosorbent assay for IgA expression

The levels of intestinal IgA were examined by enzyme-linked immunosorbent assay (ELISA). Intestinal tissue (50 mol/Lg) immersed in 1 mL (10 volumes, w/v) of phosphate-buffered saline (PBS) was incubated at room temperature for 15 min. Samples were vortexed, left to settle for 15 min, revortexed until all material was suspended, then centrifuged at 12000 r/min for 10 min. The supernatant was collected and tested on an ELISA kit for IgA (Bethyl Laboratories, Montgomery, TX, USA). Briefly, 96-well microtiter plates were coated with goat anti-mouse IgA affinity purified antibody and incubated for 60 min, then washed with PBS, and each well was incubated with 1% bovine serum albumin in PBS to block any nonspecific binding. After washing with PBS containing 0.1% Tween-20, 100 μL test samples and 100 mol/L standards were added into each well and incubated for 60 min followed by incubation with peroxidase-labeled goat anti-mouse specific IgA antibody for 30 min. Then 0.1 mol/L acetate buffer containing 1 mg/mL ortho-phenylenediamine was prepared and 3 μL of the prepared solution in 10 mL of H_2O_2 was added to each well. The reactions were stopped by adding 25 μL of 2 mol/L sulfuric acid. The absorbance of each solution was determined at a wavelength of 450 nm.

Detection of IFN-γ in the intestinal mucosa

The small intestinal mucosa homogenate was prepared as described previously[8]. The levels of IFN-γ in the homogenate were measured by sandwich ELISA (Quantikine ELISA Kits, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Software SPSS 11.0 was used for statistical analysis. Each value was expressed as the mean ± SE, and compared by using one-way ANOVA, followed by the Tukey test. $P < 0.05$ was considered statistically significant.

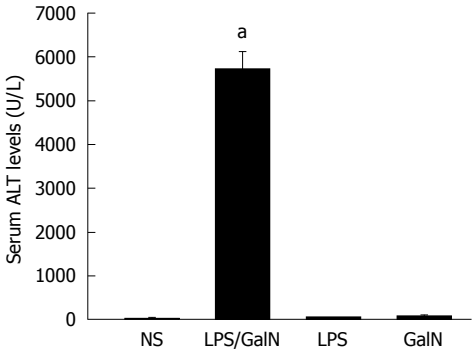

Figure 1 Serum alanine transaminase levels. Each value was expressed as mean ± SE. [a]$P < 0.05$ *vs* normal saline (NS). ALT: Alanine transaminase; GalN: Galactosamine; LPS: Lipopolysaccharide.

RESULTS

Mortality rate of mice and serum ALT levels

In the acute liver necrosis group, the mortality rate was 53.3% (8/15), compared to 0% in the other control groups. The serum ALT levels in LPS, GalN and normal groups were almost at the same level (44.3 ± 12.1, 74.2 ± 14.3, and 24.8 ± 14.7 U/L, respectively), but increased significantly in the acute liver necrosis group (5730.1 ± 383.5 U/L *vs* 24.8 ± 14.7 U/L, $P < 0.05$) (Figure 1).

Assessment of liver and proximal small intestinal injury with HE staining

In the normal group, the liver clearly showed normal structure of both the hepatic lobuli and hepatic cords. In contrast, the livers from the acute necrosis group had severe hemorrhage, hepatic necrosis, acidophilic degeneration in some residual hepatocytes, disappearance of hepatic cords, and deranged structure of hepatic lobules. Acidophilic degeneration and swelling were observed in the LPS group and edematous and spotty necrosis, as well as a few hepatic cells with acidophilic changes were found in the GalN group (Figure 2).

In normal, LPS and GalN groups, the intestinal mucosa was complete and the intestinal cells appeared ordered. In contrast, the intestinal mucosa of mice with acute liver necrosis were loosened and some of epithelial cells were edematous and necrotic (Figure 3).

IHC for IgA+ plasma cells

As shown in Figure 4, the number of IgA+ plasma cells within the lamina propria (as determined by IHC) were 495 ± 26/high power field (HPF), 282 ± 17/HPF, 429 ± 20/HPF and 406 ± 18/HPF in the normal, acute liver necrosis, LPS and GalN groups, respectively. The LPS group and GalN group had slightly lower numbers of IgA+ plasma cells than the normal group; however, the acute liver necrosis group had significantly lower numbers of IgA+ plasma cells compared to the other groups ($P < 0.05$, Figure 5A).

ELISA measurement of IgA

There was a slight decrease in the expression of IgA in LPS and GalN groups whereas no difference was noted in IgA expression in the small intestine compared with normal control. IgA expression in the small intestines from mice in the acute liver necrosis group was markedly reduced compared with the normal group ($P < 0.05$, Figure 5B).

IFN-γ in small intestinal mucosa

There were a slight increase in IFN-γ levels in LPS, GalN and acute liver necrosis groups, but no significant difference was noted compared with the normal group ($P > 0.05$, Figure 5C).

DISCUSSION

Acute liver necrosis is associated with a high mortality rate[14]. Infection is a common serious complication of acute liver necrosis and is a major cause of death[15]. A myriad of researchers have noted that secondary infections primarily originate from intestinal bacterial translocation. While the intestinal barrier has to be permeable for nutrients and macromolecules which are indispensable for growth and development, at the same time it also has to provide an effective barrier against harmful macromolecules and microorganisms to ensure local homeostasis[16]. The intestinal barrier consists of a mechanical barrier, immunological barrier, microorganism barrier, and a chemical barrier. The immunological barrier is considered the first line of antigen-specific immune defense against pathogenic microorganisms[17,18]. Recent reports indicated that immunosuppression, involving the local intestinal immunological barrier, is a major cause of intestinal bacterial translocation[19].

In agreement with previous reports[20,21], we found that injection of GalN/LPS induced increases in serum ALT and the development of severe hepatocyte necrosis. As the mortality of mice with acute liver necrosis was 53.3%, these findings indicate that the model employed to study IgA and IFN-γ was successful. In addition to the observed liver injury, loosened intestinal mucosa and some edematous and necrotic intestinal epithelial cells were also noted in the mice with acute liver necrosis. These features were not found in any of the other groups. These histological findings of intestinal mucosal injury in acute liver necrosis were consistent with other studies[14].

IgA is the most abundant immunoglobulin present on all mucosal surfaces, where it plays crucial roles in mucosal protection[3]. IgA is produced and released as a J chain-linked dimer by resident IgA+ plasma cells in mucosal tissues, including the extensive lamina propria of the intestine[22]. For decades, it has already been known that IgA plays a protective role in mucosal immunity. IgA exerts its protective effects *via* 3 primary mechanisms. First, IgA serves as an immunologic barrier which inhibits binding of organisms to mucosal surfaces. Next, the normal movement of IgA from the basilar to apical region of epithelial cells suggests that it may be effective in neutralizing intracellular pathogens. Finally, pathogens bound to IgA are taken up by macrophages *via* phagocytosis[23]. An additional property of IgA is its inability to trigger the release

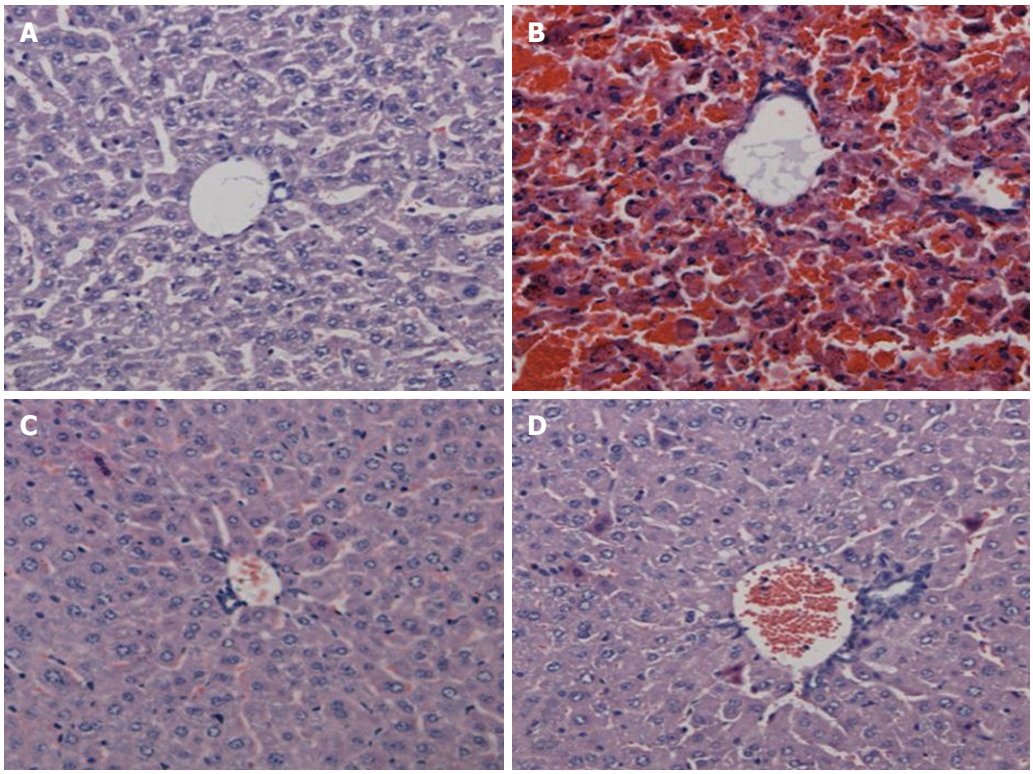

Figure 2 Hematoxylin and eosin staining of liver tissue (100 ×). A: Normal group; B: Acute liver necrosis group; C: Lipopolysaccharide group; D: Galactosamine group.

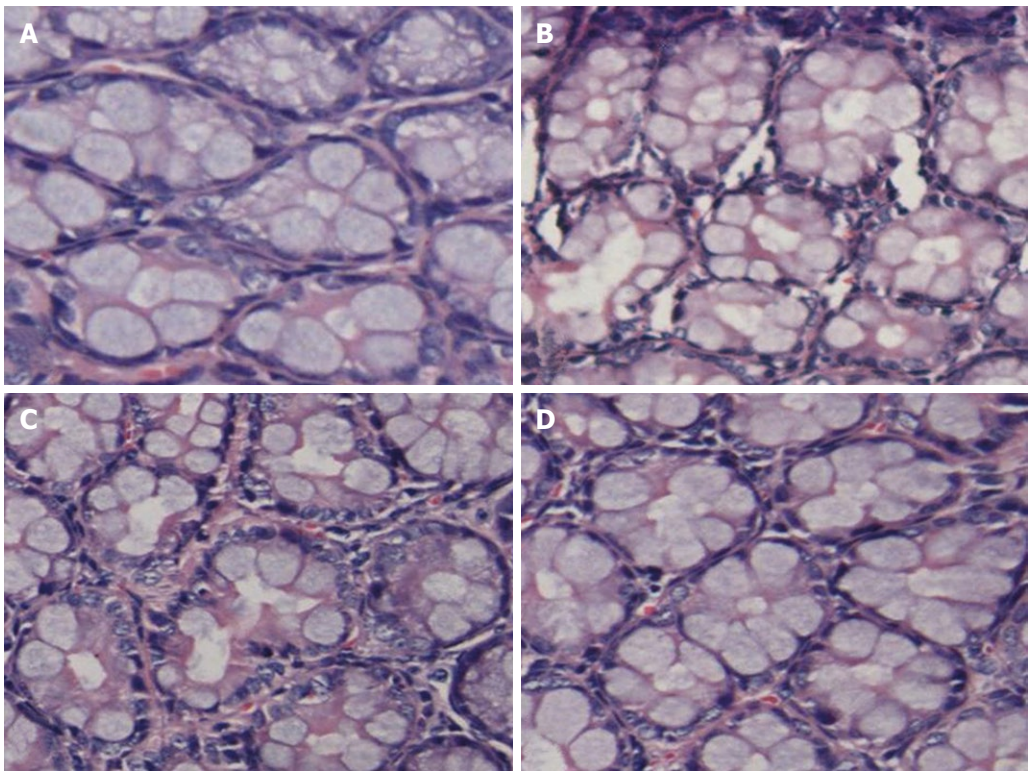

Figure 3 Morphology of intestinal samples stained with hematoxylin and eosin (200 ×). A: Normal group; B: Acute liver necrosis group; C: Lipopolysaccharide group; D: Galactosamine group.

of inflammatory mediators through receptors specific to its Fc domain[24-26].

IgA-deficient individuals have a tendency to develop infections and disorders of the gastrointestinal tract[27-29]. Zinneman *et al*[30] reported that the protective barrier of the gastrointestinal system was impaired in IgA deficiency

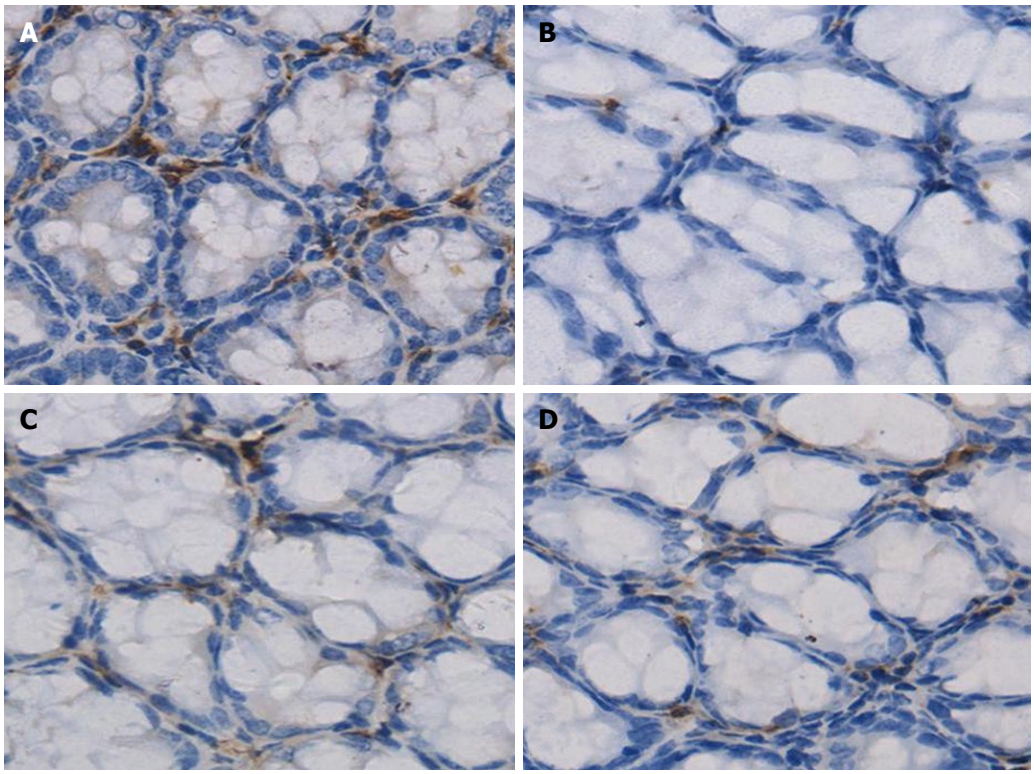

Figure 4 Immunoglobulin A+ cells determined by immunohistochemistry (400 ×). Immunohistochemistry demonstrated that Immunoglobulin A in the plasma cells within the lamina propria were stained brown. A: Normal group; B: Acute liver necrosis group; C: Lipopolysaccharide group; D: Galactosamine group.

and that protozoa such as *Giardia lamblia* can adhere to the epithelium, proliferate, and cause infection.

In the experiment presented herein, the number of IgA+ plasma cells and the IgA levels in intestinal mucosa in LPS and GalN groups showed a slight decrease but no significant difference was noted compared to the normal group. The number of IgA+ plasma cells and the IgA levels in the intestinal mucosa in the acute liver necrosis group were the lowest among all 4 study groups. The IgA+ plasma cells and the IgA levels were significantly different between the acute liver necrosis group and normal controls (Figures 5A and B). The decrease in the number of IgA+ cells and the IgA levels in the GalN/LPS group were significantly greater than the sum of the decrease in the LPS and GalN groups. It was thought that the decreased IgA+ plasma cells and decreased IgA levels in the intestinal mucosa were not the result of GalN or LPS injection, but rather of acute liver necrosis. These findings suggest that intestinal immunological barrier injury, which is a component of intestinal barrier injury, does occur in acute liver necrosis.

The mechanism of reduction in IgA+ plasma cells in acute liver necrosis is complicated and likely multifactorial. First, an increased rate of apoptosis in the subpopulation in Peyer's patches secondary to acute liver necrosis could negatively impact IgA+ plasma cell numbers[9,31]. Second, multiple organ damage, particularly the bone marrow, spleen, and mesenteric lymph nodes, in concert with mucosal edema and injury caused by acute liver necrosis, could affect the production and proliferation of IgA+ plasma cells. Third, the structural damage to the intestinal mucosa could interfere with recirculation of plasma cell precursors[32]. An accurate mechanism of IgA+ plasma cell reduction in acute liver necrosis clearly requires further study.

In this study, it was also found that the decreased IgA expression in acute liver necrosis coincided with a decline in IgA+ plasma cells. One explanation for this could be that the decrease in the number and function of IgA+ plasma cells leading to a simultaneous reduction in IgA secretion. At present, the specific pathogenesis and progression of acute liver necrosis remains unclear.

Inflammatory mediators are thought to be involved in the development and progression of acute liver necrosis. Previous studies reported that serum levels of a number of inflammatory factors, such as IFN-γ, are elevated in patients with severe liver injury[14,33]. In addition, IFN-γ is known to downregulate IgA expression[34].

In this study, IFN-γ levels in the small intestinal mucosa were slightly increased in LPS, GalN and acute liver necrosis groups, but no significant difference in IFN-γ expression was identified between the acute liver necrosis and normal control group. IFN-γ expression does not seem to explain the decrease in IgA secretion from the intestinal mucosa. Other factors involved in the reduction in IgA expression in acute liver necrosis warrant further attention.

In conclusion, this study found that mice with acute liver necrosis had a reduced number of intestinal IgA+ plasma cells and IgA expression levels indicating that mucosal immune barrier dysfunction does exist in acute

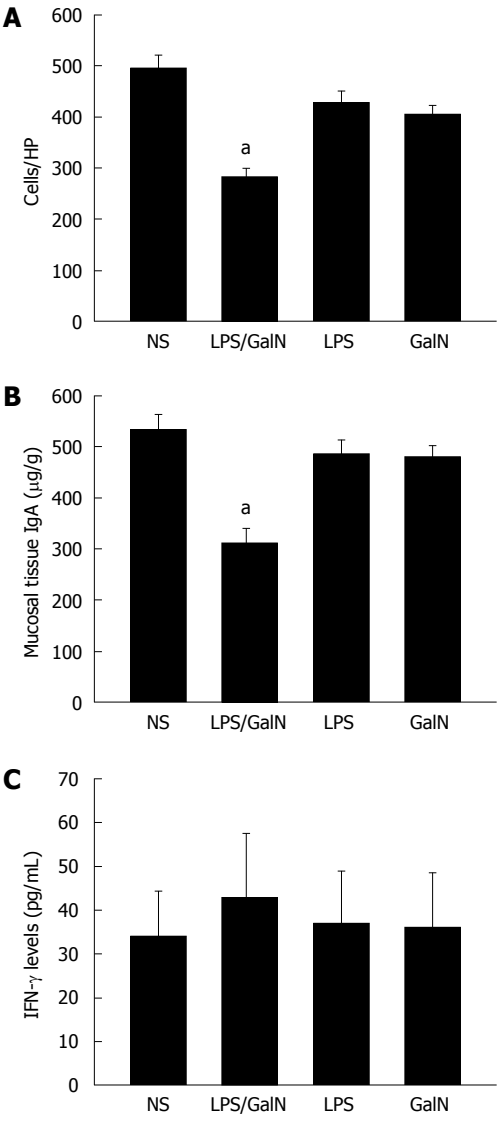

Figure 5 Immunoglobulin A+ plasma cells (A), the Immunoglobulin A expression levels (B) and interferon-γ expression (C) in intestinal mucosal tissue. $^{a}P < 0.05$ *vs* normal saline (NS). HP: High power field; GalN: Galactosamine; LPS: Lipopolysaccharide.

liver necrosis. IFN-γ expression does not seem to explain the decrease in IgA secretion from the intestinal mucosa. Further research regarding the mechanism(s) of intestinal immune barrier injury and ways to prevent this type of injury in acute liver necrosis is warranted.

ACKNOWLEDGMENTS

We are grateful to Professor Ya-Luo Dong and Professor Dong-Yan Liu for their technical guidance and help.

COMMENTS

Background

Enterogenic infection is an important cause of death in patients with acute liver necrosis. Intestinal immunological barrier injury plays a vital role in the pathophysiology of enterogenic infection. Immunoglobulin A (IgA) is an important component of the intestinal immunological barrier and is the most abundant immunoglobulin present on mucosal surfaces, where it plays crucial roles in mucosal protection.

Research frontiers

IgA is considered a first line antigen-specific immune defense against pathogenic microorganisms and plays an important role in intestinal mucosal immunity. This study found significant changes in the number of IgA+ plasma cells and IgA expression levels during acute liver necrosis.

Innovations and breakthroughs

Previous studies have mainly focused on mechanical barrier interruption in acute liver necrosis models. So far, no studies have shown the indispensable nature of the intestinal immunological barrier in acute liver necrosis. In this study, the number of IgA+ plasma cells and IgA expression in mice with acute liver necrosis were determined to investigate whether dysfunction of the immunological barrier occurred during acute liver necrosis.

Applications

IgA is an important component of mucosal immune system and is significantly reduced during acute liver necrosis. Thus, a protective or an immunoregulative treatment of intestinal immune function could be beneficial in patients with acute liver necrosis.

Peer review

The manuscript by Fu *et al* describes studies examining in mice the change of intestinal IgA+ plasma cells and the expression of intestinal IgA in acute liver necrosis. Although the underlying mechanisms that contribute to the decreased expression of intestinal IgA was not thoroughly investigated, the study addresses an issue of topical interest, providing important data.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3834

World J Gastroenterol 2010 August 14; 16(30): 3834-3840
ISSN 1007-9327 (print)

BRIEF ARTICLE

Early ileocolonoscopy with biopsy for the evaluation of persistent post-transplantation diarrhea

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Received: April 12, 2010 Revised: May 13, 2010
Accepted: May 20, 2010
Published online: August 14, 2010

Abstract

AIM: To investigate the significance of ileocolonoscopy with histology in the evaluation of post-transplantation persistent diarrhea (PD).

METHODS: We retrospectively reviewed all records of renal transplant patients with PD, over a 3-year period. All patients were referred for ileocolonoscopy with biopsy, following a negative initial diagnostic work up. Clinical and epidemiological data were compared between cases with infectious or drug-induced diarrhea.

RESULTS: We identified 30 episodes of PD in 23 renal transplant patients (1-3 cases per patient). There were 16 male patients and the mean age at the time of PD was 51.4 years. The average time from transplantation to a PD episode was 62.3 ± 53.2 mo (range 1-199 mo). Ileocolonoscopy detected mucosal abnormalities in 19 cases, whereas the intestinal mucosa appeared normal in 11 cases. Histological examination achieved a specific diagnosis in 19/30 cases (63.3%). In nine out of 11 cases (82%) with normal endoscopic appearance of the mucosa, histological examination of blinded biopsies provided a specific diagnosis. The etiology of PD was infectious in 11 cases (36.6%), drug-related in 10 (33.3%), of other causes in three (10%), and of unknown origin in six cases (20%). Infectious diarrhea occurred in significantly longer intervals from transplantation compared to drug-related PD (85.5 ± 47.6 mo *vs* 40.5 ± 44.8 mo, $P < 0.05$). Accordingly, PD due to drug-toxicity was rarely seen after the first year post-transplantation. Clinical improvement followed therapeutic intervention in 90% of cases. Modification of immunosuppressive regimen was avoided in 57% of patients.

CONCLUSION: Early ileocolonoscopy with biopsies from both affected and normal mucosa is an important adjunctive tool for the etiological diagnosis of PD in renal transplant patients.

Key words: Endoscopy; Post-transplantation diarrhea; Histology; Enteric infections; Mycophenolate mofetil-colitis

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Bamias G, Boletis J, Argyropoulos T, Skalioti C, Siakavellas SI, Delladetsima I, Zouboulis-Vafiadis I, Daikos GL, Ladas SD. Early ileocolonoscopy with biopsy for the evaluation of

persistent post-transplantation diarrhea. *World J Gastroenterol* 2010; 16(30): 3834-3840 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3834.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3834

INTRODUCTION

Diarrhea occurs frequently following renal transplantation, with reported incidences as high as 64% in large clinical trials[1-3]. Although several cases are benign and easily manageable, post-transplantation diarrhea can persist for a long period and compromise the health status of the patients. In particular, it leads to water and electrolyte disturbances, interferes with the absorption of immunosuppressive drugs, often requires hospital admission, and thus negatively affects the quality of life of the patients[4]. An association between post-transplantation diarrhea and decreased graft and patient survival has also been reported[5].

The diagnostic algorithm of post-transplantation diarrhea should take into consideration the specific characteristics of this population, particularly the presence of significant immunosuppression[6,7]. Infectious agents are often implicated; however, manifestation of enteric infections can vary considerably in this population[8,9]. Atypical presentations and severe forms of common infections frequently occur, whereas opportunistic infections with unusual microorganisms are also encountered. On the other hand, immunosuppressive regimens can cause intense and persistent diarrhea (PD)[10]. The most prominent example is toxicity of mycophenolate mofetil (MMF), which can cause enterocolitis in a substantial proportion of patients[11-14], requiring modification of the immunosuppressive regimen. However, reducing the dose of immunosuppression might lead to graft loss[15].

In the present study we have analyzed all cases of PD in renal transplant patients in our Hospital between July 2006 and June 2009. Our aim was to investigate the utility of early ileocolonoscopy, with biopsies taken both from identified lesions and blindly from normal looking mucosa, in establishing a definitive diagnosis for the diarrheal episode.

MATERIALS AND METHODS

Patient population and definitions of PD

We retrospectively reviewed the records of all renal transplant patients who presented with PD and had ileocolonoscopy as part of their diagnostic work-up in our hospital between July 2006 and June 2009.

All patients were followed at the Renal Transplantation Unit of our Hospital. Demographic, epidemiological, and clinical characteristics of the patients at the time of each diarrheal episode were retrieved from the medical files. We defined PD as an episode of diarrhea with the following characteristics: (1) change in the bowel habits with more than three movements per day and decreased stool consistency lasting longer than 2 wk; (2) an etiological diagnosis was not established after initial testing, including detailed history and clinical examination, extensive hematological, and biochemical tests, as well as stool cultures for enteric pathogens, examination for ova and parasites, and examination for *Clostridium difficile* toxins-A and B; (3) failure of diarrhea to resolve following simple dietetic modifications and non-immunosuppressive medication adjustment; and (4) further testing including ileocolonoscopy was considered necessary by the attending nephrologist, because diarrhea interfered with health status and quality of life of the patient. All patients with PD were tested with polymerase chain reaction (PCR) for cytomegalovirus (CMV) in blood; however, colonoscopy was always performed to detect endoscopic and/or histologically evident CMV-colitis.

Over the 3-year study period there was an agreed standard practice between the Renal Transplantation Unit and G.I. Endoscopy Unit of the 1st Department of Internal Medicine, to which renal transplant patients with PD are referred for ileocolonoscopy. Polyethylene glycol was used for bowel preparation. Sodium phosphate-based regimens were avoided due to their reported nephrotoxicity. Colonoscopy was performed with sedation (midazolam) and analgesia (pethidine), as required. During endoscopy, multiple biopsies were taken from all areas with mucosal abnormalities as well as blind biopsies from normal looking mucosa of the terminal ileum and throughout the colon (4-6 biopsies from right and left colon, respectively). Upper gastrointestinal (GI) tract endoscopy was performed selectively according to the clinical judgment of the treating physicians.

We defined the following categories of PD in relation with the underlying cause: (1) infectious, when a microorganism with an established role as a diarrhea-causing agent was detected by microbiological, histological, or molecular methods; (2) drug-induced, when infectious agents were excluded and histological findings consistent with pharmaceutical injury (most often MMF-related) were detected in the biopsy specimens. Histological findings highly suggestive of MMF-colitis, included: (a) mucosal abnormalities characterized by atrophy, crypt architectural distortion, flattened crypt epithelium, increased cell apoptosis and regenerative epithelial changes; and (b) edema, moderate inflammatory infiltrations with increased number of eosinophils, crypt abscesses and cryptitis, and, in the more severe cases, focal erosions or ulceration[13]. In addition, a clear beneficial effect of modification of the immunosuppressive regimen (MMF-dose reduction or switching to Myfortic or azathioprine) on the severity of PD was required to confirm a drug (MMF)-associated etiology of diarrhea; (3) Other, when a definitive cause (not associated with immunosuppressive medications or infectious agents) was established by clinical, laboratory, and histological findings; and (4) unknown, when no causative factor was identified. This group included cases with nonspecific changes either in endoscopy and/or at histology.

Statistical analysis

The SPSS software was used for the analysis. Continuous variables were analyzed by the independent *t*-test or

Table 1 Clinical and demographic characteristics of the study population[1]

	Total	Drug	Infection	Non-drug, non-infectious[2]	*P*[3]
No. of cases of persistent diarrhea	30	10	11	9	
Gender, *n* (%)					
Female	8 (26.7)	3 (30)	2 (18.2)	3 (33.3)	
Male	22 (73.3)	7 (70)	9 (81.8)	6 (66.7)	
Donor type					
Cadaveric	43.3	60	27.3	44.4	
Living	56.7	40	72.7	55.6	NS
Age at diarrheal episode (yr), mean ± SD (range)	51.4 ± 15.5 (24-76)	46.9 ± 17.1 (27-76)	52.6 ± 10 (40-70)	54.8 ± 19.4 (24-75)	NS
History of previous diarrheal episode, *n* (%)	21 (70)	4 (40)	9 (81.8)	8 (89)	0.081
Time since transplantation (mo), mean ± SD (range)	62.3 ± 53.2 (1-199)	40.5 ± 44.8 (1-142)	85.5 ± 47.6 (2-179)	58.1 ± 61.8 (6-199)	0.038
Immunosuppressive regimen[4], *n* (%)					
Mycophenolate mofetil + tacrolimus	18 (60)	6 (60)	6 (54.5)		
Mycophenolate mofetil + cyclosporine	2 (6.6)		1 (9.1)		
Mycophenolate mofetil + sirolimus	2 (6.6)	1 (10)	1 (9.1)		
Mycophenolate mofetil + everolimus	1 (3.3)	1 (10)			
Everolimus + tacrolimus	2 (6.6)		2 (18.2)		
Tacrolimus	2 (6.6)				
Mycophenolate sodium + tacrolimus	3 (10)	2 (20)	1 (9.1)		
Hospital stay (d), mean ± SD (range)	18.1 ± 30.6 (0-169)	8.5 ± 8.5 (0-22)	17.4 ± 11.6 (0-37)	29.4 ± 53.8 (0-169)	0.076
Outcome, *n* (%)					
Cessation of diarrhea	22 (73.3)	9 (90)	9 (82)	4 (44.4)	NS
Improvement	5 (16.7)			5 (55.6)	
Death/graft loss	3 (10)	1 (10)	2 (18)		

[1]Data are presented per episode of persistent diarrhea; [2]Other and unknown groups combined; [3]Comparison between infectious and drug-induced cases of persistent diarrhea; [4]All patients were taking methylprednisolone at the time of persistent diarrhea. NS: Not significant.

Mann-Whitney test (if they did not meet the criteria for parametric comparison). Categorical variables were studied by corrected χ^2 test. For all comparisons a probability level (*P*) of 0.05 was considered significant.

RESULTS

Demographic data

Over the study period, 30 ileocolonoscopies were performed for 30 separate episodes of PD in 23 renal transplant patients (Table 1). One patient had three episodes, five had two, and seventeen patients had one episode of PD. In all but one patient, the cause of PD differed between separate episodes. There was a clear predominance of males (2.3:1 male/female ratio), independently of the etiology of diarrhea (Table 1). The cause of renal failure and transplantation was polycystic kidney disease in four patients, kidney stone disease in three, whereas Henoch-Schönlein purpura, IgA nephropathy, recurrent kidney infections, renal hypoplasia, medullary cystic disease, and polyarteritis nodosa accounted for one case each. The etiology was unknown in 10 patients.

The immunosuppressive regimens that were administered at the time of each case of PD are shown in Table 1. All patients with more than one episode of PD were receiving the same immunosuppressive medications in all episodes, with the exception of one patient who was switched from MMF/tacrolimus (1st episode) to everolimus/tacrolimus (2nd and 3rd episodes) and a second patient in whom MMF/tacrolimus was changed to mycophenolate sodium/tacrolimus.

Table 2 Endoscopic[1] and histological[2] findings in renal transplant patients with persistent diarrhea

Cases	Endoscopy + histology +	Endoscopy + histology -	Endoscopy - histology +	Endoscopy - histology -
All	16	3	9	2
Drug-induced	4		6	
Infectious	7	1[3]	3	
Other	3			
No diagnosis[4]	2	2	0	2

[1]Ileocolonoscopy; [2]Including biopsies from normal-looking mucosa; [3]In this case biopsy was not taken because of typical pseudomembranous colitis in endoscopy; [4]Including cases with non-specific colitis in endoscopy or histology.

Endoscopic and histological studies

Twenty endoscopies were performed in inpatients and ten in outpatients. The cecum was reached in 26/30 colonoscopies (86.7%), with terminal ileum intubation in the vast majority of cases (22/26 with cecum intubation, 85%). We did not observe any serious complications related either to the preparation for colonoscopy, the use of sedatives/analgesics, or the procedure itself.

The diagnostic yield of ileocolonoscopy and histological examination of endoscopically obtained intestinal specimens are shown in Table 2. Biopsies were taken in all but one patient, in whom diagnosis of pseudomembranous colitis was established by typical history of prior antibiotic administration and endoscopic findings. Ileocolonoscopy revealed mucosal abnormalities in 2/3 of the patients. The most frequently encountered findings were

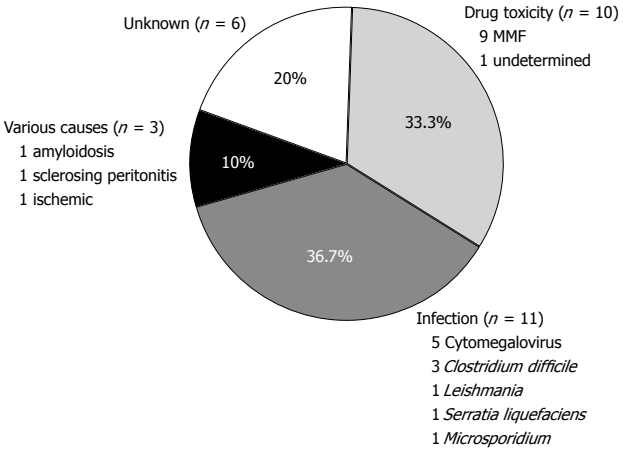

Figure 1 Causes of persistent diarrhea in renal transplant patients. MMF: Mycophenolate mofetil.

edema (loss of submucosal vascular pattern) and erythema of the mucosa, which were observed in 11 cases. More severe lesions included colonic ulceration (three cases), stenosis (two cases), submucosal hemorrhage (one case), and formation of pseudomembranes (two cases). We did not observe any endoscopic findings that were exclusively associated with infectious or drug-induced diarrhea (with the exception of pseudomembranous colitis).

Histological examination of biopsies obtained during endoscopy provided a definitive diagnosis in 19/30 cases (63.3%). More importantly, histology allowed for a specific diagnosis in nine out of 11 cases with normal endoscopic examinations (Table 2). Overall, an infectious cause was identified in 11 cases (Figure 1). The most prevalent infection was due to CMV, accounting for 16.6% of all cases. Interestingly, in 3/11 infectious cases (27%) there were no mucosal abnormalities seen on endoscopy. In three cases, diagnosis was established histologically in biopsy specimens taken from areas of normal looking mucosa (Table 2). These included two cases of CMV infection and one case infected with microsporidium. A case of leishmaniasis was diagnosed histologically by the recognition of the dot-like organisms within mucosal macrophages (Figure 2C). These were also revealed by Giemsa stain while PAS stain was negative.

In our study, we identified 10 episodes of PD (33.3%) that were related to toxicity of immunosuppressive drugs (Figure 1). All patients with drug-related diarrhea were receiving mycophenolate (eight MMF and two mycophenolate sodium) in combination with tacrolimus (eight cases), everolimus (one case), or sirolimus (one case). In the majority of drug-induced PD (6/10, 60%) the colonic mucosa looked normal on colonoscopy. Nevertheless, histological evaluation of blindly collected biopsies revealed mucosal changes consistent with MMF-colitis in all cases; thus establishing the diagnosis of drug-induced injury. These findings included mucosal abnormalities such as edema, atrophy, crypt architectural distortion, regenerative epithelial changes, and increased cell apoptosis with intraluminal apoptotic bodies (Figure 2A and B).

In our study there were three cases where a definitive diagnosis unrelated to infection or drug-toxicity was established. In the first patient, intestinal amyloidosis was diagnosed by histological examination and appropriate staining of a biopsy specimen obtained from the rectum. The second case involved a patient with sclerosing peritonitis. The pathophysiology of diarrhea was associated with external compression of the intestine by the sclerotic tissue and the accompanying motility and structural abnormalities, as diarrhea was completely abrogated following effective surgical decompression. Finally, in the third case, diarrhea was considered of ischemic origin as no other etiology was found and histology was compatible with ischemic intestinal injury. Taken together, these results show that endoscopy with histological examination of both affected and normal mucosa achieves a definitive diagnosis in the vast majority of PD in renal transplant patients.

Comparison between infectious and drug-induced PD

As our initial analysis showed that the majority of cases with PD were of infectious or pharmaceutical etiology, we then compared these two distinct groups for several characteristics. We observed no association between the type of diarrhea and the gender or age of the patient, or the type of donor (cadaveric *vs* living) (Table 1). In contrast, the time from transplantation to the PD episode differed significantly according to the etiological factor. In particular, this interval was considerably shorter in drug-related (40.5 ± 44.8 mo), as compared to infectious diarrhea (85.5 ± 47.6 mo, $P < 0.05$). There was a statistically significant difference between infectious and drug-induced PD ($P < 0.05$) in regards to their temporal distribution (Figure 3). In particular, while all but one case of infectious PD (91%) occurred later than 4 years post-transplantation, drug toxicity was usually seen at earlier time points. Accordingly, infection accounted for 14% of early episodes, whereas pharmaceutical toxicity accounted for 57%. In contrast, late episodes were caused primarily by infections (56%) and rarely by drugs (16.6%). In all, these results indicate that the time post-transplantation should be taken into consideration when searching for the etiology of PD in renal transplant patients, as different causes underlie early *vs* late episodes.

Outcome of PD

All but one case of infectious diarrhea required admission to the hospital, (91% admission rate) (Table 1). In contrast, fewer patients with drug-induced PD were admitted (60% admission rate). There was a trend towards longer hospital stay for patients with infectious diarrhea (mean hospital stay: 17.4 ± 11.6 d *vs* 8.5 ± 8.5 d for the drug-induced group, $P = 0.076$) (Table 1).

The overall outcome of PD was good, with cessation or improvement of diarrhea in 90% of cases (Table 1). There were two deaths in the infectious group, both unrelated to diarrhea. One patient with pseudomembranous colitis had a complicated clinical course due to disseminat-

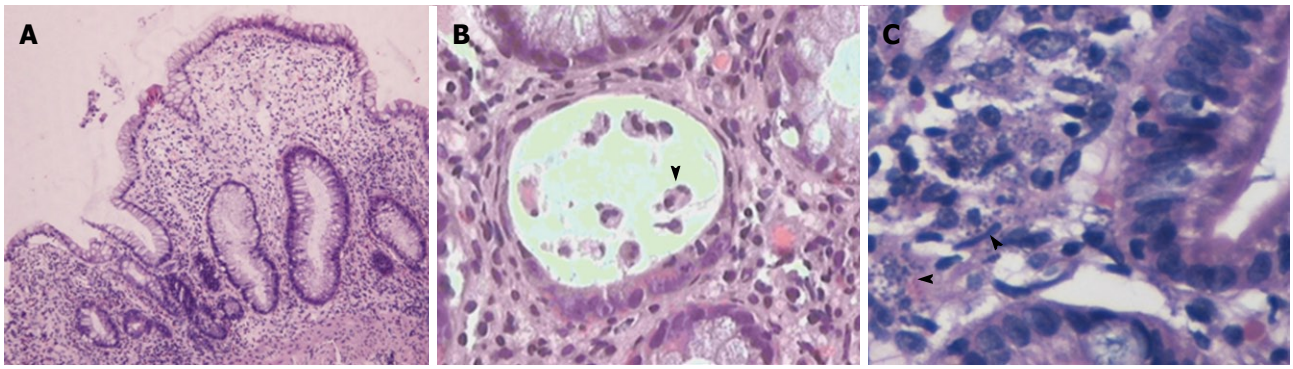

Figure 2 Histological photomicrographs from drug-induced and infectious cases of persistent diarrhea. A: Mycophenolate mofetil (MMF)-colitis, (HE stain, 200 × original magnification); B: MMF-colitis, with apoptotic bodies within the bowel lumen (arrowhead, HE stain, 400 × original magnification); C: Intestinal leishmaniasis with characteristic dot-like microorganisms within macrophages in the lamina propria (arrowheads, HE stain, 400 × original magnification).

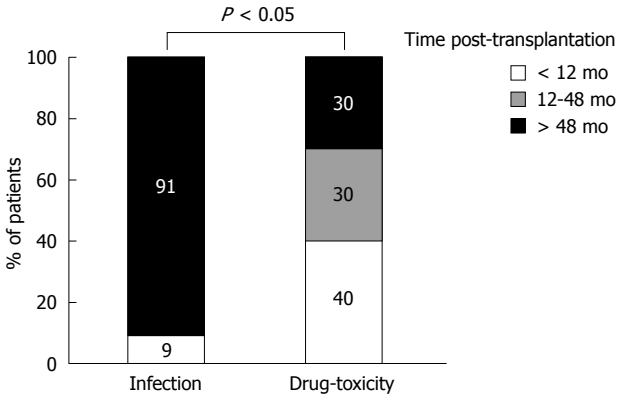

Figure 3 Distribution of infectious and drug-induced cases of persistent diarrhea in renal transplant patients according to the time post-transplantation.

ed fungal infection and was transferred to ICU where he eventually died. The other patient suffered from visceral leishmaniasis that had a fatal outcome.

Modification of immunosuppressive regimen was introduced in 12 cases. In five there was a switch from MMF to enteric-coated mycophenolate sodium, whereas in two the dose of mycophenolate was decreased with favorable outcomes in all cases. In four occasions mycophenolate had to be replaced by azathioprine. Finally, in one patient with drug-induced PD, diarrhea proved to be self-limited and required no change of immunosuppression. In our study, there was one case of graft loss in a patient with severe immunosuppression-related complications who had to stop all drugs with eventual loss of the graft.

DISCUSSION

In the present study we demonstrated that early ileocolonoscopy combined with histology of bowel mucosa, even without macroscopic abnormalities, is a critical component of the diagnostic evaluation of PD in renal transplant patients. We have shown that this approach provides a definitive diagnosis in the majority of cases, allowing prompt and specific treatment of the underlying cause, avoiding unnecessary modifications of the immunosuppressive regimen, and leading to favorable patient and graft outcome. Our data also indicate that PD is more likely due to drug-associated toxicity during the first post-transplantation year, while infectious diarrhea may occur throughout the post-transplantation period and is usually the cause of diarrhea after 4-year post-transplantation.

The majority of published studies on post-transplantation diarrhea did not take into account the severity or the duration of the episode[1-3,5]. In our study we focused on diarrhea that was judged as persistent, both in terms of long duration as well as of interference with the wellbeing of patients. We believe that these are the most clinically relevant cases and require extensive evaluation for the underlying causative agent. Our findings clearly show that there should be a low threshold for early ileocolonoscopy with histological examination in these patients. Such an approach is supported by the high percentage (80%) of definitive diagnoses that was accomplished in our study.

In a recent publication, a diagnostic algorithm for post-transplantation diarrhea was proposed, which introduced colonoscopy late in the course of evaluation and, more significantly, after modifications in immunosuppressive drugs were applied[16]. In fact, reduction of MMF is among the first measures taken in patients with post-transplant diarrhea[17]. This leads to cessation of diarrhea in a considerable proportion of cases, therefore avoiding the need for invasive tests such as colonoscopy. On the other hand, reducing the dose of immunosuppression often results in graft dysfunction[15,18,19]. In fact, in our study, the single episode of graft loss was associated with immunosuppression cessation due to severe toxicity, including drug-induced-diarrhea. Our results support the use of early endoscopy with histology in prolonged or refractory cases of diarrhea, as we were able to document non-drug-related causes in 46% of cases, thus avoiding unnecessary modifications of immunosuppressive regimens.

Early colonoscopy was suggested in a recent study on post-transplantation diarrhea, when there is strong clinical suspicion for CMV-colitis[20], including cases with positive PCR for CMV in the blood. Our findings support the use of colonoscopy with histology in this population, as it helps in establishing the localization of CMV in the intes-

tine and provides causality for chronic diarrhea. In fact, in our series, one of five cases with CMV-colitis had negative CMV-PCR in the blood, and a second one had very low number of CMV-DNA copies. Moreover, in some cases with positive CMV-PCR in the blood, colonoscopy and histology indicated absence of CMV-colitis, despite the presence of diarrhea, which was attributed to other causes.

To our knowledge there is only one published study that reported on the role of colonoscopy in renal transplant patients with diarrhea. Contrary to our study, Korkmaz *et al*[21] showed a 55% failure to establish a diagnosis with colonoscopy and/or histology. The higher rates observed in our study might be attributed to several factors. First, the severity of diarrhea in the Korkmaz study is not reported; it might, therefore, be the case that some colonoscopies were performed in milder cases with no obvious causative agent. Second, the accumulated experience on the histological lesions of MMF-colitis allowed us to use better-defined criteria for drug-induced toxicity; it is possible that such cases are included in the large number of non-specific colitis cases in the study by Korkmaz *et al*[21]. Finally, we took blinded biopsies in every patient, which was not the case in the aforementioned study. In another recent study only apparent lesions were biopsied during colonoscopy[22]. Our data clearly showed that histology of normal-appearing mucosa revealed pathognomonic findings in a considerable percentage of renal transplant patients with PD. In our study, this approach yielded a diagnosis in 27% of infection-related and in 60% of drug-induced cases of diarrhea.

Infectious agents and drugs accounted for the majority of PD cases in our cohort. This is in line with previous studies[23,24]. We detected a significant difference between the two groups (infectious *vs* drug-induced) regarding the time they occurred post-transplantation. In particular, the majority of drug-induced cases took place in the first years following transplantation. This distinction has also been observed in other studies[16]. This may be explained by the fact that intolerance to immunosuppressive regimen is expected to occur within relatively short time after their initiation[25]. In contrast, in our study, intestinal infection was diagnosed later than 4 years post-transplantation, almost exclusively. A temporal distribution of various infections post-transplantation has been reported[26]. These data, as well as our present findings, indicate that the search for PD etiology should be tailored to the individual patient, taking into consideration the time post-transplantation. In the case of an episode that takes place long after transplantation, intensive search for infectious agents is primarily required.

We were not able to establish a diagnosis in 6/30 cases (20%), including four that were classified as non-specific colitis. Follow-up revealed that diarrhea ceased or was greatly improved, indicating that self-limited infections and/or unspecified pharmacotoxicity underlay these cases. In fact, in one case *Candida albicans* was isolated from the stools, whereas in two others CMV-viremia was detected. However, since a direct proof of causality was not established, we classified these cases as non-specific colitis and not infectious. Only in two cases of unknown etiology was modification of immunosuppressive regimen considered necessary.

In conclusion, our results indicate that ileocolonoscopy has an important impact in the management of renal transplant patients with PD and should be an adjunctive tool for the causative diagnosis of PD. Endoscopy should be considered only after initial measures have failed to induce clinical improvement. These measures may include adjustment of the immunosuppressive regimen, particularly when diarrhea manifests during the initial post-transplantation months as the prevalence of drug-related causes is increased during that period. In any case, biopsies should always be taken from the lower GI tract as histology achieves a definitive diagnosis in the majority of cases, even when the intestinal mucosa appears macroscopically normal. This approach may offer the opportunity for specific treatment and lead to improved outcomes following renal transplantation.

COMMENTS

Background

Diarrhea is among the most common complications in patients who receive renal transplants and has been associated with poor outcomes in terms of quality of life as well as graft and patient survival. Infectious agents often cause diarrhea due to the universal administration of immunosuppressive regimens in this population. Immunosuppressants can themselves cause significant gastrointestinal toxicity, the most prominent example being mycophenolate mofetil-enterocolitis.

Research frontiers

Previous studies have reported diagnostic algorithms for the evaluation of post-transplantation diarrhea. Endoscopic and histological studies of the lower gastrointestinal tract have been incorporated only at the late steps of diagnostic protocols, usually when extensive clinical and laboratory work-up has been negative and modifications in the immunosuppressive scheme have been ineffective to induce diarrhea cessation.

Innovations and breakthroughs

In the present study, the authors investigated the usefulness of a standard approach for the evaluation of persistent diarrhea (PD) in renal transplant patients, which utilized an early ileocolonoscopy, i.e. as soon as limited laboratory testing came back negative. Moreover, biopsies were routinely taken both from all identified lesions but also blindly from normal looking mucosa. The present study demonstrated a high efficacy of this diagnostic scheme in establishing a definitive diagnosis for the diarrheal episode.

Applications

The application of early ileocolonoscopy with standard tissue sampling may facilitate etiologic diagnosis and targeted treatment of PD in renal transplant patients; thus avoiding unnecessary changes in the immunosuppressive regimen. This approach may be of particular importance in the late post-transplantation period, when non-drug related causes of diarrhea are increased.

Terminology

PD: an episode of diarrhea lasting longer than 2 wk and interfering with the health status and quality of life of the renal transplant patient, for which an etiological diagnosis is not established after initial clinical examination, baseline hematological and biochemical tests, as well as stool tests for infectious causes and which did not respond to simple dietetic modifications and non-immunosuppressive medication adjustments.

Peer review

The authors investigated the role of colonoscopy on persistent post-transplantation diarrhea. The results indicated that colonoscopy is a valuable diagnostic tool for evaluating transplant recipients with PD. The paper is well written and data clearly presented. The work contributes to the understanding and guides management of this important complication despite the small number of patients and selection bias.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3841

World J Gastroenterol 2010 August 14; 16(30): 3841-3846
ISSN 1007-9327 (print)

BRIEF ARTICLE

Helicobacter infection concomitant with metabolic syndrome further increase risk of colorectal adenomas

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Supported by Buddhist Dalin Tzu Chi General Hospital
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Received: April 4, 2010 Revised: May 10, 2010
Accepted: May 17, 2010
Published online: August 14, 2010

Abstract

AIM: To investigate the association of colorectal adenomas with both *Helicobacter pylori* (*H. pylori*) infection and metabolic syndrome.

METHODS: Using a cross-sectional hospital-based study, we analyzed physical examination data from 9311 healthy subjects with overnight physical examinations performed between January 2004 and December 2006. Examined data included gender, age, life style, anthropometric measurements, blood pressure, biochemical and hematological studies, *H. pylori* infection detected by esophagogastroduodenoscopy and biopsy urease tests, and colorectal adenomas detected with a complete total colonoscopy.

RESULTS: The prevalence values for *H. pylori* infection, metabolic syndrome, and colorectal adenoma were 39.2%, 18.7%, and 20.7%, respectively. Colorectal adenoma risk factors included male gender [odd ratio (OR): 2.005, 95% confidence interval (CI): 1.740-2.310, $P < 0.001$], advanced age (OR: 1.046, 95% CI: 1.040-1.052, $P < 0.001$), smoking (OR: 1.377, 95% CI: 1.146-1.654, $P = 0.001$), increased body fat (OR: 1.016, 95% CI: 1.007-1.026, $P = 0.001$), higher white blood cell count (OR: 1.038, 95% CI: 1.005-1.073, $P = 0.025$), *H. pylori* infection (OR: 1.366, 95% CI: 1.230-1.517, $P < 0.001$), and metabolic syndrome (OR: 1.408, 95% CI: 1.231-1.610, $P < 0.001$). In addition, concomitant *H. pylori* infection with metabolic syndrome further increased the probability of colorectal adenomas.

CONCLUSION: Our study revealed *H. pylori* infection with concomitant metabolic syndrome might further increase the risk of colorectal adenomas.

Key words: Biopsy urease test; Colorectal adenoma; Colorectal cancer; *Helicobacter pylori*; Metabolic syndrome

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Lin YL, Chiang JK, Lin SM, Tseng CE. *Helicobacter* infection concomitant with metabolic syndrome further increase risk of colorectal adenomas. *World J Gastroenterol* 2010; 16(30): 3841-3846 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3841.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3841

INTRODUCTION

Colorectal cancer is an extremely common malignancy and one of the leading causes of cancer mortality worldwide. Colorectal adenoma is the premalignant lesion in colorectal cancer and develops into colorectal carcinoma

through the adenoma-to-carcinoma sequence[1]. The direct etiology of colorectal neoplasms is still unknown. However, previous epidemiological studies have identified family history, dietary factors, smoking, sedentary lifestyles, and alcohol consumption as potential contributors to colorectal neoplasm development[2]. Identification of the etiology of colorectal neoplasms might assist in the development of strategies targeted toward its prevention.

Helicobacter pylori (*H. pylori*) is a human pathogen that infects the gastric mucosa and causes inflammatory process that culminate in chronic gastritis, peptic ulceration, gastric lymphoma of mucosa-associated lymphoid tissue, and adenocarcinoma[3]. *H. pylori* is a gram-negative microaerophilic bacillus, and has been classified by the International Agency for Research on Cancer as a class I human carcinogen since 1994[4]. The role of *H. pylori* in colorectal carcinogenesis has been epidemiologically examined in recent decades; however, the association has remained inconclusive. Several studies have identified an association between *H. pylori* infection and colorectal neoplasms[5-9], while others have identified a negative association between the two[10-12]. Methodological issues might account for some of the inconsistent results, including the IgG serum antibody test and incomplete colonoscopic examinations for diagnosis.

Metabolic syndrome is a clinical cluster of metabolic abnormalities. It is also referred to as insulin resistance syndrome, and is diagnosed by criteria corresponding to the modified National Cholesterol Education Program (NCEP) criteria[13]. Diagnosis is fulfilled by the presence of any three of the following conditions: higher waist circumference ($\geq$ 90 cm in men and $\geq$ 80 cm in women), elevated triglycerides ($\geq$ 150 mg/dL), lower high density lipoprotein cholesterol ($<$ 40 mg/dL in men and $<$ 50 mg/dL in women), elevated blood pressure (systolic blood pressure $\geq$ 130 mmHg or diastolic blood pressure $\geq$ 85 mmHg), and elevated fasting glucose ($\geq$ 100 mg/dL). This syndrome might be a risk factor for type 2 diabetes and cardiovascular disease[14,15]. In recent years, metabolic syndrome has also been associated with an increased risk of colorectal adenoma. However, there is very limited medical literature examining the relationship between colorectal adenoma and metabolic syndrome[16-18]. Additional information on the correlation between metabolic syndrome and colorectal neoplasms could result in the recommendation for screening of colorectal neoplasms in the patient with metabolic syndrome.

Using a cross-sectional hospital-based study, we investigated the association of colorectal adenoma with both *H. pylori* infection and metabolic syndrome. Further, the probability of colorectal adenoma in patients with both *H. pylori* infection and metabolic syndrome was evaluated.

MATERIALS AND METHODS

A total of 11 787 asymptomatic subjects were admitted to the general physical examination department of the Buddhist Dalin Tzu-Chi General Hospital for general check-ups (two-day health examination) between January 2004 and December 2006. Excluding 2476 subjects aged below 40 years, a final total of 9311 study participants (3906 males and 5405 females) were enrolled in the study. The demographic data included age, gender, medical past history, and lifestyle. Clinical data included blood pressure, fasting plasma sugar, plasma lipids levels (total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides), and hematological variables. Anthropometric measurements including height (meters), weight (kilograms), and body fat (percent; Body Composition Analyzer TBF-410, Tanita, Japan) were also examined.

Metabolic syndrome was diagnosed with the modified NCEP criteria. *H. pylori* infection was detected by the biopsy urease test (CLO test, Pronto Dry, Gastrex, Poland) using standard video esophagogastroduodenoscopy (EGD) with gastrofibroscopes (GIFXP-240, GIFQ260, Olympus Optical, Tokyo Japan). A specimen for biopsy urease testing of each subject was taken from the gastric antrum using biopsy forceps and assessed within 60 min. The agar color of the biopsy urease testing turned from yellow to red when the biopsy specimen was infected with *H. pylori*, which contained intracytoplasmic urease. Colorectal adenomas were identified by complete total colonoscopy using standard video colonoscopes (CF 240I, Olympus Optical, Tokyo, Japan) by single- and double-handed methods under intravenous 1% Propofol (Fresenius Kabi, Austria). This study was performed under the approval of our hospital Institutional Review Board.

Statistical analysis

Data for continuous variables were expressed as mean ± SD. The *t* test was applied for continuous variables when the data fitted a Gaussian distribution. If the continuous data did not fit the Gaussian distribution, the Wilcoxon rank sum test was applied. Categorical variables were tested with the χ^2 test. Stepwise logistic regression analysis was conducted for significant variable selection. Basic model-fitting techniques for regression analysis were applied to assure the quality of analysis results, including variable selection, goodness-of-fit assessment, and regression diagnostics. Statistical significance was established for two-sided *P* values $<$ 0.05. All statistical analyses were performed with the SAS® software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and R 2.6.2 (R Development Core Team, R Foundation for Statistical Computing, 2008, Vienna, Austria).

RESULTS

The median ages of the study participants were 54 years in males and 52 years in females. All subjects went through complete EGD examination, and 2.8% of participants had incomplete colonoscopy examination. The raw prevalence rates of *H. pylori* infection, metabolic syndrome, and colorectal adenoma were 39.2%, 18.7% and 20.7%,

Table 1 Baseline characteristics of the study subjects

Variable	Male	Female	*P*
n	3906	5405	
Age (yr)	54 (48, 61)	52 (47, 59)	< 0.001
Smoke, *n* (%)	884 (22.6)	49 (0.9)	< 0.001
Alcohol, *n* (%)	876 (22.4)	119 (2.2)	< 0.001
Body weight (kg)	67.6 (61.8, 74.2)	56.0 (51.3, 61.7)	< 0.001
Body fat (%)	22.7 (19.4, 26.1)	30.6 (26.8, 34.9)	< 0.001
Systolic BP (mmHg)	128 (116, 141)	122 (111, 138)	< 0.001
Diastolic BP (mmHg)	81 (73, 88)	74 (66, 82)	< 0.001
Hypertension, *n* (%)	1518 (38.9)	1575 (29.1)	< 0.001
Diabetes, *n* (%)	317 (8.1)	330 (6.1)	< 0.001
Glucose AC (mg/dL)	90 (84, 97)	88 (83, 95)	< 0.001
TCH (mg/dL)	191 (168, 215)	190 (169, 215)	0.448
WBC (× 10^3/μL)	6.31 (5.38, 7.42)	5.90 (5.02, 6.97)	< 0.001
Lymphocyte (%)	32.2 (27.0, 37.4)	33.9 (28.7, 39.2)	< 0.001
MS, *n* (%)	755 (19.3)	982 (18.2)	0.154
H. pylori, *n* (%)	1571 (40.2)	2083 (38.5)	0.096
Adenoma, *n* (%)	1053 (27.0)	870 (16.1)	< 0.001

n: Subject number; BP: Blood pressure; TCH: Total plasma cholesterol; WBC: White blood cell; *H. pylori*: *Helicobacter pylori*; MS: Metabolic syndrome.

Table 2 Multivariate logistic regression analysis of the risk factors for colorectal adenomas

Variable	β	SE	*P*	OR	95% CI
Intercept	-5.031	0.253	< 0.001	-	-
Gender (M *vs* F)	0.696	0.072	< 0.001	2.005	1.740-2.310
Age (per year)	0.045	0.003	< 0.001	1.046	1.040-1.052
Smoke (yes *vs* no)	0.320	0.094	0.001	1.377	1.146-1.654
Alcohol (yes *vs* no)	-0.010	0.093	0.915	0.990	0.826-1.187
Body fat (%)	0.016	0.005	0.001	1.016	1.007-1.026
WBC (per 10^3/μL)	0.038	0.017	0.025	1.038	1.005-1.073
H. pylori (yes *vs* no)	0.312	0.054	< 0.001	1.366	1.230-1.517
MS (yes *vs* no)	0.342	0.068	< 0.001	1.408	1.231-1.610

WBC: White blood cell; *H. pylori*: *Helicobacter pylori*; MS: Metabolic syndrome; OR: Odd ratio; CI: Confidence interval.

respectively. A total of 1923 adenomas, including 1691 tubular adenoma, 208 tubulovillous adenomas, and 24 serrated adenomas, were detected. Males were significantly older ($P < 0.001$), were more likely to smoke ($P < 0.001$), drink alcohol ($P < 0.001$), have heavier body weight ($P < 0.001$), lesser body fat ($P < 0.001$), and higher systolic and diastolic blood pressure values ($P < 0.001$). Males additionally had a higher proportion of hypertension ($P < 0.001$), diabetes ($P < 0.001$), higher fasting blood glucose levels ($P < 0.001$), higher white blood cell (WBC) counts ($P < 0.001$), lower lymphocyte percentages ($P < 0.001$), and a higher prevalence of colorectal adenoma ($P < 0.001$). There were no significant differences in total plasma cholesterol levels ($P = 0.448$), metabolic syndrome frequency ($P = 0.154$), and *H. pylori* infection frequency ($P = 0.096$) between males and females (Table 1).

Multivariate logistic regression analysis revealed that male gender (OR: 2.005; 95% confidence interval (CI), 1.740-2.310, $P < 0.001$), advanced age (OR: 1.046, 95% CI: 1.040-1.052, $P < 0.001$), smoking (OR: 1.377, 95% CI: 1.146-1.654, $P = 0.001$), increased body fat (OR: 1.016, 95% CI: 1.007-1.026, $P = 0.001$), higher white blood cell (WBC) count (OR: 1.038, 95% CI: 1.005-1.073, $P = 0.025$), *H. pylori* infection (OR: 1.366, 95% CI: 1.230-1.517, $P < 0.001$), and metabolic syndrome (OR: 1.408, 95% CI: 1.231-1.610, $P < 0.001$) were associated risk factors for colorectal adenoma. Alcohol consumption (OR: 0.990, 95% CI: 0.826-1.187, $P = 0.915$) was not a risk factor for colorectal adenoma (Table 2). Under analysis with a conditional effect plot, colorectal adenoma risk was positively associated with WBC count among paired groups of positive and negative *H. pylori*-infected patients and paired groups of positive and negative metabolic syndrome patients (Figure 1).

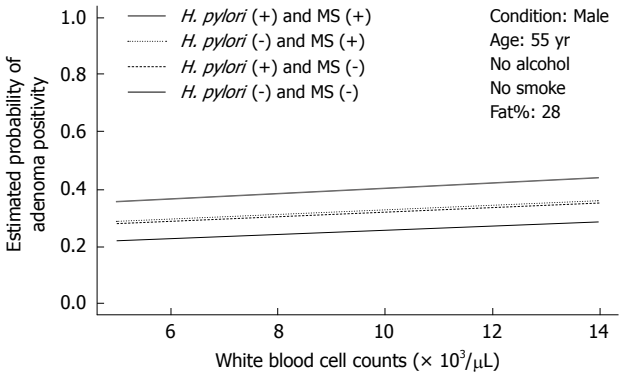

Figure 1 Conditional effect plot of *Helicobacter pylori* infection status and metabolic syndrome on the probability of adenoma positivity. The conditions were designed as non-smoking males at 55 years old and 28% body fat with the pair of both *Helicobacter pylori* (*H. pylori*) positivity and metabolic syndrome (MS) positivity *vs* another three pairs.

DISCUSSION

The results of our study supported the association of *H. pylori* infection with colorectal adenomas and were consistent with previous reports that metabolic syndrome might increase colorectal adenoma risk. It also showed that individuals with concomitant metabolic syndrome and *H. pylori* infection might have a further increased risk of colorectal adenomas.

The inconclusive results of previous studies concerning the relationship between *H. pylori* infection and colorectal neoplasm might have been due to sample bias, small sample size, inadequate consideration of potential confounding variables, and a varying frequency of cag A+ strains in the study populations[19,20]. In addition, incomplete colonoscopic studies and evaluation of *H. pylori* infection with the IgG serum test (which cannot represent real-time *H. pylori* infection) might also have contributed to the inconsistent results. The advantages of our study include large sample size, detection of *H. pylori* infection with the EGD and biopsy CLO test, and complete colonoscopy to the distal terminal ileum after good bowel preparation in 97.2% of the cases. Furthermore, patient lifestyle habits including smoking and alcohol consumption, gender, and age were also evaluated in this study. These factors might minimize potential variables during the data analysis.

However, there were also some limitations in our study. Patients in the study were selected from a population who sought routine physical examinations at our institute, and their psychosocial behaviors and lifestyle habits might differ from those in the general population, resulting in a confounding bias that could be ignored in the data analysis. Although *H. pylori* infection can be more accurately detected by biopsy CLO test than by the serum IgG method logically, in rare instances antral biopsies with CLO tests might not be representative of all gastric states of *H. pylori* infection. In addition, past historical data of diagnosis and treatment of *H. pylori* infection were not included in the analysis, although the enrolled cases were clinically asymptomatic. Additionally, blood insulin, gastrin levels, and proinflammatory cytokines were not measured. The study design also did not allow the identification of the pathologic mechanisms underlying the association of colorectal adenoma with metabolic syndrome and *H. pylori* infection.

The pathogenic mechanisms by which *H. pylori* exerts its malignant potential in the induction of colorectal neoplasms are not completely understood. A few studies have revealed that fecal shedding of viable *H. pylori* and its antigen occurs under certain circumstances[21,22], suggesting that *H. pylori* moves through the intestinal tract in direct contact with colonic mucosa, and could therefore locally activate colonic carcinogenesis. *H. pylori* was recently detected within colorectal carcinoma tissues[23]. The role of *H. pylori*-specific affinity for colorectal neoplasms requires further investigation. The presence of *H. pylori* might alter normal gastrointestinal flora as a consequence of progressive chronic gastritis with glandular atrophy and decreased acid production. This could further influence colorectal carcinogenesis. Persistent *H. pylori* exposure induces hypergastrinemia, which is a putative trophic factor for the large bowel mucosa. Cell proliferation and gastrin-induced genomic instability can increase the risk of DNA replication error and play a role in the development of colorectal neoplasms[24]. *H. pylori* infection might also result in direct damage to the colorectal mucosa or indirect damage to the epithelium through inflammatory responses. Contact between a repairing epithelium and endogenous or dietary carcinogens within the gut might transform the colorectal mucosa[25]. The CagA protein is the product of the cytotoxin-associated gene and is produced by cagA+ strains of *H. pylori*. It might locally activate colonic carcinogenesis through the induction of cytokine expression, including cytokines such as interleukin (IL)-8, which is associated with colorectal cancer[26]. In summary, *H. pylori* might result in local and distant interactions with colorectal mucosa and contribute to the pathogenesis of malignant transformation. However, further mechanistic studies are required.

Metabolic syndrome and its association with colorectal adenomas have been the subjects of recent study, and the pathogenic mechanisms for this potential association are still unclear. Insulin (a core contributor to metabolic syndrome) has been demonstrated to promote colorectal carcinogenesis in animal studies for more than 10 years[27,28]. It is postulated that insulin might exert proliferative effects on colonic tumor cells directly or indirectly *via* the insulin-like growth factor pathway[29]. Furthermore, increased production of proinflammatory cytokines and decreased production of anti-inflammatory adiponectin in adipocytes might be related to adenoma growth[30]. In addition, hypertriglyceridemia (a component of metabolic syndrome) might be involved in colorectal neoplasm pathogenesis. Triglycerides act as potent energy sources for cancer cell growth[31], and elevated serum triglyceride levels have been associated with increased synthesis of bile acids, which could promote large bowel carcinogenesis, as demonstrated in experimental studies[32]. Metabolic syndrome is associated with chronic inflammation, which might explain its possible association with colorectal adenoma. Adipose tissue and circulating levels of inflammatory cytokines [including tumor necrosis factor (TNF)-α and IL-6] are increased in obese and diabetic patients, and can induce several metabolic derangements characteristic of metabolic syndrome[33,34]. IL-6-induced C-reactive protein (CRP) could predict colon cancer occurrence; meanwhile, an elevated CRP level is a consistent feature of metabolic syndrome[35]. The findings indicate that chronic inflammation might be associated with colorectal carcinogenesis. In short, these evidence-base data suggest that metabolic syndrome might be a risk factor for colorectal neoplasm development.

In this study, concomitant with *H. pylori* infection and metabolic syndrome might further increase the risk of developing colorectal adenoma. The concomitant effect of metabolic syndrome and *H. pylori* might occur secondary to common inflammatory pathways of colorectal pathological mechanisms associated with metabolic syndrome and *H. pylori* infection. The inflammation-related factors of metabolic syndrome include IL-6, TNF-α, fibrinogen, and cyclooxygenase-2. The inflammation-related factors of *H. pylori* including IL-8, TNF-α, and the Cag A, Vac A, and babA2 proteins might display similar inflammatory effects attributable to the common inflammatory pathway. White blood cell counts are a risk factor of colorectal adenoma in the multivariate logistic regression analysis and might support this hypothesis of the involvement of the common inflammatory pathway. However, further investigations on the pathogenesis of this concomitant effect are necessary. Clinically, our results suggested that both *H. pylori* infection and metabolic syndrome should both be evaluated for the prevention of colorectal adenomas and carcinomas.

Studies have revealed that moderate alcohol consumption is related to increased insulin-sensitivity[36], while smoking exerted the opposite effect[37]. Other studies have suggested that both alcohol use and cigarette smoking were associated with increased risk of colorectal adenoma[38,39]. Cigarette smoking was related to colorectal adenomas in this study, although alcohol consumption was not. To clarify the association between alcohol consumption and colorectal adenoma, further studies are necessary.

In conclusion, this cross-sectional hospital-based study revealed a direct association of colorectal adenoma with *H. pylori* infection and metabolic syndrome. Furthermore, *H. pylori* infection concomitant with metabolic syndrome

might further increase the risk of colorectal adenoma. These results suggest that both *H. pylori* infection and metabolic syndrome should be considered important entities with regards to the prevention of colorectal adenoma and carcinoma. This is particularly important when a patient clinically presents with concomitant *H. pylori* infection and metabolic syndrome. The combined effects of metabolic syndrome and *H. pylori* infection should be further clarified.

ACKNOWLEDGMENTS

The authors greatly appreciate the financial and technical support of Dalin Tzu Chi General Hospital, and the help of the President of the School of Medicine of Japan Jichi Medical University, Professor Fumimaro Takaku, who kindly revised the manuscript.

COMMENTS

Background

Colorectal cancer is one of the leading causes of cancer mortality worldwide. Colorectal adenoma is the premalignant lesion in colorectal cancer. Identification of the etiology of colorectal neoplasms might assist in the development of strategies targeted toward its prevention. Previous epidemiological studies have identified family history, dietary factors, smoking, sedentary lifestyles, and alcohol consumption as potential contributors to colorectal neoplasm development. Recently, reports revealed that *Helicobacter pylori* (*H. pylori*) infection is associated with colorectal neoplasm, and a few reports disclosed that metabolic syndrome was also associated with an increased risk of colorectal adenoma. Based on these findings, the probability of colorectal adenoma in patients with both *H. pylori* infection and metabolic syndrome was further evaluated.

Research frontiers

Colorectal cancer is an extremely common malignancy, however, the direct etiology of colorectal neoplasm is still unknown. Epidemiologically, identification of the etiology of colorectal neoplasm might assist in development of strategies targeted toward its prevention. During latest decade, *H. pylori* infection and metabolic syndrome, respectively, were identified to be associated with colorectal neoplasms and hypotheses were provided to explain the mechanisms of their relationships. However, until now, there was no study focusing on whether concomitant *H. pylori* infection with metabolic syndrome in a patient will increase his or her risk of colorectal adenoma.

Innovations and breakthroughs

This study supported the association of colorectal adenoma individually with *H. pylori* infection and metabolic syndrome. Furthermore, *H. pylori* infection concomitant with metabolic syndrome might further increase the risk of colorectal adenoma.

Applications

These results suggest that both *H. pylori* infection and metabolic syndrome should be considered important entities with regards to the prevention of colorectal adenoma and carcinoma. This is particularly important when a patient clinically presents with concomitant *H. pylori* infection and metabolic syndrome; the increased risk of developing colorectal adenomas should be more seriously considered for preventive purpose.

Peer review

This paper reports an important study that assesses the rate of metabolic syndrome, *H. pylori*, and colonic adenomas in a large population of asymptomatic individuals. The significances of these findings are discussed in relation to previous studies, and hypothesis to explain the findings are reviewed. Strengths of the study include the size of the sample, high completion rate of colonoscopy, and the use of well-established diagnostic criteria for metabolic syndrome in this population. Limitations are acknowledged by the authors and include the single centre nature of the study, select patient population, and possible false negative results of HP testing.

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S- Editor Wang YR **L- Editor** Stewart GJ **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3847

World J Gastroenterol 2010 August 14; 16(30): 3847-3852
ISSN 1007-9327 (print)

BRIEF ARTICLE

Germline mutation analysis of *hPMS2* gene in Chinese families with hereditary nonpolyposis colorectal cancer

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Supported by The Initiation Fund for Prominent Young Researchers of Shanghai Medical College, Fudan University
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Received: March 25, 2010 Revised: May 21, 2010
Accepted: May 28, 2010
Published online: August 14, 2010

Abstract

AIM: To study the germline mutation of *hPMS2* gene in 26 unrelated Chinese hereditary nonpolyposis colorectal cancer (HNPCC) probands and to fulfill the screening strategy for HNPCC in Chinese.

METHODS: Genomic DNA was extracted from the peripheral blood. To avoid the interference of pseudogene in detection of the remaining 11 exons (exon 1-5, 9, 11-15), long-range polymerase chain reaction (PCR) was conducted to amplify the complete coding region of *hPMS2* gene firstly. Then 1/8 of the PCR products were used as template to amplify the individual exon respectively and DNA sequencing was done. Direct DNA sequencing of the conventional PCR products of exon 6, 7, 8 and 10 of *hPMS2* gene was performed. The same analysis was made in 130 healthy persons without family histories of HNPCC to further investigate the pathological effects of the detected missense mutation.

RESULTS: One HNPCC proband fulfilled Bethesda guidelines and was found to carry the germline mutation of *hPMS2* gene, which has not been reported in Chinese HNPCC families. It was a missense mutation at c.1532C>T of exon 11. It was detected in three controls as well with an occurrence rate of 2.3% (3/130). Since it could not be found in the PMS2-single nucleotide polymorphism (SNP) database, this missense mutation is a new SNP unreported up to date. Meanwhile, 260 reported SNPs of *hPMS2* gene were detected in the 26 HNPCC probands. The 2nd and 5th exons were probably the hot SNP regions of *hPMS2* gene in Chinese HNPCC families involving 53.1% of all reported SNP.

CONCLUSION: The germline mutation of *hPMS2* gene may be rare in Chinese HNPCC families. The 2nd and 5th exons are hot SNP regions of *hPMS2* gene.

Key words: Hereditary nonpolyposis colorectal cancer; *hPMS2*; Missense mutation; Single nucleotide polymorphism; Colorectal cancer

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Sheng X, Zhou HH, Zhou XY, Du X, Zhang TM, Cai SJ, Sheng WQ, Shi DR. Germline mutation analysis of *hPMS2* gene in

Chinese families with hereditary nonpolyposis colorectal cancer. *World J Gastroenterol* 2010; 16(30): 3847-3852 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3847.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3847

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominantly inherited disease with cancer-susceptibility. Perhaps it is the most common cause of hereditary colorectal cancer, accounting for 5%-10% of the total colorectal cancers worldwide[1-3]. People inheriting this predisposition are at a particularly high risk of developing colorectal cancer with an early age of onset[3,4]. The affected patients always carry germline mutations in DNA mismatch repair (MMR) genes, mostly in *hMLH1, hMSH2*, and *hMSH6*[5,6]. Less commonly, mutations in other MMR genes are present. We analyzed the abnormalities of *hMSH2/hMLH1/hMSH6* genes in a series of Chinese HNPCC families fulfilling different clinical criteria. We studied germline mutation, large genomic variations of the entire coding regions of the three genes and methylation of *hMLH1* promoter in 58 Chinese HNPCC probands, in which 24 fulfilled Amsterdam criteria (AC)[7], 15 fulfilled Japanese criteria (JC)[8] and 19 met Bethesda guidelines (BG)[7]. The total detected gene abnormality rate was only 53.4% (31/58), including 29 cases of germline mutation and 2 cases of methylation of *hMLH1* promoter[9-14]. So the aberrant MMR genes other than *hMSH2/hMLH1/hMSH6* are suspected to be involved in Chinese HNPCC.

In order to accomplish our serial studies of Chinese HNPCC, we detected *hPMS2* germline mutation in 26 Chinese HNPCC families by long-range polymerase chain reaction (LR-PCR)-based sequencing in this study, and evaluated this manner in the molecular genetics screening of Chinese HNPCC.

MATERIALS AND METHODS

Materials

Twenty-six unrelated HNPCC probands registered from January 1998 to October 2005 at the Department of Abdominal Surgery in Shanghai Cancer Center were retrieved. Five of them fulfilled AC, 10 fulfilled JC and the remaining 11 fulfilled BG. Germline abnormalities of *MSH2/MLH1/MSH6* were excluded in all the 26 probands by PCR-based sequencing. Ten milliliter peripheral blood was collected from each proband for genomic DNA preparation. The peripheral blood samples of 130 healthy volunteers without any family history of hereditary disease or development of colon cancer in early age were obtained for control. The informed consents were signed by all the probands and volunteers before blood drawing. This study was approved by the Medical Ethical Committee of Shanghai Cancer Center, Fudan University. The whole procedures of the study were in accordance with the international rules and regulations.

Table 1 Primer sequences of long-range polymerase chain reaction

Primer name	Sequence (5′-3′)	Size (bp)	Exon
LRPCR1			
For	ACGTCGAAAGCAGCCAATGGGAGTT	9964	Exon 1-5
Rev	CTTCCACCTGTGCATACCACAGGCT		
LRPCR2			
For	GGTCCAGGTCTTACATGCATACTGT	9440	Exon 9
Rev	CTGACTGACATTTAGCTTGTTGACA		
LRPCR3			
For	GCGTTGATATCAATGTTACTCCAGA	8812	Exon 11,
Rev	AGTAGTCAGGGTAAAACATTCCAGT		12
LRPCR4			
For	AAAATTAGTCAGACTTGATGGTGTG	9804	Exon
Rev	CCTTCCATCTCCAAAACCAGCAAGA		13-15

DNA extraction

Genomic DNA was extracted from the peripheral blood using the QIAGEN (Hilden, Germany) DNA extraction kit and following the manufacturer's instructions. Concentrations of the genomic DNA were determined by an ultraviolet spectrophotometer (Beckman, DU640 type).

PCR amplification and DNA sequencing

LR-PCR (exon 1-5, 9, and 11-15): Since exon 1-5, 9, and 11-15 of *hPMS2* genes were severely hampered by the presence of multiple pseudogenes with highly similar sequences. LR-PCR was conducted to preferentially amplify *hPMS2* gene and avoid the interference of the pseudogenes.

Four overlapping sets of primers were designed to amplify the complete coding region of *hPMS2* gene by LR-PCR[15,16] (Table 1). The LR-PCR amplification profile is also shown in Table 1. Then 1/8 of the four LR-PCR products were used as template to amplify the 11 exons (exon 1-5, 9, 11-12 and 13-15) individually. The primer sequences are listed in Table 2.

PCR (exon 6, 7, 8 and 10): Conventional PCR was performed to detect the four exons (exon 6, 7, 8 and 10) which were seldom influenced by pseudogenes. Four sets of primers and PCR amplification profile are listed in Table 2.

DNA sequencing: The conventional PCR products were subjected to 2% agarose gel electrophoresis, while for LR-PCR products, 1% agarose was used with 9Kb as marker. After observation of clear and expected size bands, the products were purified and used as a template for sequencing reactions with BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing primers were M13F or M13R. Automated fluorescence analysis was performed on a 3700 DNA sequence system (ABI, USA).

Bioinformatics analysis

Each result of sequencing was analyzed by DNAStar 5.08 bioanalysis software. The type of mutations and potential

Table 2 Primer sequences and polymerase chain reaction condition of individual exon of *hPMS2* gene

Exon	Primer sequence (5′-3′)	Size (bp)	AT (℃)	CN
1	M13F-ACGTCGAAAGCAGCCAATGGGAGTT M13R-CAGGTAGAAAGGAAATGCATTCAGT	475	66	28
2	M13F-ACAGTGTTGAGTCATTTCCCACAGT M13R-TTCTTAGCATAACACCTGCCTGGCA	455	66	28
3	M13F-TAGTCTGGGCTAGTAAATAGCCAGA	705	68	35
4	M13R-TATGACTTAGATTGGCAGCGAGACA			
5	M13F-CTTGATTATCTCAGAGGGATCGTCA M13R-TCTCACTGTGTTGCCCAGTCCTAAT	540	68	35
6	M13F-TGCTTCCCTTGATTTGTGCGATGAT M13R-TGAGGCAGGAGAATTGCTTGAATCT	504	67	32
7	M13F-ACCCACGAGTTTGACATTGCAGTGA M13R-GTAGAGGTTGCAGTGAGCCAAGATA	498	60	35
8	M13F-AGATTTGGAGCACAGATACCCGTGA M13R-TGCGGTAGACTTCTGTAAATGCACA	414	61	32
9	M13F-CCTTCTAAGAACATGCTGGTTGGTT M13R-ATCTCATTCCAGTCATAGCAGAGCT	279	64	45
10	M13F-AGCCCTTCCGTATTTTGTCTATTCA M13R-GCTTTAGAAGCTGTTTGTACACTGT	719	61	32
11	M13F-TCACATAAGCACGTCCTCTCACCAT M13R-GCAACAGAGCAAGACTCTGTCTCAA	1021	64	45
12	M13F-GCCAAGATTGTGCCATTGCACTGTA M13R-AGTAGATACAAGGTCTTGCTGTGTT	493	64	25
13	M13F-GTGACACTTAGCTGAGTAGTGTTGT M13R-ATGTTAGCCAGGCTGGTCTCAAACT	372	64	35
14	M13F-GGTCTGTATCTCCTGACCTCATGAT M13R-GCACGTAGCTCTCTGTGTAAAATGA	473	64	35
15	M13F-GCTGAGATCTAGAACCTAGGCTTCT M13R-ACACACGAGCGCATGCAAACATAGA	522	64	35

AT: Anneal temperature; CN: Cycle number. The sequence of M13F was 5′-GTAAAACGACGGCCAGT-3′. The sequence of M13R was 5′-AACAGCTATGACCATG-3′.

significance were determined by comparing the corresponding amino acids and proteins in the following databases (http://www.ncbi.nlm.nih.gov/; http://www.ensemble.org/homosapies; and http://www.insight-group.org).

RESULTS

Germline mutation of hPMS2 gene in HNPCC probands

Among the 26 unrelated HNPCC probands, only one (H13) was found to carry the germline mutation of *hPMS2* gene. She was a 30-year-old female BG patient. The mutation was a missense mutation at codon 511 (ACG>ATG, Thr>Met) (Figure 1). To further investigate the pathological effects of the missense mutation, we analyzed the related exon 11 in 130 controls by PCR-based sequencing. The results showed that the mutation of codon 511, consistent with the HNPCC case at c.1532C>T of exon 11 of *hPSM2* gene, was also found in three healthy controls. The occurrence rate was approximately 2.3% (3/130). It could not be found in the PMS2-SNP database (http://www.nfdht.nl; http://www.insight-group.org; and http://www.ensembl.org). Thus, the mutation at c.1532C>T of *hPSM2* gene which we detected in the HNPCC patient is an unreported new single nucleotide polymorphism (SNP).

SNP detection and analysis of hPMS2 gene

By DNA sequencing, 27 loci on the exons of *hPMS2* gene including 260 reported SNP (http://www.ensembl.org/homo_sapies) were detected in the 26 HNPCC probands. Among them, 30% (78/260) were located in the 2nd exon, 23.1% (60/260) in the 5th exon, 13.8% (36/260) in the 15th exon, 10% (26/260) in the 7th exon, and 9.2% (24/260) in the 11th exon. However, none variant was detected in the remaining exons of the 1st, 3rd, 6th, 8th, 9th and 10th. The 2nd and 5th exons were probably the hot SNP regions of *hPMS2* gene because 53.1% of the reported SNP were located in them. Distribution of the SNP of *hPMS2* gene is shown in Table 3.

DISCUSSION

HNPCC, also called Lynch syndrome, is one of the most common autosomal dominantly inherited cancer syndromes with a high risk of colorectal cancer as well as other tumors occurring in endometrium, stomach, ovary, urinary tract, pancreas, small intestine, brain and skin. People with HNPCC take about 80% risk to develop colorectal cancer in their lifetime. It accounts for 2%-15% of all colorectal cancers. Compared to sporadic colorectal cancer, HNPCC possesses its own characteristics in clinical presentations, treatment, genetic features and management of kindred[17,18]. Many countries have established the clinical diagnostic criteria for HNPCC, such as AC, JC and BG. Defects in MMR genes, mainly in *hMLH1, hMSH2* and *hMSH6* were considered to be closely related to the genetic mechanism of HNPCC. The defection would

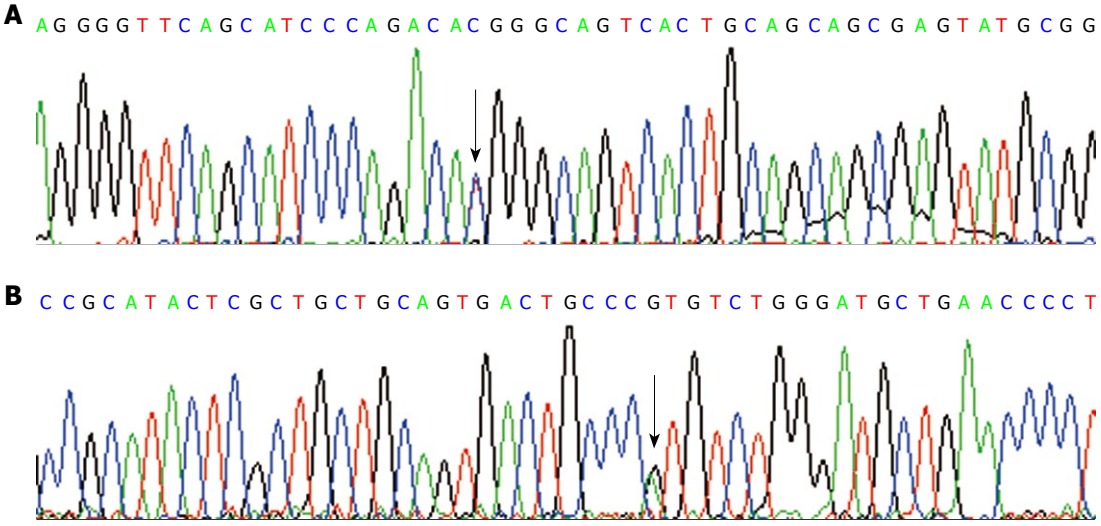

Figure 1 Missense germline mutation of exon 11 of *hPMS2* gene in the proband of H13 hereditary nonpolyposis colorectal cancer kindreds. A: The forward sequence; B: The reverse sequence. Arrow indicates the mutation site, the single basyl substitution was transversed from C to T (C>T) at the codon 511, the codon from ACG to ATG, causing the amiod acid changes from Thr>Met, the change was identified as a new single nucleotide polymorphism.

Table 3 Distribution of single nucleotide polymorphism of *hPMS2* gene in 26 probands

Exon	Nucleotide change	Amino acid change	*n*	SNP (%)
2	c.24-4C>T	-	14	78 (30)
	c.89A>C	Gln30Pro	15	
	c.117A>G	Val39Val	15	
	c.120G>A	Lys40Ly	4	
	c.121G>A	Glu41Lys	15	
	c.124T>A	Leu42Ile	8	
4	c.288C>T	Ala96Ala	8	18 (6.9)
	c.295A>C	Thr99Pro	10	
5	c.406A>G	Met136Val	10	60 (23.1)
	c.418A>G	Asn140Asp	10	
	c.429T>C	Ile143Ile	10	
	c.452G>A	Arg151His	10	
	c.478C>A	Gln160Lys	10	
	c.492C>T	Ser164Ser	10	
11	c.1408C>T	Pro470Ser	7	24 (9.2)
	c.1454C>A	Thr485Lys	11	
	c.2006+6G>A	-	7	
12	c.2007-4G>A	-	11	12 (4.6)
	c.2007-7C>T	-	1	
13	c.2253T>C	Phe751Phe	1	1 (0.4)
14	c.2324A>G	Asn775Ser	3	5 (1.9)
	c.2340C>T	Pro780Pro	2	
15	c.2466T>C	Leu822Leu	12	36 (13.8)
	c.2570G>C	Gly857Ala	2	
	c.92dupA	-	17	
	c.17G>C	-	5	

SNP: Single nucleotide polymorphism.

consequently lead to the dysfunction of MMR system, ultimately resulting in the development of neoplasm. So, detection of MMR gene mutation is the only gold criteria to make a diagnosis of HNPCC.

Within the family of MMR genes, germline mutations in the coding region of *hMSH2* and *hMLH1* could be detected in up to 45%-64% of all HNPCC families, while *hMSH6* about 10%. Previously we analyzed germline mutations and large genomic variations of the entire coding regions of *hMSH2*/*hMLH1*/*hMSH6* genes and the methylation of *hMLH1* promoter in 58 Chinese HNPCC probands, resulting in 29 germline mutations and 2 exhaustive inherited methylations of *hMLH1* promoter (excluding 3 part-methylations of *hMLH1* promoter). The total gene abnormality rate was only 53.4% (31/58). We suspected that the other MMR gene mutations might be associated with the remaining probands without *hMSH2*, *hMLH1* or *hMSH6* gene abnormalities.

The *hPMS2* gene is a member of a set of human mismatch repair genes, located on chromosome 7. It encodes the protein that plays an essential role in repairing DNA by forming an active protein complex with the MLH1 protein which interacts with MSH2 bound to mismatched bases. In 1994, Nicolaides *et al*[19] firstly found the germline mutation of *hPMS2* gene in a HNPCC patient. Since then, more and more data have proved that *hPMS2* germline mutation is involved in the development of HNPCC. In some reports, it could be detected in as high as 62% of HNPCC probands[20]. The *hPMS2* gene was suggested as the first candidate gene for testing germline mutations in HNPCC families in which *hMSH2*, *hMLH1*and *hMSH6* aberrant was excluded. However, genetic testing for germline mutation of *hPMS2* gene was technically challenging because they were severely hampered by a large family of highly homologous pseudogenes located on the same chromosome as the true *hPMS2*, such as *PMS2CL*. They shared similar sequences to *hPMS2* but had no functions. Data from literature indicated that the exon 6 to 8 and exon 10 of *hPMS2* could be easily screened by direct sequencing of genomic DNA without interference of pseudogenes. But detection of exon 1-5, 9 and exon 11-15 was complicated due to the interference of *PMS2*CL. LR-PCR was recommended as a useful method to preferentially identify *hPMS2* but not the pseudogenes. In this study, we used LR-PCR to investigate the germline mutation of *hPMS2* gene in those

HNPCC probands who did not carry *hMLH1/hMSH2/hMSH6* germline mutations investigated by the previous studies. Four overlapping sets of primers were designed to amplify the complete coding region of *hPMS2* gene by LR-PCR firstly. Then, exon-specific amplifications from the LR-PCR products were performed to obtain a clear sequence with no evidence of pseudogene contamination. We only found one missense mutation in 26 probands, which has not been reported in Chinese HNPCC families. This mutation could also be detected in the 130 control persons with an occurrence rate of about 2.3%. Since it could not be found in the PMS2-SNP database (http://www.nfdht.nl; http://www.insight-group.org; and http://www.ensembl.org), the mutation at c.1532C>T of *hPMS2* gene in our HNPCC case was an unreported new single nucleotide polymorphism (SNP). Our results showed that the germline mutation of *hPMS2* gene was probably a rare event in Chinese HNPCC, even in those probands without *hMLH1/hMSH2/hMSH6* mutations. It was consistent with the results of some other studies[21]. Interestingly, another mutation was found in the same nucleotide, c1532_1533 delCGinsAC, causing the amiod changes from Thr to Asn (http://www.insight-group.org). So, the exon 11 may be a hot SNP or mutation region of *hPMS2* gene.

The frequency of germline mutation in *hPMS2* gene was reported to be up to 62% if patients whose tumor tissues lacked protein expression of *hPMS2* or had MSI-H features, were selected[22]. Among the HNPCC families with monoallelic mutation in *hPMS2*, 65.5% were complied with BG. Recently, Niessen *et al*[23] identified 4 patients with pathogenic mutation of *hPMS2* among 97 patients with suspected Lynch syndrome who carried no germline mutation in *hMLH1*, *hMSH2* or *hMSH6*. All these 4 patients fulfilled BG and their corresponding tumor cells showed MSI-H and loss of expression of *hPMS2*. Clendenning *et al*[24] reported that a kind of frame-shift mutation of *hPMS2* occurred in 12 ostensibly unrelated Lynch syndrome patients with 20% being the deleterious mutation. However, those families with pathogenic mutation did not have significantly high incidence of Lynch syndrome associated malignant tumors, indicating that the germline mutation of *hPMS2* and occurrence of HNPCC were not concurrent sometimes. The patient with *hPMS2* gene mutation in our group also met the requirements of BG. By reviewing the family history of our mutation positive patient, we found that in her first-degree relatives, three suffered from colorectal cancer but diagnosed at age over 60 years, not in accordance with the typical feature of HNPCC. Although we are not so certain about this, the non-classical presentation of her family history, to some extent, represents the phenomenon of separation of HNPCC occurrence and *hPMS2* gene mutation.

At the same time, we detected the reported SNP in these 26 probands and found some interesting results. Most of the SNP (21/27) were in the exons and 12 were non-synonymous coding SNP(cSNP). Since these non-synonymous cSNP can induce the change of amino acid and the relationship between cSNP and pathogenesis of HNPCC still remains unclear, whether they are involved in the development of HNPCC and HNPCC related tumors needs to be further investigated.

In conclusion, the germline mutation of *hPMS2* gene is rare in the probands of Chinese HNPCC families. Since the testing of *hPMS2* gene mutation is costly and complicated, it may be not reasonable to be included in the screening strategy of Chinese HNPCC. However, the frequency of SNP of *hPMS2* gene is high and further studies are needed to identify its relationship with HNPCC.

ACKNOWLEDGMENTS

The authors are grateful to the patients who took part in this study and to the Cancer Hospital for providing blood and tumor specimens. The authors also appreciate the help from Professor Sun MH, Wang CF, Cai Q and Yan SY for the detection of germline mutations in *hMSH2*, *hMLH1* and *hMSH6* genes of the probands of the Chinese HNPCC cases, and Professor Mo SJ for supplying the Chinese HNPCC cases.

COMMENTS

Background

Germline mutations in mismatched repair genes can lead to hereditary nonpolyposis colorectal cancer (HNPCC). Previously, the authors had analyzed the abnormalities of *hMSH2*/*hMLH1*/*hMSH6* genes in a serious of Chinese HNPCC families and the total abnormality rate was only 53.4% (31/58). So the aberrant MMR genes such as *hPMS2* were suspected to be involved in Chinese HNPCC.

Research frontiers

HNPCC or Lynch syndrome, is an autosomal dominantly inherited disease with cancer-susceptibility. The testing of *hPMS2* gene mutation is costly and complicated, it may be not reasonable to be included in the screening strategy of Chinese HNPCC. However, the frequency of single nucleotide polymorphism (SNP) of *hPMS2* gene is high and further studies are needed to identify its relationship with HNPCC.

Innovations and breakthroughs

One HNPCC proband was found to carry the germline mutation of *hPMS2* gene. It was a new unreported coding SNP, which could also be detected in the control with an occurrence rate of 2.3% (3/130).

Applications

Germline mutations in genes can be used to diagnose early HNPCC and enrich the databases about HNPCC and SNP.

Terminology

HNPCC is an abbreviation of hereditary nonpolyposis colorectal cancer. Germline mutations are the mutations in genomic DNA.

Peer review

This is an interesting article which deals with a remarkably rare germline mutation, namely PMS2, which is important in the etiology of Lynch syndrome (HNPCC). Their science appears to be sound.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3853

World J Gastroenterol 2010 August 14; 16(30): 3853-3856
ISSN 1007-9327 (print)

CASE REPORT

Poorly differentiated endocrine carcinoma of the pancreas responded to gemcitabine: Case report

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Received: March 17, 2010 Revised: May 26, 2010
Accepted: June 2, 2010
Published online: August 14, 2010

Abstract

Poorly differentiated endocrine carcinoma (PDEC) of the pancreas is a rare and aggressive tumor. First-line treatment is commonly a combination of etoposide and cisplatin, but there is no consensus regarding further treatment recommendations. In this report, we describe a case of pancreatic PDEC treated with gemcitabine as third-line chemotherapy. A 62-year-old man with pancreatic PDEC was administered etoposide plus cisplatin as first-line treatment; he then received irinotecan for tumor relapse. However, because irinotecan induced ileus in this patient, we chose gemcitabine as third-line chemotherapy. After two cycles of gemcitabine (1000 mg/m^2 on days 1, 8 and 15 every 4 wk), a partial tumor response was noted by computed tomography (approximately 68% reduction in tumor size). Our patient survived for 15 mo after diagnosis. This is a rare case of unresectable pancreatic PDEC, which showed a partial response to gemcitabine after the failure of two other regimens. Gemcitabine could be an effective treatment option for pancreatic PDEC that is resistant to other treatments.

Key words: Poorly differentiated endocrine carcinoma; Pancreatic endocrine tumor; Gemcitabine; Chemotherapy

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INTRODUCTION

Pancreatic endocrine tumors (PETs) are rare neoplasms with an annual incidence of less than 1 per 100 000 people[1-6]. These tumors account for less than 1%-2% of all pancreatic neoplasms[1,7]. Poorly differentiated endocrine

carcinoma (PDEC) of the pancreas is characterized by aggressive tumor biology and poor prognosis. The biological behavior of PDEC is similar to that of small-cell lung cancer (SCLC), and metastatic pancreatic PDECs are often treated with the chemotherapy regimens that are used to treat SCLC. The combination of etoposide and cisplatin has been widely used to treat pancreatic PDEC because no promising chemotherapy regimens have been reported for this disease. Effective second- or later-line chemotherapy is still uncertain. Gemcitabine is an active agent against untreated and recurrent SCLC. In this report, we describe a case of pancreatic PDEC treated with gemcitabine as third-line chemotherapy.

CASE REPORT

A 62-year-old man with Crohn's disease had previously received treatment at a different hospital. In July 2007, his serum carcinoembryonic antigen (CEA) level was found to be elevated. A contrast-enhanced computed tomography (CT) scan of the patient's abdomen showed a tumor in the head of the pancreas and enlarged para-aortic lymph nodes. In September 2007, he underwent exploratory laparotomy, during which peritoneal dissemination was observed, and hence, a biopsy of the para-aortic lymph nodes was conducted. Based on the histological findings, small cell carcinoma of the pancreas was diagnosed.

Because the tumor was unresectable at the time of diagnosis, the patient was treated with a combination of etoposide and cisplatin as first-line chemotherapy in October 2007. The chemotherapeutic response was deemed to be partial, until multiple bone metastases to the skull, vertebrae, and pelvis were detected using CT after five cycles of chemotherapy. The patient was next administered irinotecan monotherapy as second-line chemotherapy, which started in March 2008. Irinotecan was stopped after one cycle because ileus occurred. He was referred to our hospital for further treatment in July 2008.

The patient had no family history of cancer, and the results of a physical examination were unremarkable. The laboratory findings were hemoglobin 11.5 g/dL (normal 14.0-17.0 g/dL), γ-glutamyl transpeptidase 113 IU/L (normal, 10-47 IU/L), glucose 136 mg/dL (normal, 69-104 mg/dL), CEA 12.8 ng/mL (normal, < 4.0 ng/mL), carbohydrate antigen 19-9 14 U/mL (normal, < 37 U/mL), neuron-specific enolase (NSE) 36.2 ng/mL (normal, < 10.0 ng/mL), and pro-gastrin-releasing peptide (pro-GRP) 338 pg/mL (normal, < 46 pg/mL). A contrast-enhanced CT scan of his abdomen revealed a low-density mass, 7.5 cm in diameter, in the head of the pancreas, as well as enlarged para-aortic lymph nodes at the time of admission. The pancreatic tumor did not show contrast enhancement (Figure 1A). A CT scan of his chest did not show any primary or metastatic pulmonary tumors. We reviewed an excised biopsy specimen of a para-aortic lymph node obtained at the previous hospital. Histological examination of the specimen showed small to intermediate-sized cells with a high nuclear-cytoplasmic ratio and frequent mitosis, and partial necrosis. Immunohistochemical staining revealed that these cells were strongly positive for NSE, CD56, and keratin; weakly positive for chromogranin A; and negative for vimentin, leukocyte common antigen, S-100, and CD99 (Figure 2). On the basis of the pathological findings, the para-aortic lymphadenopathy was determined to be caused by metastasis of PDEC. Therefore, pancreatic PDEC with para-aortic lymph nodes and bone metastases was diagnosed.

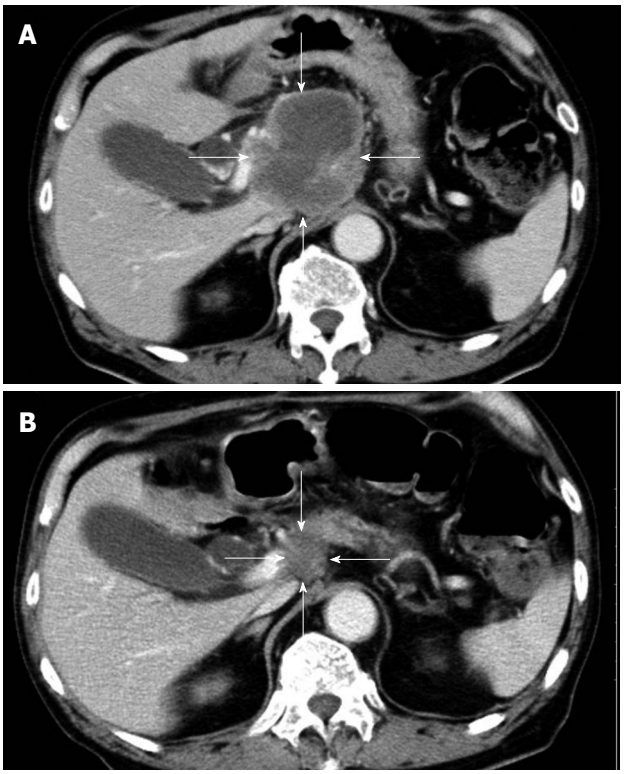

Figure 1 Contrast-enhanced computed tomography scan of the abdomen. A: There was a low-density mass, 7.5 cm in diameter, in the head of the pancreas at the time of admission. The pancreatic tumor (arrows) did not show contrast enhancement; B: A follow-up computed tomography scan showed that the pancreatic mass had reduced to 2.0 cm in diameter. The tumor (arrows) had markedly regressed 4 mo after starting chemotherapy with gemcitabine.

We chose gemcitabine as third-line chemotherapy. Starting in July 2008, the patient received 1000 mg/m^2 gemcitabine on days l, 8 and 15 every 4 wk.

After two cycles of gemcitabine, a CT scan of his abdomen showed regression of the pancreatic tumor (from 7.5 cm to 2.4 cm in diameter), and his serum NSE and pro-GRP levels had decreased to within the normal range. The chemotherapeutic response was deemed to be a partial response. After four cycles of gemcitabine, an abdominal CT scan showed a pancreatic mass that was 2.0 cm in diameter (Figure 1B). In November 2008, after day 15 of the fifth cycle, the patient requested that the therapy be stopped because of general fatigue. He died of multiple organ failure in December 2008.

DISCUSSION

Pancreatic PDEC is a rare neoplasm. Recently, Bettini *et al*[8]

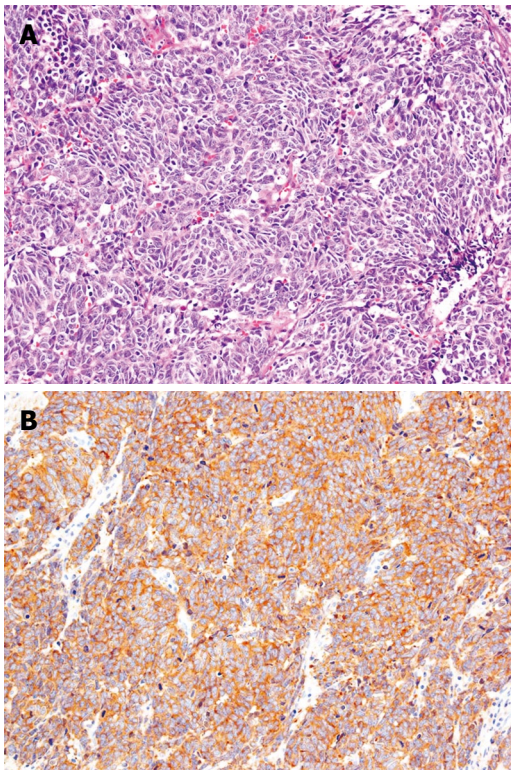

Figure 2 Histopathological findings. A: The excised para-aortic lymph node showed small to intermediate-sized cells with a high nuclear-cytoplasmic ratio. (HE stain, original magnification, × 200); B: Immunostaining for neuron-specific enolase was positive in the cytoplasm of many tumor cells (original magnification, × 200).

have reported that PDEC was diagnosed in 17 (9.4%) of 180 patients with non-functioning pancreatic endocrine tumors. PDEC is characterized by aggressive tumor biology and poor prognosis. Bettini *et al*[8] also have reported that all patients with PDEC died within 3.5 years after diagnosis (median, 11.8 mo), and that only 23.5% of the tumors were resectable at the time of diagnosis. Our patient survived for 15 mo after diagnosis. His survival time was longer than the median survival time that was reported by Bettini *et al*[8].

The standard treatment for advanced pancreatic PDEC has not yet been established. The initial approach to treatment of pancreatic PDEC is to attempt curative resection. However, liver and lymph node metastases are present in 32.5% and 59.5% of patients at the time of diagnosis[9]. Therefore, curative surgical resection cannot be achieved in most patients, and effective medical treatment to control metastatic lesions is urgently required. Systemic chemotherapy is proposed for patients with inoperable pancreatic PDEC, and adequate organ function and performance status; however, a standard chemotherapeutic regimen has not been established. In our patient, the tumor was inoperable owing to the presence of peritoneal dissemination and para-aortic lymph node metastases, and hence, systemic chemotherapy was administered to the patient.

The biological behavior of PDEC is similar to that of SCLC, and metastatic pancreatic PDECs are often treated with the same chemotherapy regimens that are used to treat SCLCs. Combination chemotherapy with etoposide and cisplatin, one of the standard regimens for SCLC, is commonly used to treat pancreatic PDEC.

Moertel *et al*[10] have reported that etoposide plus cisplatin produced good therapeutic results in patients with anaplastic neuroendocrine carcinoma (which has been defined as PDEC according to the recent WHO classification[11]), with an overall regression rate of 67% and a median regression duration of 8 mo [10]. Other investigators have reported similar results, with a median duration of response of 7-9 mo in patients with poorly differentiated endocrine tumors[12,13].

Since the report of Moertel *et al*[10], the combination of etoposide and cisplatin has been considered to be the reference treatment for PDEC; however, confirmatory studies have not been performed because of the rarity of PDEC. If this first-line chemotherapy fails to treat pancreatic PDEC, there is no consensus regarding further treatment recommendations. Irinotecan plus cisplatin is one of the standard regimens for extensive-stage SCLC[14]. In our case, the patient had been administered irinotecan monotherapy as second-line treatment before he was referred to our hospital. However, this therapy had been discontinued because ileus occurred after one cycle.

Several newer anticancer drugs, including paclitaxel[15], topotecan[16] and gemcitabine[17], have shown little activity as single agents against neuroendocrine tumors (NETs). Gemcitabine is a nucleoside analog with structural similarities to cytarabine and is widely used in the treatment of advanced pancreatic adenocarcinoma[18]. In a phase Ⅱ trial of gemcitabine for the treatment of metastatic NETs, Kulke *et al*[17] have reported that, although the treatment was well tolerated, no radiological responses were observed, 65% of the patients (n = 18) experienced disease stabilization, and that the median overall survival was less than 1 year. However, their study included various histological subtypes of NETs, and only two of the 18 patients had poorly differentiated NETs. Thus, the efficacy of gemcitabine for poorly differentiated NET of the pancreas remains unclear.

Gemcitabine is an active agent against untreated and recurrent SCLC[19-21]. The response rate to gemcitabine was reported to be 27% in patients with previously untreated SCLC[19]. In patients with previously refractory or recurrent SCLC treated with at least one chemotherapeutic regimen, gemcitabine resulted in response rates of 6%-17%[20,21].

We believe that gemcitabine is a reasonable treatment option for pancreatic PDEC, and we chose gemcitabine as third-line chemotherapy. After two cycles of gemcitabine, the pancreatic tumor showed marked regression, which resulted in a partial response. Gemcitabine has shown good efficacy as third-line chemotherapy for refractory pancreatic PDEC. The prognosis of pancreatic PDEC is extremely poor because of its highly aggressive behavior, and hence, effective second- and later-line treatments are important for improving prognosis. In light of this, gem-

citabine could be an effective treatment option for pancreatic PDEC that is resistant to other treatment.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i30.3857

World J Gastroenterol 2010 August 14; 16(30): 3857-3858
ISSN 1007-9327 (print)

Reply to "Application of contrast-enhanced intraoperative ultrasonography in the decision-making about hepatocellular carcinoma operation"

Matteo Donadon, Guido Torzilli

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Received: April 29, 2010 Revised: May 28, 2010
Accepted: June 4, 2010
Published online: August 14, 2010

Abstract

The use of contrast-enhanced intraoperative ultrasound for hepatocellular carcinoma has been already proposed as a novel technique to stage the disease during surgical resection. In the herein presented "letter to the editor", the authors underline some important points, which have been raised following the paper by Wu *et al*.

Key words: Hepatocellular carcinoma; Liver surgery; Contrast-enhanced intraoperative ultrasound; Cirrhosis

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Donadon M, Torzilli G. Reply to "Application of contrast-enhanced intraoperative ultrasonography in the decision-making about hepatocellular carcinoma operation". *World J Gastroenterol* 2010; 16(30): 3857-3858 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i30/3857.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i30.3857

TO THE EDITOR

The recent paper by Wu *et al*[1] entitled "Application of contrast-enhanced intraoperative ultrasonography in the decision-making about hepatocellular carcinoma operation" published in the January issue of *World Journal of Gastroenterology* reports some experiences of the authors in contrast-enhanced intraoperative ultrasound (CEIOUS) for hepatocellular carcinoma (HCC). This paper raises a couple of questions that, we believe, need to be asked about.

First and foremost, the authors did not mention any of the previously performed and published studies on the same topic on the application of CEIOUS for HCC[2-7]. Such studies not only represent the first pioneer investigations on CEIOUS, but up to now they are the cornerstones of this new intraoperative imaging modality, which needs to be confirmed or confuted by further studies performed by other groups. In this sense, Wu *et al*[1] have lost this opportunity.

Second, it is unclear to the readers how the authors defined a lesion as malignant based on the CEIOUS findings. This is a pivotal point. Yet, CEIOUS for HCC requires a kind of classification to interpret its findings in order to make the correct diagnosis. In particular in case of cirrhotic liver, where the finding of multiple subcentimetric nodules is common, the typical arterial phase might not be very clear because some of those nodules are high-grade dysplastic nodules or early HCC with no anticipated standard contrast enhancement. Indeed, we proposed a classification that, we believe, could help in this sense, even if it probably requires some refinements[8].

Third, the reported value of specificity for CEIOUS is very high compared with that for intraoperative ultra-

sound (IOUS) and contrast-enhanced magnetic resonance imaging (CEMRI). The impression is that the authors calculated the specificity by adding the value of CEMRI and IOUS. When CEIOUS was performed on the same population of patients who had CEMRI and IOUS, its results in terms of sensitivity and specificity might be biased by the previous radiological findings. Only a true blind performance of different diagnostic methods might allow a true comparison in terms of diagnostic accuracy.

Finally, we thank that Wu *et al*[1], because our group, developed and supported the study of CEIOUS performed many years ago, both for HCC[2,4-6] and for colorectal liver metastases[9]. Thus, any new study on the same topic further sustains its use.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2010 August 14; 16(30): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 August 14; 16(30): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

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International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
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Prague Hepatology Meeting 2010

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October 07-09
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The 7th Biannual International Symposium of Society of Coloproctology

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World J Gastroenterol 2010 August 14; 16(30): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
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Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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Format

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English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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ISSN 1007-9327
CN 14-1219/R

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Indexed and Abstracted in:
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Volume 16 Number 31
August 21, 2010
World J Gastroenterol
2010 August 21; 16(31): 3859-3986

Online Submissions
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 31 August 21, 2010

EDITORIAL

3859 Natural orifice transluminal endoscopic surgery in pancreatic diseases
Gillen S, Kleeff J, Kranzfelder M, Shrikhande SV, Friess H, Feussner H

3865 First-line eradication of *Helicobacter pylori*: Are the standard triple therapies obsolete? A different perspective
Buzás GM

3871 Therapeutic implications of colon cancer stem cells
Fabrizi E, di Martino S, Pelacchi F, Ricci-Vitiani L

REVIEW

3878 Hepatitis B virus infection and renal transplantation
Tsai MC, Chen YT, Chien YS, Chen TC, Hu TH

ORIGINAL ARTICLE

3888 Glucocorticoid receptor gene haplotype structure and steroid therapy outcome in IBD patients
Mwinyi J, Wenger C, Eloranta JJ, Kullak-Ublick GA

3897 Expression of protein S100A4 is a predictor of recurrence in colorectal cancer
Kwak JM, Lee HJ, Kim SH, Kim HK, Mok YJ, Park YT, Choi JS, Moon HY

3905 Prospective randomized controlled trial evaluating cap-assisted colonoscopy *vs* standard colonoscopy
Tee HP, Corte C, Al-Ghamdi H, Prakoso E, Darke J, Chettiar R, Rahman W, Davison S, Griffin SP, Selby WS, Kaffes AJ

3911 Anti-proliferative and pro-apoptotic effects of tectorigenin on hepatic stellate cells
Wu JH, Wang YR, Huang WY, Tan RX

BRIEF ARTICLE

3919 Mapping of liver-enriched transcription factors in the human intestine
Lehner F, Kulik U, Klempnauer J, Borlak J

3928 Liver sinusoidal endothelial and biliary cell repopulation following irradiation and partial hepatectomy
Krause P, Rave-Fränk M, Andreas Wolff H, Becker H, Christiansen H, Koenig S

3936 *Helicobacter pylori* CagA protein polymorphisms and their lack of association with pathogenesis
Acosta N, Quiroga A, Delgado P, Bravo MM, Jaramillo C

3944 Steatosis and steatohepatitis in postmortem material from Northwestern Greece
Zois CD, Baltayiannis GH, Bekiari A, Goussia A, Karayiannis P, Doukas M, Demopoulos D, Mitsellou A, Vougiouklakis T, Mitsi V, Tsianos EV

3950 Mechanism underlying carbon tetrachloride-inhibited protein synthesis in liver
Li XW, Zhu R, Li B, Zhou M, Sheng QJ, Yang YP, Han NY, Li ZQ

3957 Usefulness of CT angiography in diagnosing acute gastrointestinal bleeding: A meta-analysis
Wu LM, Xu JR, Yin Y, Qu XH

3964 Multiple primary malignant tumors of upper gastrointestinal tract: A novel role of ^{18}F-FDG PET/CT
Sun L, Wan Y, Lin Q, Sun YH, Zhao L, Luo ZM, Wu H

3970 Use of pre-, pro- and synbiotics in patients with acute pancreatitis: A meta-analysis
Zhang MM, Cheng JQ, Lu YR, Yi ZH, Yang P, Wu XT

3979 Living donor liver transplantation using dual grafts: Ultrasonographic evaluation
Lu Q, Wu H, Yan LN, Chen ZY, Fan YT, Luo Y

CASE REPORT

3984 Multiple bowel intussusceptions from metastatic localized malignant pleural mesothelioma: A case report
Liu H, Cheng YJ, Chen HP, Hwang JC, Chang PC

World Journal of Gastroenterology
Volume 16 Number 31 August 21, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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http://www.wjgnet.com

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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
August 21, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
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Room 903, Building D, Ocean International Center,
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Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3859

World J Gastroenterol 2010 August 21; 16(31): 3859-3864
ISSN 1007-9327 (print)

EDITORIAL

Natural orifice transluminal endoscopic surgery in pancreatic diseases

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Received: May 8, 2010 Revised: May 31, 2010
Accepted: June 7, 2010
Published online: August 21, 2010

Abstract

Natural orifice transluminal endoscopic surgery (NOTES) is a surgical technique that has received considerable interest in recent years. Although minimal access surgery has increasingly replaced traditional open abdominal surgical approaches for a wide spectrum of indications, in pancreatic diseases its widespread use is limited to few indications because of the challenging and demanding nature of major pancreatic operations. Nonetheless, there have been attempts in animal models as well as in the clinical setting to perform diagnostic and resectional NOTES for pancreatic diseases. Here, we review and comment upon the available data regarding currently analyzed and performed pancreatic NOTES procedures. Potential indications for NOTES include peritoneoscopy, cyst drainage, and necrosectomy, palliative procedures such as gastroenterostomy, as well as resections such as distal pancreatectomy or enucleation. These procedures have already been shown to be technically feasible in several studies in animal models and a few clinical trials. In conclusion, NOTES is a rapidly developing concept/technique that could potentially become an integral part of the armamentarium dealing with surgical approaches to pancreatic diseases.

Key words: Natural orifice transluminal endoscopic surgery; Pancreatic disease; Chronic pancreatitis; Pancreatic resection; Pancreatic drainage

Peer reviewer: Dr. Kemal Kismet, MD, 4th General Surgery Department, Ankara Training and Research Hospital, Ankara, 06430, Turkey

Gillen S, Kleeff J, Kranzfelder M, Shrikhande SV, Friess H, Feussner H. Natural orifice transluminal endoscopic surgery in pancreatic diseases. *World J Gastroenterol* 2010; 16(31): 3859-3864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3859.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3859

INTRODUCTION

Flexible endoscopy has traditionally been limited to the intestinal lumen. However, in recent years various attempts to also provide endoscopic access to the peritoneal cavity for diagnostic and therapeutic procedures have been made. Two novel developments in gastrointestinal endoscopy and surgery have facilitated these attempts: (1) the establishment of endoscopic retrograde cholangiopancreatography in the 1970s and endoscopic ultrasound in the 1980s offered gastrointestinal endoscopists not only purely diagnostic but also therapeutic options; and (2) simultaneously, minimal access surgery increasingly replaced traditional open abdominal surgical approaches for a wide spectrum of indications. These developments led to a new and innovative, interdisciplinary way of accessing the

peritoneal cavity through the natural orifices of the body by means of transluminal endoscopic approaches to the abdominal cavity: natural orifice transluminal endoscopic surgery (NOTES). These new techniques avoid the need for abdominal incisions and may offer potential benefits, such as being less invasive and possibly more cost-effective than the traditional open or laparoscopic surgery for certain indications. In addition, NOTES may offer specific advantages for selected patient populations. For example, this technique seems especially relevant to those patients with high surgical risk, e.g. the morbidly obese patient or patients with multiple prior abdominal interventions or surgical wound infections. Since the method was first described by Kalloo *et al*[1] in 2004, surgeons and gastroenterologists have worked on transluminal access and intraabdominal surgical procedures[2].

In America and Europe, collaborative organizations of surgeons and gastroenterologists, the Natural Orifice Surgery Consortium for Assessment and Research™ (NOSCAR™)[3] and the EURO-NOTES Foundation (www.euro-notes.org), have been established to encourage and document the further development of NOTES. However, before establishing this new method and bringing it into general clinical practice, it must be confirmed to be safe and to provide real advantages for patients, thus avoiding the mistakes that were made when laparoscopic surgery was introduced a few decades ago. Therefore, the American Society for Gastrointestinal Endoscopy (ASGE) and the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) Working Group met in 2006 to define in a white paper the hurdles and challenges (e.g. safe methods for closure of the gastric incision, avoidance of infections, *etc*.) to be addressed in the coming years[4].

The first experimental laparoscopy was reported in 1901 by the German surgeon Georg Kelling[5], who insufflated gas into the abdomen of dogs, but it was only 84 years later in 1985 that Erich Mühe performed the first laparoscopic cholecystectomy. Since this was rejected by the German Surgical Society, it took another two years until the French gynecologist, Philippe Mouret, reported a laparoscopic cholecystectomy with only four trocars, and that event finally triggered the interest in modern minimal access invasive surgery[6]. However, in the years that followed, many barriers to laparoscopic surgery had to be overcome. Critical elements of a new surgical technique include the development of appropriate instrumentation, requiring collaboration of medical professionals, engineers and the industry. Learning from the introduction of laparoscopic surgery, NOTES should only be implemented if all important aspects including feasibility and safety have been sufficiently evaluated, and indications have been clearly defined[7].

Training performed in a clean and safe environment, with performance analysis generating learning curves, improves patients' safety and outcomes and maximizes the benefits of implementation of new procedures such as NOTES[8]. Besides animal laboratory training, newly developed training phantoms for NOTES have been described[9].

Despite these important issues, NOTES techniques have developed rapidly over the past few years, mainly due to a close collaboration between surgeons and gastroenterologists and extensive experimental animal research. Multiple trials regarding the different access sites to the peritoneal cavity and endoscopic interventions in the abdomen have been described. The basic experience with this new procedure has been largely with animal studies; human case reports are rare, but the number is increasing steadily.

Using NOTES, surgeries like cholecystectomy[10,11], gastrojejunostomy[10,12], antireflux surgery[13], appendectomy[14], and splenectomy[15], as well as several gynecologic procedures including tubal ligation[16], oophorectomy[17] and partial hysterectomy[17], have been performed successfully in animal models *via* different approaches such as transgastric/transcolonic/transvaginal using current commercial endoscopes. Retroperitoneal interventions such as nephrectomy have also recently being described[18].

PURE OR HYBRID NOTES

According to the NOSCAR committee, pure NOTES is defined as flexible endoscopic procedures performed by crossing the respective lumen[5,19]. Natural orifices to the abdominal cavity that are actually used are the transgastric route (*via* the mouth), the transvaginal route, the transsigmoidal access *via* the anus, and the transurethral path. The use of single port surgery for percutaneous access is viewed controversially, and considered only if flexible endoscopes are used. The use of rigid instruments and even transanal endoscopic micro-surgery (TEM) are not considered as pure NOTES procedures.

Some difficulties result from these access sites as follows; firstly: penetrating the transluminal barrier with the endoscope, secondly: avoidance of contamination of the abdominal cavity, and thirdly: the closure of the entrance point. Once having passed the transluminal barrier further challenges arise. Intraoperative manipulations are possible but often limited by the unidirectional force exertion, the lack of haptic and tactile sensations and the limited triangulation with just one instrument. Medical scientists, engineers and industrial companies are working on various solutions, such as double channel endoscopes and bending instruments. Finally, the closure of the transluminal entrance has to be assured. This seems to be easier with the transvaginal and transurethral routes (which are also less prone to contamination) than with transgastric or transsigmoidal access. Nevertheless, all routes have their specific difficulties. Potential advantages of this new technique are the lack of incisional problems, e.g. pain, hernia, wound infections, as well as less adhesions and better cosmetic results.

Hybrid NOTES procedures include endoscopic surgery with the aid of laparoscopic vision or instruments for operation or access closure. The hybrid technique is actually the most commonly used form. Pure NOTES interventions are rare, and thus hybrid NOTES may serve as a temporary approach to further develop pure NOTES techniques. Parallel to the NOTES working group, the

New European Surgical Academy (NESA) founded the interdisciplinary working group for Natural Orifice Surgery (NOS) to develop surgical procedures using the natural body openings, e.g. by using a new surgical instrument, the Transdouglas Endoscopic Device (TED), a flexible multichannel instrument enabling single-entry "scarless" operations[20]. Whether pure NOTES, hybrid procedures or NOS, all these techniques are expected to move forward towards a less invasive surgical discipline.

The role of NOTES in pancreatic diseases has been analyzed in a relatively small number of experimental and clinical studies[21]. NOTES procedures might play a potential role in the diagnosis and therapy of pancreatic diseases, specifically in those areas where endoscopic and/or laparoscopic approaches have already been established, and are - at least in some centers - part of the clinical routine.

CURRENT SURGICAL PROCEDURES FOR PANCREATIC DISEASES

Open pancreatic surgery

There are various surgical procedures available for different pancreatic diseases. Resections include pancreatic head resections (classical, pylorus-preserving and duodenum-preserving partial pancreatoduodenectomies), segmental resections, distal resections, total pancreatectomies, enucleations and others. In addition, palliative procedures such as biliodigestive anastomosis and gastric bypass procedures are frequently carried out, as well as special procedures such as necrosectomy or pancreatic pseudocyst drainage (cysto-gastrostomy or cysto-jejunostomy). Open pancreatic surgery is still the gold standard but is now being challenged by endoscopic or laparoscopic approaches for a number of indications as discussed below.

Diagnostic approach for pancreatic tumors

Diagnostic laparoscopy has a limited role in potentially resectable tumors to evaluate local resectability, and to exclude distant metastases. In addition, in patients with locally non-resectable tumors who are scheduled for neoadjuvant therapy, laparoscopy is generally recommended to confirm diagnosis and to rule out occult metastasis[22,23].

Endoscopic treatment of pancreatic diseases

Endoscopic retrograde cholangiopancreatography (ERCP) offers a number of options in the diagnosis and management of pancreatic and biliary duct obstruction. However, ERCP as a diagnostic measure has been replaced to a large extent by modern imaging, e.g. MRI/MRCP. In addition, biliary or pancreatic duct drainage has a limited role in pancreatic and biliary diseases, being mostly restricted to the palliative setting. The development of endoscopic ultrasonography (EUS) offers further diagnostic accuracy for some pancreatic diseases, e.g. small tumors, neuroendocrine or cystic lesions/tumors. Nonetheless, there have been several novel therapeutic applications requiring an endoscopic approach. To cite an example, endoscopic ultrasound-guided celiac plexus block or pancreatogastrostomy and pancreatobulbostomy with stent insertion into the pancreatic duct for pain relief in patients with chronic pancreatitis[24,25]. Even procedures targeting pancreatic tumors with radiofrequency ablation[26], photodynamic therapy[27], and brachytherapy[28] using an endoscopic approach have been recently described in pilot studies. However, while there is a clear trend towards development of novel endoscopic procedures in the therapy of pancreatic diseases, evidence-based data are mostly lacking, and if present, point towards a more surgical approach, at least for some indications[29].

Laparoscopic pancreatic surgery

Drainage and necrosectomy: Internal drainage of pancreatic pseudocysts can be accomplished by traditional open or minimal access laparoscopic or endoscopic approaches. Minimal access surgery to drain pseudocysts can be performed with comparable morbidity and has become the standard of care in many cases; endoscopic approaches have similar success rates[30,31]. Open surgical necrosectomy for the treatment of infected pancreatic necrosis has relatively high morbidity and mortality rates; therefore minimal access laparoscopic as well as endoscopic or radiologic approaches are more commonly being used nowadays[32].

Bypass operations: Open (versus laparoscopic) gastrojejunostomy has been the standard palliative treatment in patients with unresectable pancreatic cancer with gastric outlet obstruction. It has a good functional outcome and relieves symptoms in many patients (if the patients were not treated by prior endoscopic stent therapy). Laparoscopic gastrojejunostomy has nowadays been proven as an effective palliation with rapid recovery in these advanced cases. Even transumbilical single-incision laparoscopic anastomoses have been reported as feasible and safe[33].

In cases of biliary obstruction (and in the case when endoscopic stent placement is not the treatment of choice), open biliodigestive anastomosis (hepaticojejunostomy) *vs* double bypass surgery (biliodigestive anastomosis and gastric bypass) has been a topic of discussion. However, laparoscopic hepaticojejunostomy is a relatively complex surgical procedure and only few reports are available for adult patients[34,35].

Laparoscopic pancreatic resections

Distal pancreatectomy: Laparoscopic distal pancreatectomies with or without preservation of the spleen have been performed and described since 1996[36]. The available data confirm that laparoscopic distal pancreatectomies are safe operations with similar or shorter operative times, blood loss, complication rates, and length of hospital stay for benign or noninvasive lesions of the pancreas in experienced hands[37,38]. As long as the resection margins are negative and the lymph node clearance is within accepted standards, this can also be performed for malignant lesions. Even though laparoscopic distal pancreatectomies are safe and feasible, most centers still carry out this resection as an open procedure[36].

Enucleation of pancreatic lesions: Laparoscopic enucleation of smaller lesions, especially with regard to neuroendocrine tumors, has also been described to be a feasible and safe approach[39]. It is associated with reduced postoperative hospital stay and comparable rates of pancreatic fistula in comparison to open surgery, although controlled trials and larger series are lacking to support these early observations.

Pancreaticoduodenectomy: Despite their early description by Gagner *et al*[40] in 1994, partial pancreaticoduodenectomies are considered extremely technically demanding for the laparoscopic approach. Recently published analyses describe laparoscopic partial pancreaticoduodenectomy as feasible, safe, and effective. Performed by highly skilled surgeons, even malignant lesions can be resected with negative margins and adequate lymph node dissection[41]. On this background, it remains to be seen whether laparoscopic pancreaticoduodenectomy can become the new surgical standard in the years to come[42].

NATURAL ORIFICE TRANSLUMINAL ENDOSCOPIC SURGICAL INTERVENTIONS IN PANCREATIC DISEASES

Diagnostic

Transgastric diagnostic endoscopic peritoneoscopy has been proven to be safe and feasible[43]. The first human clinical trial was performed on a group of ten patients with pancreatic masses. In four of these cases, peritoneal or liver biopsies were taken. Clinically significant contamination of the peritoneal cavity from the transgastric route was not observed[44]. In a recent study, 20 patients underwent laparoscopy and afterwards transgastric endoscopic peritoneoscopy, with comparable results for both procedures in 19 of 20 patients[45]. Safe and reliable gastric closure is now perhaps the only limitation to routine clinical implementation of this approach.

Therapeutic-non resection

Drainage and necrosectomy: In recent decades many interventional attempts to improve symptoms of chronic pancreatitis have been performed, such as decompression of the pancreas by stenting or stone extraction, as well as evacuation and drainage of pseudocysts. Endoscopic cystogastrostomy and cystoduodenostomy are important steps towards pure NOTES interventions[46]. In the reported case of a seven-year-old child, a hybrid NOTES cystogastrostomy was performed successfully through an existing gastrocutaneous fistula[47].

Therapy of necrotizing pancreatitis has changed in recent decades. Open approaches have increasingly been replaced by minimal access necrosectomies[48]. Minimal access approaches are often performed *via* an endoscopic transgastric access and therefore these procedures build the bridge to NOTES[49]. Indeed, transgastric/transduodenal necrosectomy has been carried out successfully in a number of studies with good long-term maintenance of the initial success and this approach has arguably been termed a currently practiced NOTES procedure[50].

Bypass operations: There have been no reports regarding pure NOTES operations for gastric and/or biliary bypasses. Hybrid NOTES for Roux-en-Y gastric bypass has been shown to be technically feasible in human cadavers[51].

EUS-guided therapeutic strategies in the therapy of pancreatic lesions: The EUS-guided injection of different substances seems to be a potential therapeutic option for cystic and also malignant pancreatic lesions. For example, the injection of ethanol into the pancreas in a swine model has been described and resulted in a localized concentration-dependent tissue necrosis without complications, which might arguably be used in the therapy of cystic lesions of the pancreas[52]. EUS-guided photodynamic therapy (PDT) with photosensitizing agents, as well as radio frequency ablation, has been shown to be safe and effective in ablation of pancreatic tissue, achieving local pancreatic tissue destruction[26,53]. EUS-guided injection of paclitaxel provided high and sustained localized concentrations in the porcine pancreas, leading to the assumption that this technique might be a potential minimal access therapeutic option for unresectable pancreatic tumors[54].

Therapeutic-resection

Distal pancreatic resections: Ryou *et al*[55] demonstrated in 2007 the technical feasibility of hybrid NOTES distal pancreatectomy in five pigs, and Matthes *et al*[56] demonstrated the feasibility of a pure NOTES distal pancreatectomy also in 2007. Allemann *et al*[57] reported on the initial experience in five pigs using a transvaginal retroperitoneal NOTES approach for distal pancreatectomy without any intraoperative complications. In a first randomized controlled trial of NOTES *vs* laparoscopic distal pancreatectomy in a porcine model, Willingham *et al*[58] demonstrated that there were no clinical or survival differences between NOTES and laparoscopy, although the laparoscopic operations were significantly faster (Table 1).

Enucleation of pancreatic lesions and partial pancreaticoduodenectomy: Only one actual study has been carried out concerning the feasibility of pancreatic tumor enucleation *via* a transgastric route in a porcine model[59]. No animal or human NOTES partial pancreaticoduodenectomies have currently been reported in the literature.

CONCLUSION

Transgastric/transduodenal drainage of pancreatic pseudocysts as well as necrosectomies are performed regularly in humans and have been shown to be safe and feasible, with a potential clinical benefit. Transgastric diagnostic peritoneoscopy for the staging of pancreatic cancer is also safe and feasible, and has been experimentally performed in humans. Pancreatic left resections, tumor enucleations and EUS-guided application of radiofrequency ablation,

Table 1 Pancreatic resections *via* natural orifice transluminal endoscopic surgery

	Yr	Access	Operation	Model	*n*	Type of study	Pure NOTES
Matthes *et al*[56]	2007	Transgastric	Distal pancreatectomy	Swine	6	Feasibility, nonsurvival	+
Ryou *et al*[55]	2007	Transcolonic/transvaginal	Distal pancreatectomy	Swine	5	Nonsurvival (3), survival (2)	-
Willingham *et al*[58]	2009	Transgastric	Distal pancreatectomy	Swine	28	Survival	-
Allemann *et al*[57]	2009	Transvaginal	Distal pancreatectomy	Swine	5	Nonsurvival	+

NOTES: Natural orifice transluminal endoscopic surgery.

photodynamic therapy or application of chemotherapeutics seems to be feasible in porcine models. The oncological outcome of these interventions remains unclear. Biliary and/or gastric bypass operations, as well as partial pancreaticoduodenectomies, have rarely or never been performed using NOTES either in animal models or in humans.

In summary, NOTES may play an increasing role in the drainage of pancreatic pseudocysts and in necrosectomy, in the staging of pancreatic masses and also in the palliative treatment of unresectable pancreatic tumors. Other minimal access pancreatic procedures may be a long-term aim in ongoing development. It is obvious that technical issues, including instrumentation, visualization, intra-abdominal manipulation and gastric closure need further refinement.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3865

World J Gastroenterol 2010 August 21; 16(31): 3865-3870
ISSN 1007-9327 (print)

EDITORIAL

First-line eradication of *Helicobacter pylori*: Are the standard triple therapies obsolete? A different perspective

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Received: February 23, 2010 Revised: May 29, 2010
Accepted: June 5, 2010
Published online: August 21, 2010

Abstract

Studies concerning the eradication of *Helicobacter pylori* have resulted in a proliferation of meta-analyses. To date, there are 303 meta-analyses cited in PubMed, 113 dealing with the therapy of the infection. A chronological analysis of the results of meta-analyses performed between 1998 and 2010 shows that first-line standard triple therapies achieved eradication rates on an intention-to-treat basis of around 80%; prolonging treatment to 14, but not 10 d should improve the results. The proton pump inhibitors have a similar efficiency, and giving a double dose is more efficient than the standard doses of these drugs. Triple and quadruple therapies proved to be equivalent. Based on meta-analytical data, the decrease in efficiency over time cannot be substantiated: eradication rates < 80% followed from the introduction of triple therapies. As alternatives, ranitidine bismuth citrate-, levofloxacin- or furazolidone-based therapies were shown to obtain the same eradication rates as standard triple regimens. Sequential therapies and quadruple non-bismuth-based therapies were superior to standard triple therapies but their use is limited to certain countries. In the author's opinion, and from a meta-analytical viewpoint, standard triple therapies cannot yet be considered obsolete. Furthermore, non-inferiority trials are proposed for the future, including assessment of local contemporary antimicrobial resistance profiles and the CagA and CYP2C19 status of the enrolled patients.

Key words: Antibiotics; Eradication; *Helicobacter pylori*; Meta-analysis

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Buzás GM. First-line eradication of *Helicobacter pylori*: Are the standard triple therapies obsolete? A different perspective. *World J Gastroenterol* 2010; 16(31): 3865-3870 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3865.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3865

BACKGROUND: META-ANALYSES IN THE EVALUATION OF *HELICOBACTER PYLORI* ERADICATION

The discovery of *Helicobacter pylori* (*H. pylori*) led to a tremendous scientific output with 28 441 papers published between 1983 and May 2010 (http://www.pubmed.com, accessed on 27 May, 2010). Randomised controlled trials (RCT) have emerged as the main method for assessing the efficiency of *H. pylori* eradication. The guidelines are based on the most recent results of RCTs[1-6]. It became clear that RCTs could not cover all aspects of anti-*H. pylori* treatments and thus, studies on eradicating the bacterium prompted many meta-analyses, developing

into a fashionable genre in scientific literature. In contrast with human studies, meta-analyses do not require ethical/institutional approval, they are much cheaper, while they only need a detailed literature survey - which is much easier in the age of the internet than before - and a professional statistical background. Meta-analysis itself is an evolving field of medical statistics: starting from simple summary statistics it applies increasingly sophisticated procedures. Its methodology is described in textbooks[7] and is available in software packages. Gastroenterology journals were keen to publish meta-analyses, which created an abundance of analyses, superfluous information, and even errors and confusion.

In spite of the high worldwide prevalence of *H. pylori*-related conditions, the eradication of the infection has not been the subject of megatrials with tens of thousands of cases, as has been the case with hypertension, hyperlipidemias or diabetes[8-10]. To date, there are 303 meta-analyses which have been published worldwide on the topic of *H. pylori*, 113 dealing with its eradication: these can be classified by subject as shown in Table 1. In this editorial, the current position of the standard triple therapies will be discussed from a meta-analytical perspective[11-34].

Table 1 Topics of meta-analyses of *Helicobacter pylori* eradication between 1992-2010

Topics of meta-analysis	No. of studies
First-line triple therapies	25
PPI-based therapies	22
Ranitidine bismuth citrate-based therapies	3
Bismuth-based quadruple therapies	2
Non-bismuth-based quadruple therapies	1
Histamine H_2 receptor blockers + two different antibiotics	5
Rescue (second- and third-line) therapies	3
Sequential therapies	4
Alternative therapies	4
Eradication in functional dyspepsia	20
Eradication in peptic ulcer	10
Effect of CagA status on eradication	1
Gastric cancer prevention	5
Eradication in children	2
Eradication in prevention of NSAID-ulcers	1
Eradication in extradigestive diseases	5
Effect of probiotics on eradication results	5
Eradication and antimicrobial resistance	4
Effect of CYP polymorphism on eradication	2
Adverse effects of eradication	4
Multiple topics	11
Total	113

PPI: Proton pump inhibitors; NSAID: Nonsteroidal anti-inflammatory drugs; CYP: Cytochrome P450 isoenzyme.

ARE FIRST-LINE STANDARD TRIPLE THERAPIES OBSOLETE?

According to the Maastricht I consensus[1], in naive *H. pylori*-infected patients, a 7-d triple therapy consisting of any of the available proton pump inhibitors (PPI) b.i.d. + amoxicillin 1000 mg b.i.d. and clarithromycin 500 b.i.d. or metronidazole 400 or 500 mg q.i.d. is recommended. In the United States, the same regimens are given for 10-14 d[2]. The recommendations date from 1996 and 1998, and remained unchanged in subsequent guidelines[3,4]. The Second Asia-Pacific[5] and the revised Japanese consensus[6] both recommended 7-d regimens including PPI + amoxicillin 1000 mg + clarithromycin 500 mg or metronidazole 400 mg b.i.d., or PPI + clarithromycin 500 mg + metronidazole 400 mg b.i.d.

Meta-analyses of the first-line standard triple therapies demonstrated eradication rates of around 80% on an intention-to-treat (ITT) and over 85% on a per protocol (PP) basis[11-17], with the different PPIs achieving similar results[18,21-23,27]. The duration of treatment did[16] or did not[29] influence the rates of eradication. High-dose PPIs were more efficient than standard doses[18,30] (Table 2).

Before the consensus era, successful eradication was considered to be the curing of the infection in more than 80% of cases on an ITT basis. This level was proposed by Graham et al in 1989 and later even higher rates (85%-90%) were considered realistic[35,36]. This rate was later accepted by the guidelines and consensus conferences. Some experts observed that the efficiency of standard triple therapies over the range of 7-10-14 d has decreased in recent years (i.e. < 80%)[34-37] and proposed that they should possibly be abandoned as being no longer effective. In a recent overview of the topic, the results of large trials document this decrease[35]. These studies, however, seem to be grouped rather arbitrarily, not as systematic reviews and no meta-analytic workup was performed. In fact, as shown in Table 2, many meta-analyses are based on studies performed between 1993 and 2000 and show that the eradication rates of first-line standard therapies are around 80%. Looking at the 95% CI, it is obvious that in a variable proportion of the studies, the rate is well under 80%, thus the decrease in efficiency of PPI-based triple therapies is not a new phenomenon: it existed from the introduction of these regimens[11-18]. An analysis of European congress abstracts published between 1997 and 2004 revealed no decrease in the efficiency over time of first-line therapies[28]. Most of the data came from a Spanish centre, using standardized data extraction, with study quality assessment and upgraded statistical methodology, resulting in high-quality meta-analyses[14-16,18-24].

REASONS FOR ERADICATION FAILURES

The main, but not the only, culprit for the lower eradication rates is antimicrobial resistance. Meta-analyses showed that macrolide resistance reduced the success rate of standard triple therapies by 20%-55%, and nitroimidazole resistance by 25%-50%[38-41]. Antimicrobial resistance, however, is always a local, and yet a uniform county/country/continental or even global phenomenon; therefore, determining of the local resistance rates must occur at the same time as the eradication trials - however, this rarely happens. Geographically, regions with low rates of eradication are not always the same as those with high antimicrobial resistance rates. Unfortunately, antimicrobial resistance

Table 2 First-line standard triple therapies for *Helicobacter pylori* eradication: chronological order and results of meta-analyses

Author	Databases, abstracts[1]	Study period	No. of studies	No. of patients	Eradication rate [ITT, (%) mean + 95% CI]	Duration of treatment (d)	Comments
Bazzoli *et al*[11]	Medline	1993-1996	14	507	LAC: 80.6 OAC: 69.6	7	L and O are equally efficient
Laheij *et al*[12]	Medline, abstracts	1983-1998	644	53228	PPI + A + C: 80.09 (NS)	7-14	
Huang *et al*[13]	Medline, PubMed, abstracts	1986-1998	82	6123	PPI + A + C: 89.5.6 (86.9-92.0) PPI + A + M: 90.8% (87.0-94.5)	7	C 500 mg b.i.d. achieved the best result
Gisbert *et al*[14]	PubMed	1986-1999	22	2862	PPI + A + C: 81 (76-85) PPI + A + N: 84 (79-89)	7	PAC and PAN have similar efficiency
Gisbert *et al*[15]	PubMed + abstracts	1995-1999	12	1170	RBC + A + C: 76.6 (72-81) RBC + CN: 87.2 (83-91) PPIAC: 73.7 (69-78) PPIAN: 74.9 (71-84)	7	RBC + AC and PPI + AC have similar efficiency, RBC + CN has higher efficiency than PPI + CN
Calvet *et al*[16]	Medline + abstracts	1990-1999	21	1349	PPI + 2AB 76 (68-86) 82 (77-86) 84 (79-8)	 7 10 14	Triple therapies of 14 d are superior to 7, but not 10 d regimens
Janssen *et al*[17]	Medline + abstracts	1994-2000	47	3541	RBC + A + C: 81 (71-96) RBC + N + C: 88 (78-94) PPI + A + C: 79 (24-95) PPI + N + C: 79 (42-100)	5-10	PPI + AC and NC are equally effective, RBCNC is superior to RBCAC
Vallve *et al*[18]	Medline + abstract	1996-2000	13	2391	Single dose PPI: 77.7 (72-77) Double dose PPI: 83.9 (81-85)	7	Single dose PPI triple regimens are less efficient
Vergara *et al*[19]	Medline + abstracts	1995-2002	134	3293	O: 74.7 (NS) L: 74.7 (NS) R: 77.9 (NS) E: 87.9 (NS)	7	PPIs are similar in standard triple therapy
Gené *et al*[20]	PubMed, abstracts	1995-2002	5	1118	Triple therapy: 79 (74-81) Quadruple therapy: 80 (77-84)	7-10	The effectiveness of triple and quadruple therapies is similar
Gisbert *et al*[21]	Medline, Embase, CINAHL, CCTR	1996-2002	7	2226	RAC: 79 (76-82) OAC: 77 (74-80) LAC: 77 (75-79)	7-14	R, O and L achieved similar results
Gisbert *et al*[22]	Medline, congress abstracts	1997-2003	12	1137	P + 2AB: 83 (78-88) O, L + 2AB: 81 (77-86)	7	P, O and L achieved similar results
Gisbert *et al*[23]	Medline, congress abstracts	1999-2003	4	816	E + 2AB: 85 (81-89) O + 2AB: 82 (78-86)	7	E + 2AB has comparable efficacy with O + 2AB
Gisbert *et al*[24]	Medline, Embase, CINAHLISIWS + congress abstracts	1997-2004	14	4435	RBC + C + A: 79.5 (72.2-83.7) PPI + C + A: 78.1 (73.6-84.1) RBC + C + N: 87.4 (82.8-93.6) PPI + C + N: 79.9 (73.6-84.8)	7-10-14 7-10-14 7-10-14 7-10-14	RBC or PPI + A + C are comparable, RBC + C + N is superior to PPI + C + N
Padol *et al*[25]	Medline, Embase, CCTR	1996-2005	17	1569	PPI + 2AB PM: 88.9 (81.2-97.6) HomEM: 70.9 (64.3-77.4) HetEM: 82.7 (75.3-89.2)	7-14	O, but not L and R effect is influenced by CYP2C19 status
Suzuki *et al*[26]	PubMed	1998-2005	14	1529	CagA +: 84% (79-89%) CagA-: 73% (65-82)	7-14	Presence of CagA is predictive for a successful eradication
Wang *et al*[27]	Medline, Embase, CCTR	2000-2005	11	2159	E + 2AB: 86% PPI + 2AB: 81%	7	E, O and P are of comparable efficiency
Buzás *et al*[28]	Abstracts	1997-2004	75	15634	PPI + 2AB: 81.4% (78.5-84.5) RBC + 2AB: 78.5% (70.5-84.3) PPI + 2AB + bismuth: 82.6% (76.0-89.2)	7	PPIs, RBC + 2AB and quadruple regimens are equally efficient as first-line therapies
Fuccio *et al*[29]	Medline, Embase, CCTR, abstracts	1996-2007	21	4831	PPI + 2AB: 75% (72-77) 80.7% (75.2-85.7) 78.2% (74.3-82.6)	 7 10 14	Extending triple therapy to 10-14 d is not useful
Villoria *et al*[30]	PubMed, ISIWS, Embase, CCTR CINAH, abstracts	1990-2007	6	1703	High-dose PPI: 82% (78-84) Standard dose PPI: 74% (NS)	7	High-dose PPIs are 8% more effective than standard doses in 7 d therapies
Zhao *et al*[31]	Medline, PubMed, Embase, ISIWB, CCTR, Chinese Databases	1999-2007	20	3330	PMs: 91.6 (83-99) HetEMs: 85.5 (79.6-92.3) HomEMs: 74.6 (70.1-79.8)	7-10-14	O and L effects are dependent on CYP2C19 genotype, R effect is not dependent

Essa *et al*[32]	PubMed, Embase, CCTR + abstracts	1990-2008	9	1054	Triple therapies: 76.8 (72.2-81.2) Concomitant quadruple therapy: 89.7% (86.8-92.1)	5-10 7	Concomitant quadruple therapy is superior to standard triple therapy
Luther *et al*[33]	Medline, Embase, Google Scholar, CCTR, ACP Journal Club	1996-2009	9	1679	PPI + AC: 77.0 (71-84) PPI + 2AB + Bi: 78.3 (71.7-84.6)	7	Triple and quadruple therapies yielded similar suboptimal results

[1]Abstracts of the Digestive Diseases Week, United European Digestive Week and European Helicobacter Study Group annual meetings. Abbreviations: A: Amoxicillin; AB: Antibiotic; Bi: Bismuth compound C: Clarithromycin; CCTR: Cochrane Controlled Trials Register; CI: 95% confidence interval; E: Esomeprazole; ISIWB: Institute of Scientific Information Web of Science; ITT: Intention-to-treat; HetEM: Heterozygous extensive metabolizers; HomEM: Homozygous extensive metabolizers; L: Lansoprazole; N: Nitroimidazole; P: Pantoprazole; PM: Poor metabolizers; PPI: Proton pump inhibitor; O: Omeprazole; R: Rabeprazole; RBC: Ranitidine bismuth citrate.

determinations are largely neglected even in developed countries. It seems that the 27 years from the discovery of *H. pylori* were not enough for the medical community to understand that chronic gastritis and peptic ulcers are infectious diseases and doctors must think as infectionists in their therapeutic judgements. A recent study from California stated that the "epidemic" of antimicrobial-resistant infections was related to insufficient funding, surveillance, control, prevention, research and development and misguided regulation of antibiotic use, including in agriculture and especially for food animals[42]. In fact, none of the guidelines cited[1-6] or experts/opinion leaders[43] contraindicate explicitly the use of clarithromycin or metronidazole; they only outline the levels of antimicrobial resistance in which these compounds should be avoided.

Polymorphism of the CYP2C19 isoenzyme has been shown to result in significant differences of eradication rates between homozygous and heterozygous extensive metabolizers and poor metabolizers of omeprazole and lansoprazole, but not of rabeprazole; however, almost all the data are from Japan and China[29,31].

CagA positive status seems to affect eradication rates favorably, at least in Europe and North America[26]. Tailoring treatment after the determination of both CYP2C19 and CagA status yielded a 96% eradication rate *vs* 70% with standard triple therapy in Japan, without an increase in the final cost of successful eradication[44].

Eradication rates show significant geographical variations: a Canadian systematic review and meta-analysis revealed that although PPI-based triple regimens are recommended worldwide as first-line treatment, there are regional differences in success rates between Asia, Africa, North and South America and Europe that are not completely explained by antimicrobial resistance rates and local prevalence of the infection[45,46]. In our meta-analysis of European congress abstracts, we also found continental variations, without an east-west or north-south gradient[28]. Genetic differences of *H. pylori* strains infecting these populations might influence eradication rates but this has not yet been investigated.

ALTERNATIVE THERAPIES

Several alternatives to standard triple therapies have been proposed. Ranitidine-bismuth citrate (RBC) emerged in 1991 as a highly efficient drug in association with amoxicillin and clarithromycin; 3 meta-analyses showed that RBC-based triple therapies achieve similar rates of eradication to PPI-based regimens, and when given with nitroimidazoles are superior to PPI-based combinations[15,17,24]. The lack of worldwide availability and a fall in the product's promotion have led, however, to a limited use of this valuable compound. Levofloxacin given to 1926 cases in 11 studies as part of first-line triple therapy was superior to standard regimens (odds ratio 1.56, CI: 1.25-1.94)[31] and it was also found to be efficient and safe according to a Chinese meta-analysis[47]. Moxifloxacin, given in 4 studies to 772 patients, achieved 84.1% eradication as compared to the 73.6% of the standard therapy (relative risk: 1.13, CI: 1.01-1.27)[48], but resistance values forecast that the quinolones will suffer the same fate as macrolides. Furazolidone is cheap and useful in first-line treatment: when given with PPI + one antibiotic, it achieved eradication in 81.4% of cases, better than standard regimens (71.7%, odds ratio: 2.34, CI: 0.76-3.92), but this nitrofuran derivative has limited availability[49]. Three meta-analyses showed that 10-d sequential therapy is superior to standard regimens, but almost all studies are Italian: these data must be confirmed in other countries/populations before considering it a first-line therapy[50-52]. The non-bismuth concomitant quadruple therapies are also better than the standard regimens, and less complex than sequential therapy[33,36]. All of the regimens have their pros and cons: we still lack an ideal first-line therapy.

PROPOSAL FOR THE FUTURE

From a meta-analytical point of view, the decrease in the efficiency of standard triple therapies over time cannot be substantiated: sets of studies obtaining an eradication rate of less than 80% have existed from the beginning. Further studies are needed before abandoning them as being no longer effective. While there is no new antibiotic on the horizon that works against *H. pylori*, instead of the multistep approach of small pilot studies to identify effective new therapies, I would like to propose adequately powered non-inferiority trials with a pre-defined margin (10%, 15% or perhaps 20%) in which standard triple therapies are compared with the available alternatives, taking the antimicrobial resistance profile, CagA and CYP2C19

status into consideration. The design and methodology of non-inferiority trials are available[53]. If the inferiority of standard triple therapies is confirmed in this way, they can be abandoned and deleted from the guidelines. Until then, in this author's opinion, standard triple therapies should be given in most countries/regions as first-line therapies, according to the local guidelines. Furthermore, as leading authorities have stated, there is still much to be learned about the association of *H. pylori* with human disease and optimal therapy of these conditions[43].

ACKNOWLEDGMENTS

The secretarial help of Mrs. Jolán Józan (Semmelweis University, Department of Physiology, Budapest) and correction of the English manuscript by Mr. Douglas Arnott (EDMF Translation Services, Etyek, Hungary) are gratefully acknowledged.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3871

World J Gastroenterol 2010 August 21; 16(31): 3871-3877
ISSN 1007-9327 (print)

EDITORIAL

Therapeutic implications of colon cancer stem cells

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Received: March 12, 2010 Revised: May 8, 2010
Accepted: May 15, 2010
Published online: August 21, 2010

Key words: Colon cancer initiating cells; Colon carcinogenesis; Target therapy

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Fabrizi E, di Martino S, Pelacchi F, Ricci-Vitiani L. Therapeutic implications of colon cancer stem cells. *World J Gastroenterol* 2010; 16(31): 3871-3877 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3871.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3871

Abstract

Colorectal cancer is the second most common cause of cancer-related death in many industrialized countries and is characterized by a heterogenic pool of cells with distinct differentiation patterns. Recently, the concept that cancer might arise from a rare population of cells with stem cell-like properties has received support with regard to several solid tumors, including colorectal cancer. According to the cancer stem cell hypothesis, cancer can be considered a disease in which mutations either convert normal stem cells into aberrant counterparts or cause a more differentiated cell to revert toward a stem cell-like behaviour; either way these cells are thought to be responsible for tumor generation and propagation. The statement that only a subset of cells drives tumor formation has major implications for the development of new targeted therapeutic strategies aimed at eradicating the tumor stem cell population. This review will focus on the biology of normal and malignant colonic stem cells, which might contribute to our understanding of the mechanisms responsible for tumor development and resistance to therapy.

COLONIC EPITHELIAL STEM CELLS

The adult colonic epithelium has a well-defined architecture organized into crypts, dynamic structures which are constantly self-renewing[1]. Each crypt unit is maintained by adult multipotent stem cells (SCs), located at the bottom of the structure itself, that are able to simultaneously self-renew and generate a population of transiently amplifying cells which in turn generate more mature cells. Three differentiated cell types mediate the function of colonic epithelium: the colonocytes, also termed absorptive enterocytes, the mucus-secreting goblet cells and the enteroendocrine cells.

Adult SCs are defined by several key functional properties including: self-renewal, potential for multilineage differentiation and tissue regeneration. Two different models have been proposed to localize the intestinal SCs; the "+4 position" model and the "stem cell zone" model[2]. According to the former, the intestinal SCs are located at +4 position relative to the bottom of the small intestine crypt, just above the non-cycling Paneth cells. These cells are actively cycling and, through asymmetric division, give rise to their differentiated progeny. The more recent "stem cell zone" model states that small undifferentiated cycling

cells, termed crypt base columnar (CBC) cells, are the true intestinal SCs. These cells are interspersed between the Paneth cells in the small intestine or located at the very bottom of the crypt in the colon.

Despite the fact that colonic crypts have long been known to harbor a functional stem cell compartment, the identification, isolation and characterization of colonic crypt SCs has been hampered by the absence of reliable molecular markers. Over the last 30 years, many studies have been performed to indirectly localize intestinal SCs within the colonic crypts by using techniques such as long-term retention of DNA label[3] or histone-GFP marking[4], both based on the "immortal strand hypothesis" proposed by Cairns[5]. According to this theory, SCs might selectively retain their old DNA strands, while donating the newly synthesized DNA strands to their progeny. However, this hypothesis is currently a subject of controversy due to the demonstrated absence of asymmetric genetic material segregation in hematopoietic stem cells[6]. Recently, several molecules have been proposed as markers of SCs in the intestine including the RNA-binding protein musashi-1 (Msi-1)[7] and Hes-1, a transcriptional repressor transactivated by Msi-1[8]. Hes-1 and Msi-1 were shown to be co-expressed by the putative SCs at the crypt base, but immunoreactivity was also observed in a broader population of cells.

Other putative biomarkers have been evaluated for distinguishing the SC population within the colon, such as members of the integrin superfamily of transmembrane glycoproteins including α2 and β1 subunits[9]. Additionally, Eph-B receptors have been described as important regulators of migration and proliferation in the intestinal epithelium[10]. However, the inhibition of Eph-B2/Eph-B3 signaling has been shown to reduce the number of proliferating cells without altering the stem cell number, suggesting that Eph-B receptors are unlikely to be independent biomarkers of colonic SCs.

Another possible intestinal stem cell marker recently identified is the polycomb protein Bmi-1, known to be involved in the maintenance of hematopoietic and neural stem cells[11]. In the small intestine, this factor is expressed in cells with stem cell features located near the crypt bottom[12].

The colonic epithelium is replaced every five days[13]. This high rate of tissue renewal depends on a complex interplay between processes involving cell proliferation, differentiation, migration, adhesion and cell death that are finely coordinated by a relatively small number of highly evolutionarily conserved signaling pathways including BMP, Sonic hedgehog, Notch and Wnt, the latter playing a critical role in the regulation of epithelial SCs in the intestinal tract.

Wnt signaling is required for self-renewal of gut SCs; it is involved in intestinal embryogenesis and adult intestinal epithelial cell proliferation[14,15]. The unique role played by the Wnt pathway in the physiology of the intestine led to the identification of the Wnt target gene *Lgr5* as a biomarker of intestinal SCs in mouse small intestine and colon. The *Lgr5* gene encodes a leucine-rich repeat containing G-protein coupled receptor, also known as Gpr49. Lgr5 expression is restricted to cycling CBC cells and it has been demonstrated that Lgr5-expressing cells differentiate into the expected functional lineages of the colonic epithelium[16].

Interestingly, the ability of single $Lgr5^+$ SCs to establish long-term culture and to generate crypt-villus organoid, without requiring a mesenchymal niche, has been also described[17].

Transcriptome analysis of $Lgr5^+$ epithelial cells isolated from the bottom of the small intestinal crypts led to the identification of a gene signature for these $Lgr5^+$ SCs[18]. Not surprisingly, many genes on the list were Wnt target genes whose expression was confirmed to be restricted to cells at the base of the crypts, as revealed by in situ hybridization. By this technique olfactomedin-4 (OLFM4) was also identified as a highly-specific and robust marker for $Lgr5^+$ SCs, even though its expression was not under the control of Wnt. The *OLFM-4* gene encodes a secreted molecule with unknown function, originally cloned from human myeloblasts[19], which is enriched in human colon crypts[20]. Due to the very low expression levels of Lgr5, OLFM-4 has been recently proposed as a more faithful SC marker highly expressed in CBC cells in human small intestine and colon[21].

COLORECTAL CANCER STEM CELL IDENTIFICATION

Tumors are composed of a heterogeneous mixture of cancer cells at various levels of differentiation, very similar to the structure of an organ. Recently, the "cancer stem cell" model of tumorigenesis has proposed that within the tumor mass there is a predetermined cell population with a "stem cell" phenotype, able to perpetuate the cancer, while the rest of the tumor cells are incapable of self-renewal. Even though it has long been assumed that mutations within adult colonic stem cells may induce neoplastic transformation, the proof of existence of colorectal cancer stem cells (CRC-SCs) has been hindered in the past years by difficulties in identifying a specific biomarker for this rare cell population. Only recently, new evidence has been provided that supports the existence of CRC-SCs, confirming that the tumorigenic cell population of CRC can be isolated on the basis of the expression of specific cell surface biomarkers. The standard analysis to ascertain the existence of a subpopulation of cancer stem cells (CSCs) is the demonstration that these cells can transfer the tumor in immunocompromised mice and replicate the phenotypic heterogeneity of the parental tumor. Several recent studies have evaluated the functionality of specific CRC-SC biomarkers by using a combination of flow cytometry to identify a "putative" SC population and xenograft models involving immunodeficient mice to determine their tumor initiating potential[22]. In the first two studies, CD133, also known as Prominin-1, was employed to identify the tumorigenic cell population within CRC[23,24].

O'Brien *et al*[23] isolated CD133$^+$ cells from seven primary colon cancers and ten extracolonic (metastatic) sites. When transplanted into the renal capsule of NOD/SCID mice, CD133$^+$ cells readily developed tumors displaying morphologic features equivalent to those of the parental cancer. Tumor phenotype was further maintained upon serial transplantation[23]. Similarly, in the second study, a population of CD133$^+$ cells, accounting for approximately 2.5% of tumor cells, was isolated from colon cancer specimens and perpetuated *in vitro* as floating colonies or "tumor spheres". These tumor spheres, expressing the epithelial adhesion molecule BerEp4, but not differentiation markers such as cytokeratin 20 (CK20), were enriched in a tumorigenic population and could be maintained for serial *in vitro* passages[24]. Both studies demonstrated the expression of CD133 also in normal colon tissue, although at a lower frequency, reinforcing the hypothesis that CD133$^+$ CRC-initiating cells in cancer samples might result from oncogenic transformation of normal colonic SCs. Subsequently, Dalerba *et al*[25] proposed CD44 and the epithelial surface antigen (EpCAM) as CRC-SC-specific markers, with further enrichment by CD166. Purified CD44$^+$/EpCAM^{HIGH} cells injected into NOD/SCID mice resulted in high frequency generation of tumor xenograft. In contrast, CD44$^-$/EpCAM^{LOW} cells lack tumor-initiating activity[25]. Further subfractionation of the CD44$^+$/EpCAM^{HIGH} cell population by using the mesenchymal stem cell marker CD166 increased the success of tumor xenograft. Finally, in a more recent study, aldehyde dehydrogenase 1 (ALDH) has been proposed as a promising new marker for normal and malignant human colonic SCs[26]. As few as 25 ALDH1$^+$ cancer cells, isolated by flow cytometry, were able to generate tumor xenografts. Notably, a subsequent isolation of cancer cells using a second marker (CD44 or CD133 serially) produced a modest further enrichment of tumor-initiating ability.

Significant controversies exist over the functional role of these CRC-SC markers. Major questions have been raised regarding CD133. Indeed, Shmelkov *et al*[27], using a transgenic mouse model in which the CD133 promoter drove *LacZ* reporter expression, demonstrated that CD133 was expressed by both mature and undifferentiated colonic epithelial cells, suggesting that CD133 is not restricted to the SC compartment[27]. Moreover, using confocal microscopy the authors reported that in primary colon cancer samples from humans and mice, CD133 was expressed in all epithelial, EpCAM$^+$ cells in the malignant tissue and that CD133 expression was excluded from the non-epithelial cell components of the tumor. Thus, they proposed that the inability of CD133$^-$ to generate tumors could be simply due to their non-epithelial nature. Furthermore, the same authors demonstrated that both CD133$^+$/EpCAM$^+$ and CD133$^-$/EpCAM$^+$ cell populations, isolated from liver metastasis of colon cancer, were able to generate tumors upon serial transplantation into NOD/SCID mice. Taken together these data appear to be in contrast with those previously published. However, the different techniques employed do not allow a direct comparison between the studies. Indeed, it is possible that primary and metastatic tumors may have a different expression pattern for CD133. Moreover, when Shmelkov *et al*[27] analysed primary tumors they did not perform the same functional assessment of tumor-initiating activity as was done in previous reports.

Regardless of the ongoing debate as to CD133 as a CRC-SC marker and the lack of evidence for a functional role in tumorigenesis of this marker, clinical data support the significance of this molecule in CRC, particularly as an independent negative prognostic marker[28]. Furthermore, the combined evaluation of CD133 and nuclear β-catenin can identify high risk cases of low stage CRC[29]. A comparison of expression of the three markers CD133, CD44 and CD166 that have been associated with CRC-SCs revealed that the expression of CD133 correlates with that of CD166, whereas both do not correlate with CD44, confirming that CD133 is, alone, the best marker to predict poor patient survival[30]. In a more recent report, Artells *et al*[31] observed longer relapse-free interval and increased overall survival in patients with lower levels of CD133.

To address the clinical relevance of CD133 for CRC metastasis in patients, Horst *et al*[32] analyzed CD133 expression in a matched case-control collection of 54 pairs of CRC with and without synchronous liver metastasis. They demonstrated that there is a strong correlation between high CD133 expression and synchronous liver metastasis. However, the authors did not observe any effect, when knocking down CD133, on colon cancer cell line proliferation, migration, invasion and colony formation. Thus, they conclude that CD133 is a marker with high prognostic impact for CRC, even though it seems to have no relevant functional role as a determinant of tumor progression.

Taken together these data confirm the need to identify biomarkers for CRC-SCs in order to improve our understanding of the mechanisms underlying tumor growth and progression. In this regard, Barker *et al*[33] have shown that deletion of APC in Lgr5$^+$ expressing cells leads to their transformation within days, suggesting that Lgr5 may mark not only normal intestinal stem cells, but also a limited population of CSCs. Simultaneously, using knock-in *LacZ* reporter mice within the Prominin-1 (*Prom1*) locus, Zhu *et al*[34] have shown that Prom1$^+$ cells, located at the base of the crypts in the small intestine, co-express Lgr5, generate the entire intestinal epithelium and are susceptible to neoplastic transformation.

From a clinical point of view, a recent study showed that Lgr5 was markedly over-expressed in the majority of advanced CRCs compared with normal mucosal tissue[35]. As expected, in situ hybridization analysis confirmed the expression of Lgr5 in CBC cells in both small intestine and colon. This Lgr5 expression, which was variable among CRC cases, correlated significantly with lymphatic and vascular invasion, lymph node metastasis and tumor stage, suggesting the involvement of this marker in tumor progression.

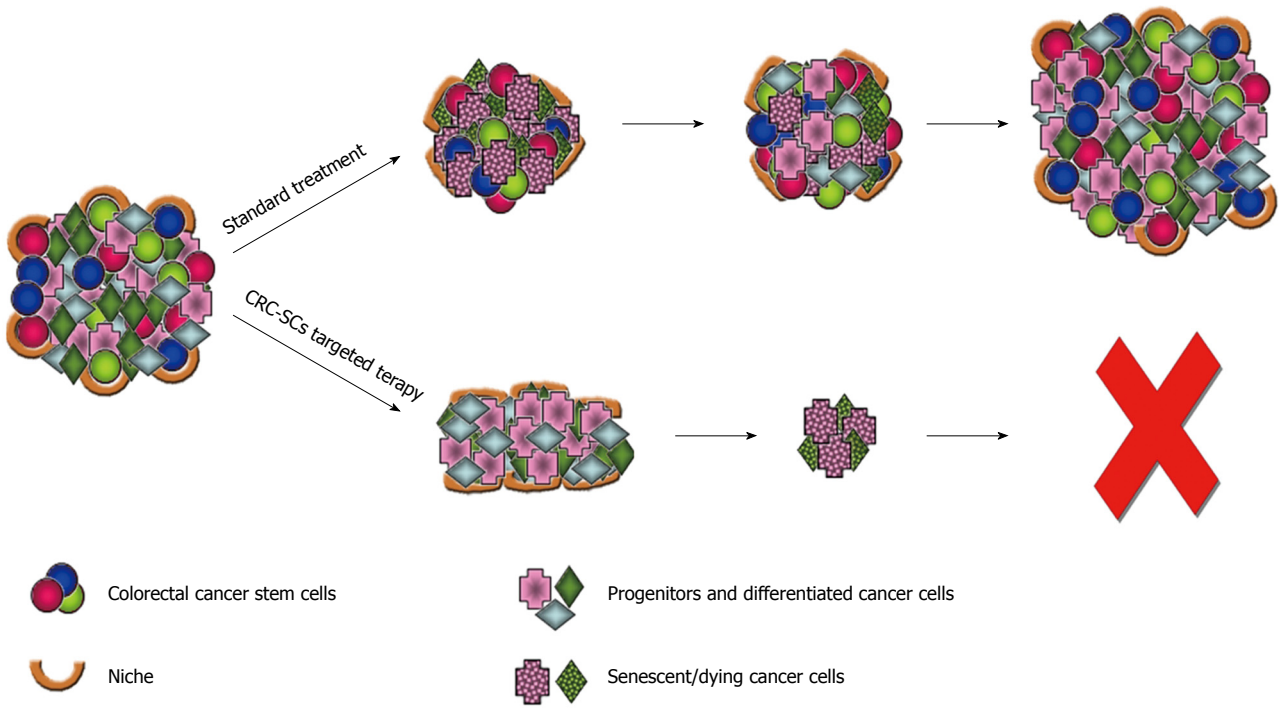

Figure 1 Therapeutic implication of cancer stem cells. The failure of current standard therapies in tumor eradication can be explained by assuming that colorectal cancer stem cells (CRC-SCs) are able to survive treatments leading to an only transitory clinical remission. Therapeutic strategies that specifically target the CRC-SC pool, by eliminating the self-renewing component of the tumor mass, could be more effective in eradicating the tumor and reducing the risk of relapse and metastasis.

A similar correlation has been described for the "stemness" gene *Bmi1* by Du *et al*[36]. Real-time analysis of 98 samples of CRC showed that high expression of Bmi-1 is directly correlated with poor patient survival.

IMPLICATION OF CRC-SC MODEL FOR THERAPY

The CSC model has important implications for cancer therapy. Many current cancer therapies target the most rapidly dividing cells, which represent the majority of the tumor cell population. This can result in a remarkable but frequently transitory clinical remission. Failure of conventional treatment options to eliminate the CSC compartment might result in tumor relapse and, more importantly, in the proliferation of therapy-resistant and more aggressive tumor cells, which ultimately reduce patient survival (Figure 1). Several features of CSCs may make them hard to eliminate. CSCs are relatively quiescent and this allows them to escape from chemotherapeutic regimens that typically target actively cycling cells. Moreover, as shown for their normal counterpart, CSCs have been proposed to exhibit high level expression of multidrug transporter family genes, likely resulting in more efficient efflux of chemotherapeutic drugs and innate multidrug resistance[37]. In addition, signaling pathways that regulate self-renewal of normal colonic SC population, such as Wnt, Hedgehog or Notch, are dysregulated in CRC leading to tumor development. The development of an efficient therapeutic approach would therefore require the identification of distinctive molecular pathways active in CSCs and the identification of agents that can either block CSC proliferation or induce CSC differentiation, thus enhancing sensitivity to chemotherapeutic drugs.

Together with resistance to chemotherapy, CSCs are frequently resistant to standard radiotherapy regimens. In this respect, it has been recently demonstrated that resistance to radiation of $CD133^+$ glioblastoma SCs can result from elevated expression of DNA damage response genes[38]. Radiotherapy for glioblastoma is associated with an increase in the proportion of the $CD133^+$ fraction. Similarly, Dylla *et al*[39] showed that CRC-SCs are enriched in residual tumors following chemotherapy and remain capable of rapidly regenerating tumor from which they were derived. The authors have further demonstrated that resistance is mediated, at least in part, by ALDH1 enzyme activity.

Together with intrinsic factors, the microenvironment, or niche, may influence the ability of CSCs to proliferate, migrate or invade. The niche is an anchoring site for CSCs, and adhesion molecules or microenvironmental soluble molecules, including growth factors and cytokines, can significantly contribute to the refractoriness to therapy.

Todaro *et al*[40] have recently demonstrated that the up-regulation of interleukin-4 (IL-4) in $CD133^+$ CRC-SCs is an important mechanism that protects these tumorigenic cells from apoptosis. $CD133^+$ CRC-SCs produce IL-4 as an autocrine growth factor promoting tumor resistance to chemotherapeutic agents such as 5-fluorouracil and oxaliplatin. Growth inhibition by these agents was significantly increased when cells were first treated with antibodies

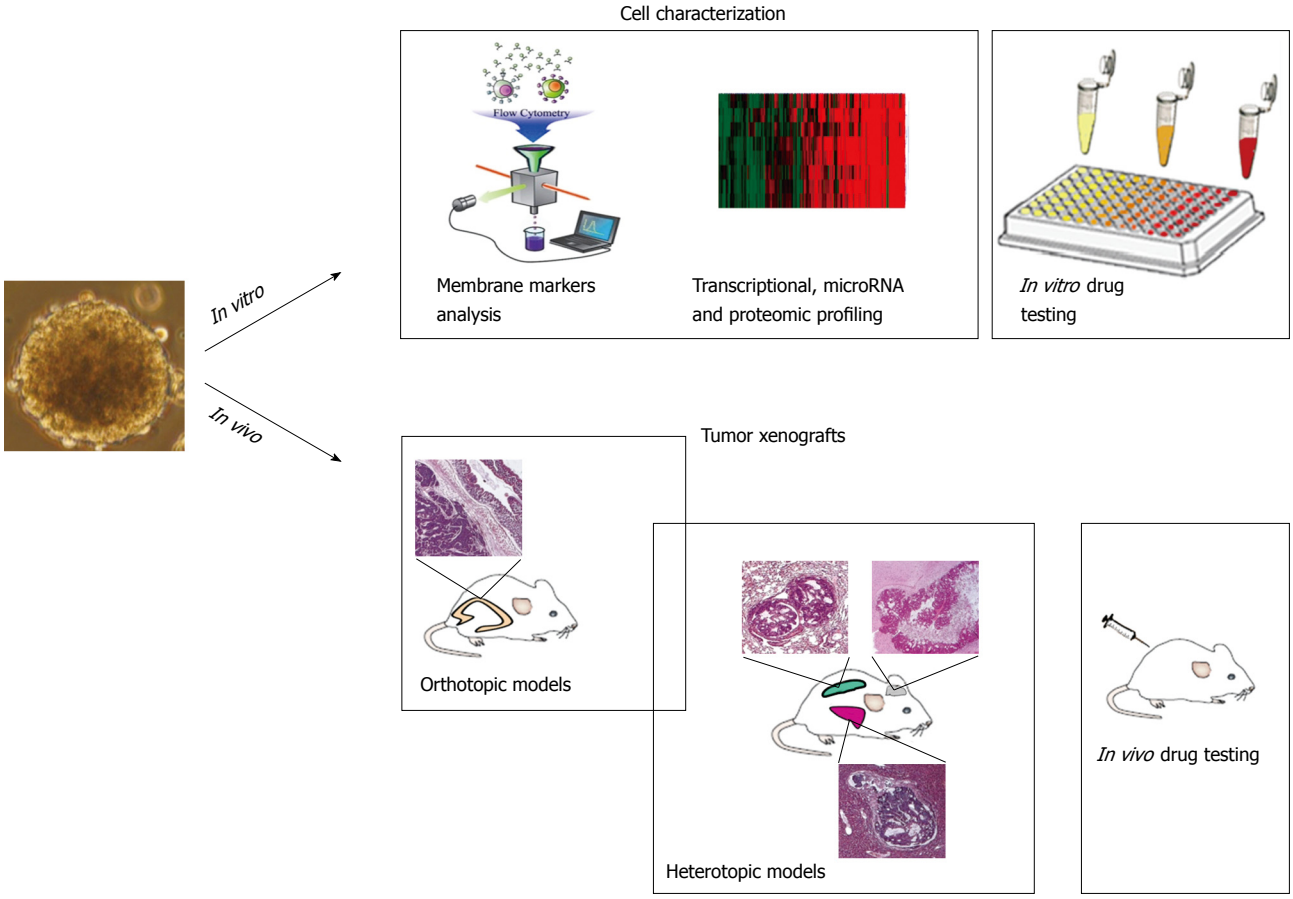

Figure 2 Colorectal cancer stem cells as a tool for drug discovery. *In vitro*, colorectal cancer stem cells (CRC-SCs), isolated from the tumor specimen, are propagated as "tumor spheres". Membrane marker analysis together with transcriptional, microRNA and proteomic profiling lead to the identification of molecular targets that are expressed by this cell population. These findings can be used to evaluate the cytotoxic ability of new compounds. *In vivo*, CRC-SCs can be orthotopically and heterotopically injected into immunocompromised mice generating tumors that mimic the cytoarchitecture of the parental tumors. The use of such mouse models of CRC allows for drug testing analyses in order to eradicate the primary tumor and avoid the formation of incurable metastases.

blocking the IL-4 signal. This phenomenon was confirmed in xenografts in which the administration of anti-IL-4 antibodies significantly reduced tumor growth after chemotherapy.

When human chemotherapy-resistant CRC cell lines (HT29/5FU-R and HT29/OxR) were developed following exposure of HT29 to increasing doses of 5-fluorouracil and oxaliplatin, there was a marked enrichment of $CD133^+$ and $CD44^+$ double positive cells. Phosphorylated and total insulin-like growth factor receptor (IGF-IR) levels were also increased in the resistant cell lines and their derived-tumors showed significantly greater growth inhibition in response to an IGF-IR mAb than parental cells, demonstrating that IGF-IR activation provided for enhanced sensitivity of CRC-SCs[41]. Other potential targets include specific targeting of symmetric stem cell division. To study new approaches to develop drugs that target CSCs, Boman *et al*[42] used computer modelling. They demonstrated that exponential increase in both SC and non-SC populations in CRC development involves an enhanced symmetric SC division. This finding suggests that any systemic therapy designed to effectively treat CRC and other cancers must act to control or eliminate symmetrical CSC division in tumors, while minimally affecting normal SCs[42].

Another important aspect to be considered with regard to the designing of novel and more effective therapies against CRC is that CRC-SCs represent an excellent tool for the preclinical evaluation of new anticancer therapies both *in vitro* and *in vivo*, where they generate xenografts that phenocopy the human tumor of origin (Figure 2). Currently available mouse models of CRC are based on chemically-induced tumors, genetically engineered animals and tumor implants in immunocompromised mice, and none of them faithfully replicates all the aspects of human tumor development. Reliable mouse models of human CRC are essential to understand the mechanisms underlying tumor development and pathogenesis. In this respect CSCs represent an innovative and powerful tool in cancer research by conjugating the advantages of *in vitro* amplification, high grafting efficiency and, more importantly, the ability to closely reproduce the original tumors in terms of histology and drug sensitivity.

Thus, a systematic approach to identify and challenge the CSC survival machinery appears to be mandatory in order to develop novel and more efficient stem cell-based therapies. Genome-wide analyses of cancer have revealed the existence of a great genetic variation among individual tumors, which makes extremely complex the use of an ex-

clusively genomic approach to cancer biology. At the same time, it is increasingly clear that tumors share common features in terms of protein pathway level, suggesting that a pathway-orientated perspective would represent the most effective approach to drug discovery and therapy. In a recent study, Fang *et al*[43] generated $CD133^+$ tumor sphere cultures from several colon cancer specimens and performed mass-spectrometry-based quantitative proteomics in order to identify cell surface proteins enriched on culture tumor cells. These cells retain the expression of cell surface markers such as CD133, CD166, CD44 and EpCAM as well as other stem cell-associated proteins including nestin, Bmi1 and Msi-1, thus confirming the value of this *in vitro* model for biological analysis of CSC populations as well as for drug screening experiments. Therefore, integrated strategies based on high-throughput proteomic, drug screening and gene expression-based approaches will allow in the near future the identification and targeting of survival signaling pathways in CSCs.

CONCLUSION

Increasing evidence shows that CSCs may play a critical role in tumor development and progression. CSC resistance to conventional therapies may explain why it is difficult to completely eradicate cancer and why recurrence is often inevitable. Thus, the identification and molecular characterization of CSCs is critical to develop therapeutic strategies that specifically target this rare population of cells and that are likely to be effective in eradicating tumors and in reducing the risk of relapse and metastasis.

ACKNOWLEDGMENTS

The authors wish to thank the Italian Association for Cancer Research for supporting the colon cancer stem cell research.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3878

World J Gastroenterol 2010 August 21; 16(31): 3878-3887
ISSN 1007-9327 (print)

REVIEW

Hepatitis B virus infection and renal transplantation

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Received: March 11, 2010 Revised: June 1, 2010
Accepted: June 8, 2010
Published online: August 21, 2010

Abstract

Although the prevalence of chronic hepatitis B virus (HBV) infection has declined in renal transplant recipients (RTRs), it remains a relevant clinical problem with high morbidity and mortality in long-term follow up. A thorough evaluation, including liver biopsy as well as assessment of HBV replication in serum (i.e. hepatitis B e antigen and/or HBV DNA) is required before transplantation. Interferon should not be used in this setting because of low efficacy and precipitation on acute allograft rejection. The advent of effective antiviral therapies offers the opportunity to prevent the progression of liver disease after renal transplantation. However, as far as we are aware, no studies have compared prophylactic and preemptive strategies. To date, the majority of RTRs with HBV-related liver disease have had a high virological and biochemical response to lamivudine use. However, lamivudine resistance is frequent with a prolonged course of therapy. Considering long-term treatment, antiviral agents with a high genetic barrier to resistance and lack of nephrotoxicity are suggested. The optimal strategy in RTRs with HBV infection remains to be established in the near future.

Key words: Hepatitis B; Renal transplantation; Lamivudine resistance

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Tsai MC, Chen YT, Chien YS, Chen TC, Hu TH. Hepatitis B virus infection and renal transplantation. *World J Gastroenterol* 2010; 16(31): 3878-3887 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3878.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3878

INTRODUCTION

Since the first successful renal transplantation in 1954, there has been an exponential growth in publications dealing with the care of renal transplant recipients (RTRs). Hepatitis B virus (HBV) infection is an established cause of morbidity and mortality in RTRs[1-4]. Although numerous studies have reported the impact of HBV status on patients and graft outcome in RTRs, the role in pathogenesis of liver damage is still unclear. In recent decades, as improvement in renal transplantation has been the result of better immunosuppression, organ preservation, and patient selection[5,6]. In addition, substantial improvement in the understanding of HBV virology and the natural course of infection, combined with highly sensitive HBV DNA assays and the advent of effective antiviral drugs with different mechanisms of action, has led to better therapeutic strategies for chronic HBV infection. It is of

increasing interest to determine the long-term outcome of HBV in RTRs. Hence, the aim of this study is to review the available literature concerning the natural course and clinical manifestations of HBV-related liver disease in RTRs. Furthermore, treatment of RTRs with HBV infection is discussed.

PREVALENCE RATES OF HBV INFECTION IN RTRs

The prevalence rates of hepatitis B surface antigen (HBsAg) seropositive status among RTRs varies among countries (Table 1), and it has been decreasing over time[3,7,8]. Mathurine and colleagues have reported that the prevalence of HBsAg decreased significantly (24.2% before 1982 *vs* 9.1% after 1982, $P < 0.001$)[3]. Recently, Santos *et al*[7] also have shown a marked decline in the prevalence of HBV infections over the last 15 years (6.2% in 1994 *vs* 2.3% in 2006). In our previous study[9], the prevalence of HBV infection in RTRs was 9.2% (51/554), which is lower than that reported previously in Taiwan in 1994 (20.9%, 14/67)[10]. The decreasing prevalence of HBV infection in this population can be attributed to the use of HBV vaccination, the use of erythropoietin for anemia and the consequent decreased need for blood transfusions during the pre-transplantation period.

TRANSMISSION OF HBV INFECTION BY RENAL TRANSPLANTATION: DONOR EVALUATION

The possibility of HBV transmission by organ transplantation can be predicted from the serological status of both donor and recipient. It is generally accepted that transplanting an HBsAg-positive allograft into an HBsAg-negative recipient carries a significant risk of *de novo* infection[11,12]. A recent meta-analysis has shown that recipients who are seropositive for HBsAg show an increased risk for mortality and graft failure compared with seronegative recipients[2]. This has resulted in a policy at many organ procurement organizations to restrict the use of kidneys from HBsAg-positive deceased donors. However, the rapid increase in patients with end-stage renal disease has exacerbated the shortage of donor organs. The ban on using kidneys from HBsAg-positive donors has been challenged under certain circumstances. A study from Thailand[13] has reported that 14 anti-HBsAg-positive patients received kidneys from HBsAg-positive donors and 27 HBsAg-positive patients received kidneys from HBsAg-negative donors. Hepatitis B hyperimmune globulin (HBIG) and lamivudine were not used at any time. The 10-year survival of these patients was not significantly different (92.8% *vs* 62.5%, $P = 0.14$). In a study from Turkey, Berber *et al*[14] have reported seven kidney transplants from HBsAg-positive donors. All of the recipients were HBsAg-negative and hepatitis B surface antibody (HBsAb)-positive. Prophylactic lamivudine treatment after transplantation was given. None of the patients who received a kidney from an HBsAg-positive donor developed clinical HBV infection in a mean 42-mo follow-up period. Jiang *et al*[15] have reported a prospective non-randomized controlled study: 373 HBsAg-positive RTRs received a kidney from HBsAg-positive ($n = 65$) or HBsAg-negative ($n = 308$) donors. Using a standardized immunosuppressive and antiviral regimen (400 U HBIG once and twice for recipients with HBsAg-negative or HBsAg-positive grafts, respectively; 400 U HBIG weekly for 3 mo and lamivudine 100 mg daily for 6 mo for recipients with HBV DNA-positive grafts), there was no significant difference in liver injury and patient survival. Therefore, it is suggested that kidney allografts from HBsAg-positive donors can be used safely in HBsAb-positive recipients with preexisting naturally acquired or vaccination-induced immunity to HBV. HBV viral status should be monitored, and HBIG and/or lamivudine should be prescribed, depending on donor HBV DNA status.

Table 1 Frequency of hepatitis B surface antigen-positivity in renal transplant recipients

Authors	HBsAg rate, % (*n*)	Reference year	Country of origin
Hu *et al*[10]	20.9 (14/67)	1994	Taiwan (China)
Mathurin *et al*[3]1	15.3 (128/834)	1999	France
Lee *et al*[4]	12.9 (62/477)	2001	Taiwan (China)
Chan *et al*[30]	13.2 (67/509)	2002	Hong Kong (China)
Morales *et al*[8]2	2.2 (76/3365)	2004	Spain
Aroldi *et al*[20]	14.2 (77/541)	2005	Italy
Santos *et al*[7]3	3 (37/1224)	2009	Portugal
Tsai *et al*[9]	9.2 (51/554)	2009	Taiwan (China)

[1]Prevalence of hepatitis B surface antigen (HBsAg) was 24.2% before 1982 and 9.1% after 1982 ($P < 0.001$); [2]Prevalence of HBsAg was 3.0% in 1990 and 2.0% in 1998; [3]Prevalence of HBsAg was 6.2% in 1994, 4.1% in 1998, 3.2% in 2002, and 2.3% in 2006.

NATURAL HISTORY OF HBV DISEASE IN RTRs

Defining the natural history of HBV infection among RTRs is difficult because of the unique characteristics of this population and chronic HBV infection has a long natural course, and it might need 10-20 years to see the key complications, most notably cirrhosis and hepatocellular carcinoma (HCC). The natural history of hepatitis B in the immunocompetent population has been described by Liaw *et al*[16]. Chronic HBV infection is a dynamic state of interactions between HBV, hepatocytes and the immune system of the patient. Therefore, the natural course of chronic HBV infection can be divided into four phases: immune tolerant, immune clearance, residual inactive, and reactive immune clearance phases. The reactive phase can be viewed as a variant of the immune clearance phase. However, in the population of RTRs, therapeutic immunosuppression post-transplantation might affect the host immune responses against HBV. In addition, most centers are reluctant to perform liver biopsies before and after renal transplantation due to concern about platelet

dysfunction. Furthermore, antiviral therapy is now widely available, which might change the course of hepatitis and stop disease progression. All these factors limit the clinician's understanding of the progression of liver disease in RTRs.

Fornairon *et al*[17] have reported a large cohort study of 151 HBsAg-positive RTRs. The spontaneous annual clearance rates of HBsAg, hepatitis e antigen (HBeAg), and HBV DNA were 0.1%, 3% and 3%, respectively, which are lower than in the general population. A high rate of persistent viral replication and reactivation, as defined by HBeAg reappearance, was observed in 30% after renal transplantation, which was significantly higher than in the general population. Degos *et al*[18] have demonstrated reactivation of HBV replication in 11 of 12 (92%) initially HBV-DNA-negative, HBsAg-positive RTRs after 3-12 mo and an increase in HBV DNA in 6 of 11 (55%) initially HBV-DNA-positive patients. In our study of 51 HBsAg-positive RTRs during a mean follow-up of 58 mo, 34 patients (67%) had higher HBV DNA levels at the end of follow-up than at baseline, which was associated with cirrhosis development[9].

Liver biopsy is a gold standard for the diagnosis of hepatic disease. A variety of studies has shown more severe courses in RTRs infected with HBV compared with non-infected patients. In 1985, Parfrey *et al*[19] performed a prospective study of 20 HBsAg-positive RTRs who underwent serial liver biopsy. There was a marked tendency to progression, and 82% of patients who had virus only, reactive hepatitis, or chronic persistent hepatitis on initial biopsy, subsequently developed chronic active hepatitis or cirrhosis on final biopsy. Fornairon *et al*[17] have reported the largest single-center evaluation of follow-up liver biopsies in HBsAg-positive RTRs: 310 biopsies in 131 RTRs, with two or more biopsies in 101 patients. At the time of renal transplantation, normal liver histology was found in 39%, chronic persistent hepatitis in 25%, chronic active hepatitis in 25%, and miscellaneous findings in 11% of patients. After a mean interval of 66 mo, histological deterioration was observed in 85.3% of these 101 patients who underwent serial liver biopsies, with occurrence of cirrhosis in 28% and chronic active hepatitis in 42%. Only 6% showed normal liver biopsy in the second biopsy. These results were in accordance with the findings in other smaller series. Aroldi *et al*[20] have reported 34 HBsAg-positive RTRs who underwent serial liver biopsy: 24 (71%) showed progression of liver fibrosis in which 15 patients evolved to cirrhosis, and six of them died of liver failure.

The reactivation of HBV infection in RTRs can also occur in HBsAg-negative but HBsAb- and hepatitis B core antibody (HBcAb)-positive patients. However, it is difficult to distinguish these from *de novo* infection. There have also been a few reports in the literature about the reactivation of HBV infection in RTRs with previously resolved HBV infection[21-23]. Blanpain *et al*[21] have reported two RTRs with cured HBV infection at the time of transplantation, as defined by the absence of HBsAg and the presence of HBsAb and HBcAb in the serum, who presented with HBV reactivation at 7 mo and 3 years after transplantation, respectively. Degos *et al*[18] have detected HBV DNA in seven of 35 RTRs (20%) following transplantation, who either had no serological evidence of HBsAg or HBV DNA before transplantation; a finding that suggests that immunosuppression can amplify even minimal residual HBV DNA. Based on this evidence, transplant physicians should be aware of the risk of HBV reactivation in patients with cured HBV infection before transplantation.

OUTCOME OF RTRs WITH HBV INFECTION: MORTALITY AFTER TRANSPLANTATION

Mortality is a reliable end-point in the natural course of HBV after renal transplantation. However, the impact of HBsAg on survival in RTRs has been controversial, especially in early studies. Some initial studies that have focused on 5-year survival rates generally have failed to show a difference between HBsAg-positive and -negative RTRs[24-27]. However, more recent studies with a large sample size and longer follow-up have suggested a detrimental effect of HBsAg on patient and graft survival[3,4,28,29]. Lee *et al*[4] have reported that the 10-year survival rate was much higher in the HBsAg-negative group (82.8%) than in the HBsAg-positive group (51.4%) ($P < 0.005$). Multivariate analysis has revealed that HBV infection is an independent risk factor for patient mortality. The major cause of death was liver failure in the HBsAg-positive group: 62.5% (10/16) *vs* 23.3% (7/30) in the HBsAg-negative group. Mathurin and colleagues also have reported that 10-year survival was significantly higher in non-infected (80% ± 3%) than in HBsAg-positive recipients (55% ± 6%). Multivariate analysis has shown that, in patients transplanted before 1982 (year of HBV vaccination), HBsAg was an independent factor for poor survival ($P < 0.0001$)[3]. In a case-control study by Mathurin, survival was significantly lower at 10 years in patients infected by HBV than in matched patients (55% ± 6% *vs* 80% ± 4%, $P = 0.004$). Furthermore, in a study by Chan from Hong Kong[30], HBsAg-positive patients who underwent renal transplantation before 1996, without the availability of lamivudine, had a markedly inferior survival rate compared with that in HBsAg-negative RTRs. Recently, a meta-analysis performed by Fabrizi *et al*[2], which pooled 6050 patients, indicated clearly that HBsAg in serum was an independent risk factor for death after renal transplantation (relative risk: 2.49, $P < 0.0001$).

OUTCOME IN RTRs WITH HBV INFECTION: MORBIDITY AFTER TRANSPLANTATION

Graft survival

The influence of chronic HBV infection on graft survival remains controversial. In a large cohort study by Foraniron[17], better allograft survival was described in HBsAg-

positive than in HBsAg-negative patients (P = 0.0006). Also London *et al*[31] have found a beneficial effect of HBsAg positivity on graft survival. In contrast, Lee *et al*[4] have reported an inferior 10-year graft survival for HBV- or hepatitis C virus (HCV)-infected RTRs, although this was not significant (44% and 50%, P = NS, respectively) compared with non-infected patients (74%). Recently, Mathurin *et al*[3] have observed that 10-year graft survival (36%) in HBsAg-positive RTRs was significantly lower compared with that in non-infected patients (63%). Their case-control study also showed that 10-year graft survival was significantly lower in patients infected by HBV than in matched control patients (36% ± 5% *vs* 61% ± 5%, P < 0.001). Finally, a 2005 meta-analysis has indicated that HBsAg positivity was associated with an increased risk of allograft loss (relative risk: 1.44, 95% CI: 1.02-2.04)[2].

FACTORS AFFECTING PROGRESSION IN HBV-RELATED DISEASE AFTER RENAL TRANSPLANTATION

In chronic HBV patients, viral factors (viral load, genotype and genomic mutations), host factors (age, sex, and immune status), and other factors (alcohol consumption, cigarette smoking, exposure to aflatoxin, and other viral superinfections) contribute to the progression of liver disease[32]. However, in RTRs with HBV infection, the factors that affect HBV-related progression have not been identified. Fairley *et al*[33] have found that the presence of HBV DNA and/or HBeAg in serum prior to renal transplantation was associated with an increased probability of death from liver disease: five of 10 patients in this group died of chronic liver disease; but only one of 15 patients who were HBV DNA and/or HBeAg negative prior to transplantation died of liver disease (P = 0.002). In our previous study[9], 13 of 51 RTRs developed cirrhosis in a period of 57 mo. Among these, HBV DNA levels at baseline could not predict cirrhosis development. However, the elevation of serum HBV DNA ($\geq 10^5$ copies/mL) after renal transplantation was a significant risk factor for development of cirrhosis.

There are at least eight major genotypes of HBV (A-H) with distinct geographical distribution. Genotype A and C variants can induce more severe liver disease than genotype B and D in general immunocompetent chronic HBV patients[34-36]. To date, a paucity of data exists to discuss the HBV genotype that affects liver disease in RTRs. Only our previous study has demonstrated that there is no significant association between genotype and cirrhosis development in RTRs[9]. We do not know the reason for this inconsistency with results from general chronic HBV populations. However, genotype B was the predominant genotype in the RTRs (45/51, 88.2%) of our study, which is considerably higher than that (60%) in the general population[35]. This difference might have been because these patients were selected before renal transplantation for their relatively benign clinical course of hepatitis B.

Chu *et al*[37] have suggested that older RTRs with HBsAg and/or HCV antibody carriers have a greater chance of developing HCC and mortality than younger patients do. In that study, 173 RTRs with serum HBsAg and/or HCV antibody were divided into three groups: older age (≥ 55 years, n = 3); middle age (18-55 years, n = 160); and younger age (≤ 18 years, n = 10). The incidence of HCC and the risk of death due to liver disease post-transplantation in the older, middle and younger age groups were 100%, 3.75% and 0% (P < 0.001), and 100%, 19.8% and 0% (P < 0.001) respectively. These results were in agreement with the findings from the study of Aroldi[38], in which older age (> 40 years) was independently associated with poor survival in RTRs (relative risk: 2.8).

Recent studies have indicated that precore and core promoter mutations are significantly associated with advanced liver disease in chronic HBV carriers[39-41]. For immunosuppressed patients, Günther *et al*[42] have performed serial HBV sequences by polymerase chain reaction (PCR) and DNA sequencing in nine HBsAg-positive RTRs. Seven of them showed either persistent or increasing amounts of the HBV core gene deletion mutants. All seven patients developed cirrhosis, and five died from end-stage liver disease. The incidence of complications was higher than in recipients without mutations. In our previous analysis[9], there was no significant association between core promoter mutations (T1762/A1764) and advanced liver disease. However, we have found that the development of T1762/A7164 mutants can predict an increase in HBV DNA, which was associated with cirrhosis development after renal transplantation. Hence, we hypothesized that immunosuppressive agents induce HBV DNA replication, especially in recipients with T1762/A1764 mutants, which results in cirrhosis development. We think that a smaller case number and/or the use of interventional antiviral therapy might cause the absence of a significant correlation between T1762/A1764 mutants and cirrhosis development. In a study by Preikschat *et al*[43], development of cirrhosis and end-stage liver disease after renal transplantation was associated with persistence and accumulation of specific HBV mutant populations. Their results demonstrate that viruses are characterized by a set of mutations rather than by a single mutation: deletions/insertions in core promoter plus deletion in the C gene and/or deletion in the pre-S region.

THERAPY OF HEPATITIS B IN RTRs

With the improving results of renal transplantation techniques and care, liver disease has emerged as an important cause of morbidity and mortality. Before the advent of effective antiviral agents, chronic liver disease developed in > 80% of HBsAg-positive RTRs, and 37%-57% of mortality in these subjects was attributed to liver complications[17,19,44,45]. Therefore, hepatitis B was considered to be a contraindication for organ donation in some western countries in the era before antiviral agents. However, the past decade has witnessed important developments in the therapy of hepatitis B. The availability

Table 2 Efficacy summary of antiviral monotherapy in renal transplant recipients with hepatitis B virus infection

Antiviral agent	Authors	Reference year	Patients	Duration (mo)	Biochemical response[1] (%)	Virological response[2] (%)	Resistance (%)
Lamivudine	Rostaing *et al*[73]	1997	6	6	4/5 (80)	6/6 (100)	0
	Jung *et al*[74]	1998	6	8	6/6 (100)	6/6 (100)	0
	Fontaine *et al*[75]	2000	26	16	NA	26/26 (100)	8/26 (31)
	Lee *et al*[82]	2001	13	12	NA	10/13 (77)	1/4 (25)
	Park *et al*[83]	2001	10	35	8/10 (80)	7/10 (70)	1/10 (10)
	Chan *et al*[30]	2002	26	32	14/14 (100)	26/26 (100)	11/26 (42)
	Thabut *et al*[95]	2004	14	65	8/14 (57)	14/14 (100)	8/14 (57)
	Kamar *et al*[96]	2004	18	37	13/18 (72)	6/18 (33)	12/18 (67)
Adefovir	Fontaine *et al*[90]	2005	11 (LAM-R)	15	9/11 (82)	1 (9)	0
Entecavir	Kamar *et al*[92]	2008	8 (ADV-R)	16	NA	5/8 (63)	NA

[1]Biochemical response was calculated at the end of the treatment as alanine aminotransferase normalization; [2]Virological response was calculated as the disappearance of hepatitis B virus (HBV) viremia at the end of the treatment. HBV DNA was quantified in all studies by non-polymerase chain reaction assay (PCR), hybridization techniques with a limit ranging being 5-6 $\log_{10}$ copies/mL, except for the studies by Thabut, Kamar, Gwak and Fontaine (2005) who used a PCR-based assay. LAM-R: Lamivudine-resistance; ADV-R: Adefovir-resistance; NA: Not available.

of lamivudine in 1998 marked a new era of oral therapy. According the study by Chan *et al*[30], the survival of HBsAg-positive RTRs treated preemptively with lamivudine was similar to that of HBsAg-negative controls, whereas HBsAg-positive RTRs without lamivudine treatment had significantly inferior survival (relative risk of death: 9.7, $P < 0.001$). A study by Ahn *et al*[46] has investigated the clinical outcome of 2054 RTRs to establish the efficacy of lamivudine treatment in HBsAg-positive recipients ($n = 66$). Lamivudine given to 27 recipients markedly improved 10-year patient and graft survival compared to those who did not take lamivudine. Notably, the 10-year patient survival rates were similar between pre-transplant HBsAg-negative and -positive patients (88.2% *vs* 85.3%). Hence, the overall prognosis of patients with HBsAg positivity has probably improved given the increased efficacy of antiviral therapy. Currently, there are seven agents approved for the treatment of chronic hepatitis B by the US Food and Drug Administration[16,47]. They are conventional interferon (IFN)-α, pegylated IFN, lamivudine, adefovir, entecavir, telbivudine and tenofovir. In the non-renal transplant population, IFN-based therapy is modestly efficacious in inducing HBeAg loss or seroconversion (30%-40%) in HBeAg-positive patients[48-51] and entecavir, telbivudine and tenofovir are the most potent in HBV DNA suppression. At 1 year, ⩾ 60% of HBeAg-positive and ⩾ 85% of HBeAg-negative chronic hepatitis B patients achieved undetectable HBV DNA after therapy with these three agents[52-54]. Drug resistance occurs most frequently with lamivudine, followed by telbivudine, adefovir and tenofovir, and is very low with entecavir[52,53,55-59]. However, the optimal antiviral agent for the treatment of RTRs with chronic hepatitis B infection is still unclear because of limited data on the use of these agents in RTRs. Table 2 summarizes antiviral agents used in RTRs with HBV infection.

IFN

IFN has antiviral, antiproliferative and immunomodulatory effects, such as increased cytotoxic T lymphocytes and natural killer cell immune response to viral proteins[60-62]. There have been only a few successful studies of monotherapy with IFN in RTRs. Post *et al*[63] have reported a 38-year-old RTR who developed symptomatic hepatitis. Tests for HBeAg and HBV DNA were both positive. After treatment with 1 MU IFNα three times weekly for 3 wk, followed by an increase to 3 MU three time weekly for a total of 16 wk, hepatitis clinically resolved, with HBeAg seroconversion and HBV DNA negativity. The renal allograft function remained excellent throughout the course of therapy with IFN. Grotz *et al*[64] have reported HBsAg positivity in RTRs suffering from hepatitis flare up. Their patient was HBeAg seropositive, with detectable HBV DNA. IFN was given at a dose of 3 MU three times a week for 14 wk. At the end of treatment, HBeAg seroconversion developed, with a decrease in HBV DNA. However, an acute rejection episode occurred after IFN therapy, and: renal biopsy revealed acute interstitial alterations. Prednisone was prescribed and renal function recovered. Although IFN therapy has been shown to terminate effectively viral replication in some RTRs with HBV infection, the use of IFN for the treatment of hepatitis B in RTRs has been reported infrequently. Reluctance to use IFN in RTRs could be related to concern about precipitating acute allograft rejection, direct nephrotoxicity, and tubulointerstitial nephropathy with glomerular alterations, which are frequently irreversible and steroid resistant[65-68]. As a result, IFN should not be used for treating HBV infection in this setting.

Lamivudine

Lamivudine is a nucleoside analogue that has a potent inhibitory effect on HBV replication by competitive inhibition of viral reverse transcriptase and termination of proviral DNA chain extension. It has been approved worldwide for the treatment of chronic hepatitis B both in non-immunosuppressed[69,70] as well as liver[71] and renal transplantation patients[30,72-83]. Although several authors have reported the use of lamivudine for the treatment of hepatitis B in RTRs, these were small, uncontrolled trials. To assess the efficacy of lamivudine, Fabrizi *et al*[84] have performed a meta-analysis of 14 clin-

ical prospective cohort studies that included 184 RTRs. The mean overall clearance of HBV DNA and HBeAg was observed in 91% and 27%, respectively; and alanine aminotransferase normalization occurred in 81%, with lamivudine resistance being reported in 18%. Frequency of HBeAg loss and lamivudine resistance was positively associated with increased duration of therapy ($r = 0.51$, $P = 0.039$; $r = 0.620$, $P = 0.019$, respectively).

Adefovir

Adefovir has demonstrated safety and efficacy in treatment-naïve patients and those with lamivudine-resistant HBV infection[55,85]. Safety and efficacy also have been proven in immunodeficient patients including HIV-positive patients[86] and after liver transplantation[87]. However, nephrotoxicity and Fanconi-like syndrome with phosphaturia and proteinuria were reported when adefovir was evaluated at higher daily doses (30 mg)[88]. Of note, adefovir dose must be adjusted in accordance with creatinine clearance in patients with impaired renal function[89]. To date, a paucity of studies exists concerning the use of adefovir in RTRs[90,91]. Fontaine *et al*[90] have described the use of adefovir in 12 RTRs with lamivudine-resistant HBV infection. The daily dosage was 10 mg initially and then adjusted based on renal function. After the 12 mo, the median decline in serum HBV DNA was from 8.76 to 2.97 $\log_{10}$ Eq/mL, without virological breakthrough. The efficacy was similar to that reported in the general population. Notably, there has been no experience in the use of adefovir in treatment-naïve RTRs with HBV infection.

Entecavir, telbivudine and tenofovir

In immunocompetent patients, entecavir, telbivudine and tenofovir are the most potent antiviral agents, followed by lamivudine and then adefovir. Entecavir is associated with the lowest rate of drug resistance (< 1% in 5 years among previous treatment-naïve patients)[58,59], and therefore, might be the preferred treatment, since long-term therapy will be needed in most RTRs. To date, there are no published data concerning the use of entecavir, telbivudine and tenofovir in RTRs. Only one study has demonstrated the efficacy and safety of entecavir in RTRs with HBV infection[92]. Eight RTRs, who have become adefovir or lamivudine-resistant, were administered entecavir (0.5-1 mg/d). After a median follow-up of 16.5 mo, there was a significant decrease in HBV DNA viral loss (3.86 to 2.94 $\log_{10}$ copies/mL, $P = 0.004$). Clinical tolerance of entecavir was very good without any rejection episode. Furthermore, there were no statistically significant changes in creatinine level, estimated creatinine clearance, or daily microalbuminuria. Hence, from currently available data, we suggest that entecavir is preferable to lamivudine, to minimize development of potential drug resistance, unless there are concerns about cost or unavailability of entecavir.

Drug resistance

Antiviral resistance is defined as the selection of HBV mutations that confer reduced susceptibility to a drug, which results in primary or secondary treatment failure. Clinically, the emergence of drug resistance is indicated by viral breakthrough, which is defined as a > 1 $\log_{10}$ increase in serum HBV DNA from nadir in a patient who had an initial virological response. Usually, subsequent biochemical breakthrough or raised alanine aminotransferase (ALT) values occurred in > 90% of patients[93], with some hepatic decompensation among patients with advanced fibrosis[94]. In chronic hepatitis B immunocompetent patients, lamivudine is associated with the highest rate of resistance, reaching nearly 70% after 5 years of continuous therapy[16]. Similarly, there was an increasing incidence of lamivudine resistance with longer treatment in RTRs. Thabut *et al*[95] have reported 14 RTRs with a median of 65 mo of lamivudine therapy. Lamivudine resistance appeared in eight patients (57%). In the study of Kamar[96], virological breakthrough was observed in 12/18 (67%) RTRs after 37 mo of lamivudine treatment. Chan *et al*[97] have reported 29 RTRs who received almost 60 mo of lamivudine therapy; 14 (48.3%) patients developed lamivudine resistance at 10-35 mo. Among these, hepatic flares were observed in 11 (79%). Therefore, in 2007, the concept of a roadmap was set up by an internal group of experienced hepatologists and virologists[98]. The panel recommends monitoring of serum HBV DNA levels to identify outcomes of therapy and to reduce the risk of viral resistance. In our analysis (unpublished data), we set forth the roadmap concept into 19 lamivudine-treated RTRs to monitor the efficacy and resistance of lamivudine. At week 24, there were seven patients with inadequate virological response (defined as HBV DNA levels $\geqslant$ 2000 IU/mL ($\geqslant$ 4 $\log_{10}$ copies/mL) at week 24 after antiviral treatment). Three patients developed YMDD mutations at week 52 and therapy was switched to adefovir-based therapy due to virological breakthrough. In the remaining four patients, two developed YMDD mutation at week 104 and shifted to adefovir-based therapy. These results provide a new insight into the roadmap concept for HBV therapy in RTRs.

Timing of antiviral agent initiation

There is lack of an algorithm for therapeutic approaches to antiviral treatment in RTRs with HBV infection. Several points remain to be defined regarding the management of HBV-related liver disease after renal transplantation. The first question is the timing of introduction of the antiviral agent. There are two principal approaches to prevent HBV reactivation after renal transplantation: prophylactic and preemptive strategies. To the best of our knowledge, there have been no studies in the renal transplant setting that have compared directly the prophylactic and preemptive approaches. A prophylactic strategy involves the administration of antiviral agents to patients at increased risk of developing HBV reactivation prior to transplantation; a preemptive strategy permits prompt treatment after the detection a marked increase in serum HBV DNA. Chan *et al*[30] have confirmed that preemptive lamivudine therapy improves survival of RTRs with HBV infection. There is also some evidence that prophylactic

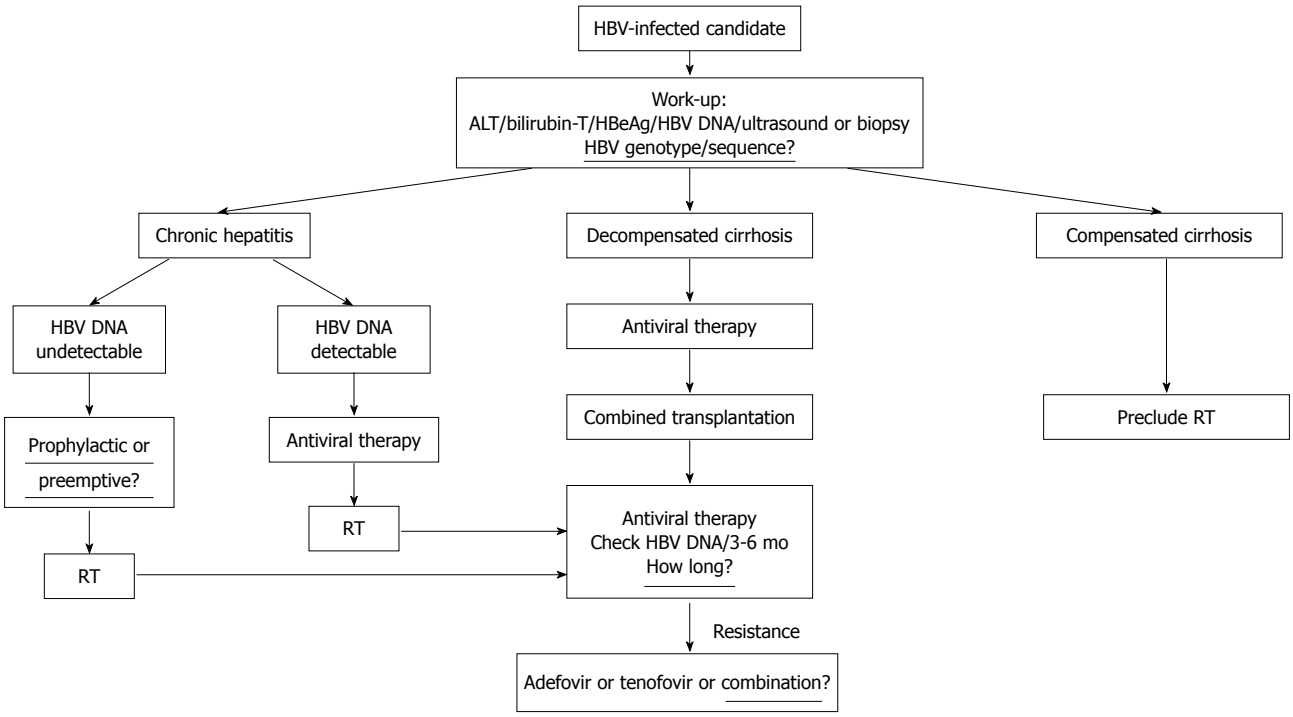

Figure 1 Algorithm for renal transplant candidates with hepatitis B virus infection. The font-underline in figure 1 means that there is no optimal strategy for renal transplant recipients (RTRs) with hepatitis B virus (HBV) infection so far, and they remain to be defined. ALT: Alanine aminotransferase; HBeAg: Hepatitis e antigen.

lamivudine might be beneficial in RTRs[78,83]. Whatever the prophylactic or preemptive strategy, it can be confirmed that salvage treatment after hepatic dysfunction with HBV recurrence is less effective[81].

Duration of treatment

The next unanswered question is whether lifelong treatment is needed in this special patient group. In the era of lamivudine, prolonged therapy is associated with drug resistance. One study has reported that lamivudine resistance was observed in three of 14 (21%) patients after 1 year of treatment, and in eight of 14 (57%) patients after 2 years[95]. Withdrawal of lamivudine is associated with a high risk of relapse, replication of the wild strain, and liver failure, all of which suggest prolonged therapy is necessary. Rostaing *et al*[73] have found that when lamivudine therapy was stopped for four patients after 6 mo, it was associated with a biochemical and virological relapse within the following weeks. However, a study by Chan *et al*[30], in which lamivudine was discontinued in 12 low-risk RTRs (> 9 mo therapy, HBV DNA and HBeAg negative, stable immunosuppression) five patients achieved treatment success, with two maintaining undetectable serum HBV DNA for > 18 mo. It seems that discontinuation is safe in selected patients, to minimize the emergence of drug resistance. Recently, Huang *et al*[99] have reported that the liver-related mortality rate was not significantly higher in patients who discontinued lamivudine treatment compared with continuously treated patients (both, 0%), in a total of 20 HBsAg-positive transplant recipients (discontinued, $n = 9$; continued, $n = 11$). However, these studies all had limitations of small case numbers and short follow-up periods. In the era of adefovir, entecavir, telbivudine and tenofovir, there are only a few studies concerning their efficacy in RTRs with HBV infection. Further large cohort studies with long-term, and/or combination, and/or high-dose antiviral therapy in HBsAg-positive RTRs are required.

Although there are no definite algorithms about antiviral therapy of HBV in RTRs, the 2007 American Association for the Study of Liver Diseases guidelines[100] issued some suggestions. For chronic hepatitis B patients who require immunosuppressive therapy, prophylactic antiviral therapy is recommended for HBV carriers at the onset of a finite course of immunosuppressive therapy. With baseline HBV DNA < 2000 IU/mL, antiviral therapy should be continue for 6 mo after completion of immunosuppressive therapy. In patients with baseline HBV DNA ⩾ 2000 IU/mL level, treatment should be continued until the endpoints are reached. However, there is still a lack of direct evidence for these guidelines to be applied in RTR patients.

CONCLUSION

The reported prevalence of chronic HBsAg carriers receiving renal transplantation is lowering, but it is not negligible, especially in endemic areas for HBV infection. HBV confers a high risk of morbidity and mortality in long-term follow up in RTRs. HBsAg-positive donors can be safely used in anti-HBs-positive recipients. To date, there is no optimal strategy for RTRs with HBV infection. Many points remain to be defined (Figure 1). Lamivudine therapy is effective in serological and virological responses in RTRs, and the tolerance is good. However, resistance to lamivudine frequently occurs with prolonged therapy.

Considering long-term treatment, antiviral agents with a high genetic barrier to resistance and lack of nephrotoxicity (e.g. entecavir) are suggested. Long-term follow-up studies are required in the near future.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3888

World J Gastroenterol 2010 August 21; 16(31): 3888-3896
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Glucocorticoid receptor gene haplotype structure and steroid therapy outcome in IBD patients

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Supported by The Swiss IBD Cohort Study (SNF Grant 33CSC0-108792); the Swiss National Science Foundation (Grant 32-120463/1); the Zurich University Research Priority Programme "Integrative Human Physiology" (ZIHP); the Center of Clinical Research at the University Hospital Zurich; and the Novartis Foundation for Biomedical Research
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Received: March 11, 2010 Revised: May 14, 2010
Accepted: May 21, 2010
Published online: August 21, 2010

Abstract

AIM: To study whether the glucocorticoid receptor (*GR*/*NR3C1*) gene haplotypes influence the steroid therapy outcome in inflammatory bowel disease (IBD).

METHODS: We sequenced all coding exons and flanking intronic sequences of the *NR3C1* gene in 181 IBD patients, determined the single nucleotide polymorphisms, and predicted the *NR3C1* haplotypes. Furthermore, we investigated whether certain *NR3C1* haplotypes are significantly associated with steroid therapy outcomes.

RESULTS: We detected 13 *NR3C1* variants, which led to the formation of 17 different haplotypes with a certainty of > 95% in 173 individuals. The three most commonly occurring haplotypes were included in the association analysis of the influence of haplotype on steroid therapy outcome or IBD activity. None of the *NR3C1* haplotypes showed statistically significant association with glucocorticoid therapy success.

CONCLUSION: *NR3C1* haplotypes are not related to steroid therapy outcome.

Key words: Inflammatory bowel disease; Steroid therapy; Glucocorticoid receptor; Pharmacogenetics; Haplotype analysis

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Mwinyi J, Wenger C, Eloranta JJ, Kullak-Ublick GA. Glucocorticoid receptor gene haplotype structure and steroid therapy outcome in IBD patients. *World J Gastroenterol* 2010; 16(31): 3888-3896 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3888.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3888

INTRODUCTION

Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial disorders, which are characterized by chronic recurrent inflammation of the gastrointestinal tract[1]. The molecular pathogenesis of IBD is not fully elucidated, although an exaggerated mucosal immune response triggered by intestinal bacteria

in genetically susceptible individuals appears to play an important role[2]. The combined prevalence of CD and UC is estimated to be 100 to 200 per 100 000 individuals in developed countries[3]. IBD shows extensive variation in individual clinical presentation and outcomes, which is likely to be caused by differences in genetic susceptibility, environmental factors, intestinal bacteria and activation of the intestinal immune system[3].

Although great advances have been made in the management and therapy of IBD, curative therapy does not yet exist. The anti-inflammatory agents mesalazine (5-aminosalicylic acid, 5-ASA) and sulfasalazine, in combination with glucocorticoids (GCs), are common first line therapy options in induction and maintenance of UC remission. Severe cases of UC are treated intravenously with GCs or cyclosporine. CD is mainly treated with GCs and/or antibiotics, and azathioprine (AZA), 6-mercaptopurine (6-MP), or the anti-folate methotrexate (MTX) are often added to maintain the state of remission[4]. GC-resistant or -dependent disease courses can be treated with anti-TNF-α antibodies, such as infliximab and adalimumab. GCs are often used in the initial treatment of most cases of moderate to severe active UC or CD. However, 20% of patients develop GC resistance within one year of treatment[5,6]. Non-response to GCs often leads to the need for a surgical intervention as a result of a poor therapy outcome. For example, it has been reported that 38% and 29% of steroid-resistant CD and UC patients, respectively, required surgery within one year after beginning GC treatment[5].

Glucocorticoids are potent inhibitors of T cell activation and cytokine secretion, primarily *via* binding to the cytoplasmically located glucocorticoid receptor (GR) as ligands. Due to ligand binding, homodimers consisting of two activated GRs are formed that translocate into the nucleus. The complex subsequently binds to specific glucocorticoid response elements (GREs) within the regulatory regions of GR target genes[7]. The mechanisms by which GC resistance develops, are not fully understood. Three possible mechanisms have been proposed. First, decreased plasma levels of GCs through overexpression of the drug efflux system P-glycoprotein (*MDR1*). Second, an altered function of GR or, third, excessive synthesis of pro-inflammatory cytokines induced by activation of pro-inflammatory transcription factors may reduce the affinity of GR to its ligands and lead to the development of GC resistance[4].

GR is known to be expressed as several polymorphic variants[8]. Several mutations in the *NR3C1* gene have been found to modulate individual GC sensitivity in *in vitro* investigations and in studies with healthy individuals[9,10]. In the present study we evaluated the association between the *NR3C1* gene haplotypes and therapeutic outcome of GC administration in a well-sized cohort of 181 patients with IBD. The aim was to comprehensively determine abundant GR variants by sequencing all protein-coding *NR3C1* exons (exons 2 to 9) and the first 50 bp of the neighbouring intronic regions in all individuals. We hypothesized that *NR3C1* gene polymorphisms may influence GC sensitivity and thus might serve as predictive markers for treatment success with GCs in IBD patients.

MATERIALS AND METHODS

Patients

One hundred and eighty-five clinically diagnosed Swiss IBD patients were recruited at the centers participating in the Swiss Inflammatory Bowel Disease Cohort Study (SIBDCS)[11]. All patients gave their informed consent for inclusion into the study. An ethical approval was obtained from the Medical Ethical Committees of the University Hospital Lausanne, Switzerland, and all local study sites. All patients had been treated with steroids and past steroid therapy outcome had been recorded. The standard employed criteria for the steroid therapy success or failure are available on the website www.epact.ch. Briefly, an insufficient response upon appropriate treatment in terms of doses and duration was considered a steroid therapy failure. EDTA-blood samples were stored at the central tissue repository at the Institute of Pathology, University of Bern, Switzerland. The SIBDCS data center at the University Hospital of Lausanne, Switzerland, provided data on past and current disease characteristics and GC therapy outcome. Diagnosis of IBD (CD or UC) was confirmed by the study investigators based on clinical presentation, endoscopic findings and histology.

Sequencing reactions

DNA was extracted from EDTA-blood using the QIAcube robotic workstation and a standard procedure (QIAamp DNA Mini Kit, QIAGEN, Switzerland). The PCR and sequencing primer design was based on the NCBI reference sequence (GenBank accession number NT_029289). Primers for genomic DNA were designed to span all expressed exons (2 to 9) and at least 50 bp of flanking intronic sequences at both 5'- and 3'-ends. The DNA sequences of purified PCR fragments were obtained with an ABI 3730xl sequencing machine. Details of the PCR primers can be found in the Table 1. Optimized PCR conditions, and methods used for subsequent purification and sequencing of the fragments are available upon request.

Haplotype analysis

The PHASE software was used to calculate the haplotypes based on the detected single nucleotide polymorphisms (SNPs) and mutations in the *NR3C1* gene. PHASE predicts *in silico* haplotypes on the basis of a Bayesian inference algorithm[12,13]. Haplotype calculations were performed on 181 individuals, from which sequence data of adequate quality were obtained. To allow referral to specific haplotypes, a frequency-based priority criterion was used to name them (e.g. *GR_1* for the most often occurring haplotype, Table 2).

Calculation of linkage disequilibria

Linkage disequilibria (LD) were calculated using the r^2 statistics. Calculations were performed using the software package Haploview (www.haploview.com).

Statistical analysis

To detect differences in haplotype distribution between

Table 1 Oligonucleotides used as polymerase chain reaction primers to amplify the *NR3C1* exons

Primer name	Primer sequence	Nested PCR	Primer name	Primer sequence
GR 2_F	CACTTAGGTTGTCTACCTTTCCTAC	Y	GR 2_Fa	TTCAAAAGGCCACTTAAACTTATTC
GR 2_R	GATAGAAACTACTCTTCTGGTAAC	Y	GR 2_Ra	CCTTGGAGATCAGACCTGTTG
		Y	GR 2_Fb	CTGTGCCCAGTTTCTCTTGC
		Y	GR 2_Rb	CAGCCAGATCTGTCCAAAGC
		Y	GR 2_Fc	TTGGAAACTCCTTCTCTGTGG
		Y	GR 2_Rc	AATGTGGCATGCTGAATGG
GR 3_F	CATTAGAGGACCTAGGAGCCAC	N		
GR 3_R	GAAGTGAACCAGAACCACACC	N		
GR 4_F	TGAATTCAGTGTGTGTAAGAAGAAC	N		
GR 4_R	TTGCACTGTTTTCAGTTTGTTG	N		
GR 5_F	CACCTGTATTCACCTGACTCTCC	N		
GR 5_R	TTTTTTCTCCTTTTCCATGTCAC	N		
GR 6_F	GCCCCAAGCACTCATAACTC	N		
GR 6_R	TCAGATGACAGAAGAAAACTGTGTC	N		
GR 7_F	AATCTTGGTGTCACTTACTGTGC	N		
GR 7_R	CCAAGATGCAGGAAGTTTAAGG	N		
GR 8_F	CACCAACATCCACAAACTGG	Y	GR 8_Fa	TTGGTCAGTGGGAACATC
GR 8_R	CCACCAGTTCTTCTTACACACAC	Y	GR 8_Ra	ATGGTGGCTTGTGCCTAC
GR 9a_F	TGATGACGACTCAACTGCTTC	N		
GR 9a_R	ATCTGGGGAATTCCAGTGAG	N		
GR 9b_F	TCCTAAAAGGGCACAGCTTC	N		
GR 9b_R	CAATCATTTGCTTTTTGAATGC	N		

PCR: Polymerase chain reaction; GR: Glucocorticoid receptor.

Table 2 Predicted haplotypes found to be in best reconstruction for 181 inflammatory bowel diseases patients

Haplotype number[1]	Haplotype composition[2] Reference: GGGATGGCCATGT	Absolute haplotype frequency (n = 362)	Relative haplotype frequency	Number of haplotypes not included[3]
GR_1	1111111111111	165	0.456	
GR_2[4]	1111112111211	90	0.249	
GR_3[4]	1111211111111	72	0.199	2
GR_4[4]	1112111111111	8	0.022	
GR_5	2211112111221	6	0.017	
GR_6	1111112111212	4	0.011	
GR_7	1111112111111	3	0.008	
GR_8	1111111111211	2	0.006	1
GR_9	1111112121212	2	0.006	
GR_10	1111221111111	2	0.006	2
GR_11	2211112111211	2	0.006	1
GR_12	1111111111212	1	0.003	1
GR_13	1111112211212	1	0.003	
GR_14	1111211112111	1	0.003	
GR_15	1111212111111	1	0.003	
GR_16	1121111111111	1	0.003	
GR_17	2211111111111	1	0.003	1

[1]Haplotypes are arranged in the order of decreasing frequency; [2]1 indicates the reference allele at a certain position, 2 indicates the variant allele; [3]Number of haplotypes not included in the subsequent association analysis (steroid therapy outcome, tables 4, 7 and 8) due to likelihood values ≤ 0.95; [4]Haplotype *GR_2* corresponds to haplotype b, *GR_3* corresponds to haplotype c and *GR_4* corresponds to haplotype d in Figure 2.

groups with different GC therapy outcomes, the Chi-Square test or the Fisher's exact test was used. It was analyzed whether one or two copies of a specific haplotype were associated with a particular therapy outcome compared to the GR wild-type carriers. If the number of subjects per group was large enough, heterozygous carriers with one wild-type allele and homozygous carriers of one distinct haplotype were analyzed together against homozygous wild-type carriers. The latter calculations were only performed for haplotypes which occurred in a reasonably large (> 40) number. The statistical analysis was performed using the software package SPSS 17 (SPSS Inc., Chicago, IL).

RESULTS

NR3C1 sequence variability

DNA samples from 185 IBD patients (CD or UC) were initially sequenced for the *NR3C1* coding exons 2 to 9

Table 3 Frequencies of single nucleotide polymorphisms detected in the glucocorticoid receptor gene (*NR3C1*)

SNP number	Alternative name[1]	Variant number[2]	DNA position[3]	DNA region	cDNA position[4]	Nucleotide reference	Nucleotide variant	Amino acid exchange	Allele frequency (n = 362)	Reported allele frequencies[2]
1	2.1	*rs6189*	3943266	Exon 2	558	G	A	E22E	0.025	0.002-0.034
2	2.2	*rs6190*	3943264	Exon 2	560	G	A	R23K	0.025	0.002-0.034
3	2.3	*rs72542742*	3942647	Exon 2	1177	G	A	A229T	0.003	0.002
4	2.4	*rs56149945*	3942244	Exon 2	1580	A	G	N363S	0.025	0.000-0.046
5	3.1	*rs4986593*	3856773	Intron 3		T	C		0.213	0.008-0.228
6	4.1	*rs61753484*	3852751	Intron 4		G	C		0.006	0.000-0.009
7	5.1	*rs6188*	3843271	Intron 5		G	T		0.290	0.000-0.500
8	6.1	*rs6194*	3841288	Exon 6	2256	C	T	H588H	0.006	0.000-0.091
9	8.1	*rs258751*	3825207	Exon 8	2526	C	T	D678D	0.006	0.000-0.149
10	8.2	*novel SNP*	3824968	Intron 8		A	G		0.003	NA
11	8.3	*rs258750*	3824816	Intron 8		T	C		0.307	0.091-0.362
12	8.4	*rs10482704*	3824690	Intron 8		G	T		0.017	0.000-0.027
13	9.1	*rs6196*	3824417	Exon 9	2790	T	C	N766N	0.022	0.058-0.325

[1]Defines both the exon/intron localization and the single nucleotide polymorphism (SNP) number; [2]According to the National Center for Biotechnology Information (NCBI) SNP database; [3]According to the NCBI genomic reference sequence NT_029289.11; [4]According to the NCBI cDNA reference cDNA Sequence NM_000176.2. NA: Not applicable.

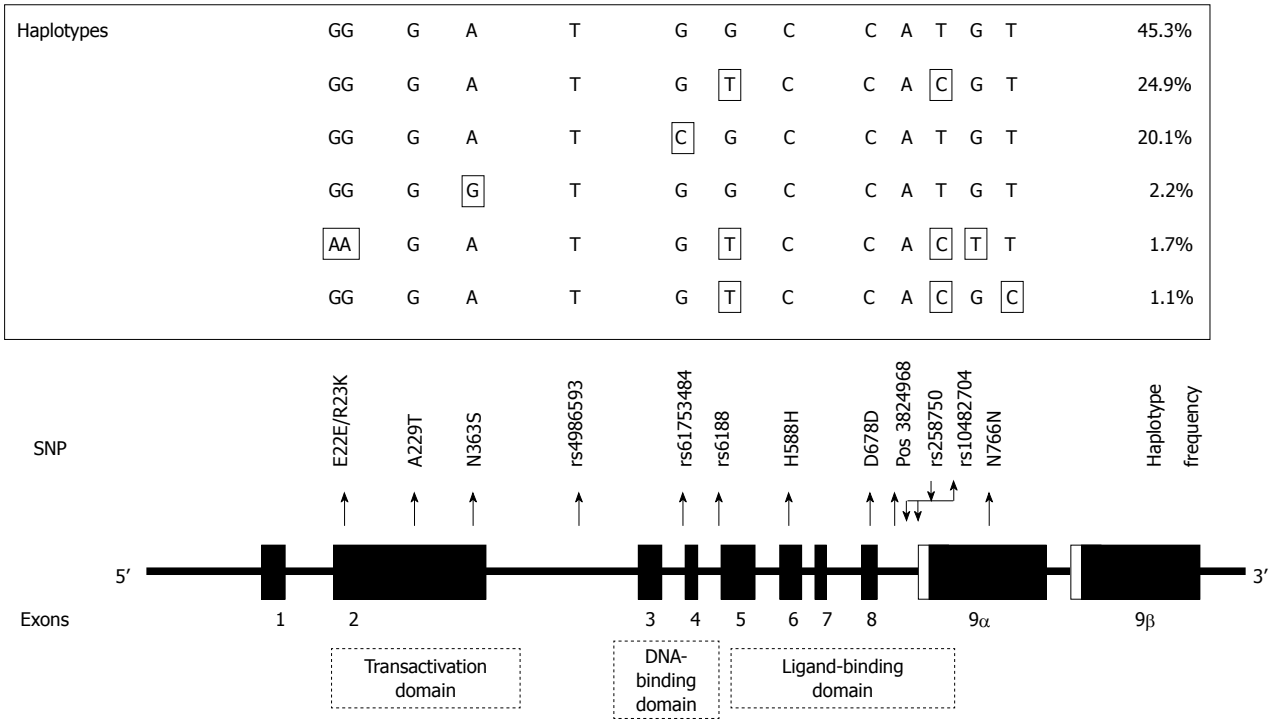

Figure 1 Most frequently occurring *NR3C1* haplotypes and their single nucleotide polymorphisms composition. The localization of the variant nucleotides in the *N3RC1* gene is indicated. All detected non-synonymous single nucleotide polymorphism (SNP) (R23K, A229T, N363S) flank the N-terminal transactivation domain. Only four out of 17 predicted haplotypes occur at a frequency higher than 2%.

and 50 bp of the neighbouring intronic sequences. The sequencing results of 181 individuals were of adequate quality and further used for SNP and haplotype analyses. The sequence data were screened for genetic variations in the *NR3C1* gene, using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nih.gov) and the GenBank entry NT_029289 as the reference sequence.

In Table 3 we list the allele frequencies of all detected SNPs within the IBD cohort under study. Thirteen variants were detected, which were-with exception of one mutation (*rs6196*, $P < 0.01$)-in Hardy-Weinberg equilibrium. All variants were single nucleotide substitutions. Six variants were detected within the intronic regions, whereas seven variants were found in exons (Figure 1). Three of the seven variants detected within the coding regions of the *NR3C1* gene resulted in non-synonymous amino acid exchanges, while four of them did not lead to changes in the GR amino acid sequence. Eight variants occurred with an allelic frequency of more than one percent (*rs56149945*, *rs6189*, *rs6190*, *rs4986593*, *rs6188*, *rs258750*, *rs10482704*, *rs6196*). All non-synonymous amino acid exchanges (R23K, A229T, N363S) were found in the N-terminal half of GR, flanking the N-terminal transactivation domain[14]. The intronic variant found at

SNP		1 [2.1]	2 [2.2]	3 [2.3]	4 [2.4]	5 [3.1]	6 [4.1]	7 [5.1]	8 [6.1]	9 [8.1]	10 [8.2]	11 [8.3]	12 [8.4]	13 [9.1]	
Exon		2	2	2	4	5	6	7	8	9	10	11	12	13	
[1]	SNP Pos	E22E	R23K	A229T	N363S	rs4986593	rs61753484	rs6188	H588H	D678D	pos3824968	rs258750	rs10482704	N766N	Haplotype frequencies
a (wt)		G	G	G	A	T	G	G	C	C	A	T	G	T	0.4529
b		G	G	G	A	T	G	T	C	C	A	C	G	T	0.2485
c		G	G	G	A	C	G	G	C	C	A	T	G	T	0.2006
d		G	G	G	G	T	G	G	C	C	A	T	G	T	0.0219
e		A	A	G	A	T	G	T	C	C	A	C	T	T	0.0166
f		G	G	G	A	T	G	T	C	C	A	C	G	C	0.0108
g		G	G	G	A	T	G	T	C	C	A	T	G	T	0.0083
h		G	G	G	A	T	G	T	C	T	A	C	G	C	0.0055
i		G	G	G	A	T	G	G	C	C	A	C	G	T	0.0053
j		A	A	G	A	T	G	T	C	C	A	C	G	T	0.0046
k		G	G	G	A	C	C	G	C	C	A	T	G	T	0.0036
l		A	A	G	A	T	G	G	C	C	A	T	G	T	0.0035
m		G	G	G	A	C	G	T	C	C	A	T	G	T	0.0028
n		G	G		A	T	G	T	T	C	A	C	G	C	0.0028
o		G	G	A	A	T	G	G	C	C	A	T	G	T	0.0028
p		G	G	G	A	T	G	G	C	C	A	C	G	C	0.0027
q		G	G	G	A	T	C	G	C	C	A	T	G	T	0.0020
r		G	G	G	A	T	G	G	C	C	G	T	G	T	0.0016
s		G	G	G	A	C	G	G	C	C	G	T	G	T	0.0012
t		G	G	G	A	C	G	T	C	C	A	C	G	T	0.0011
u		G	G	G	A	C	G	G	C	C	A	C	G	T	0.0005
v				G	A	C	G	G	C	C	A	T	G	C	0.0002
w		A	A	G	G	T	G	G	C	C	A	T	G	T	0.0002
x		G	G	G	A	T	G	G	C	C	A	T	G	C	0.0001
y		G	G	G	G	T	G	T	C	C	A	C	G	T	0.0001

Non-synonymous coding SNP
Synonymous coding SNP
Intronic SNP

Figure 2 *NR3C1* haplotypes predicted by PHASE in the cohort of 181 inflammatory bowel diseases patients. [1]Counter (a to y) for the 25 theoretically arising haplotypes in the inflammatory bowel diseases cohort. SNP: Single nucleotide polymorphism

DNA position 3824968 has not been previously listed in the NCBI SNP database.

Haplotype analysis

The 13 *NR3C1* variants described above were included in the haplotype calculations using the computer program PHASE. All 181 individuals were included in the haplotype prediction analysis (Figure 2). Twenty-five *NR3C1* haplotypes were predicted by PHASE to exist in the studied cohort. Furthermore, PHASE determined 17 different distinct haplotypes, which were found to be in best reconstruction for the cohort (Table 2). Six out of these 17 haplotypes occurred at a frequency higher than 1% (Figure 1). PHASE was only able to determine the haplotype structure of 174 individuals out of 181 subjects with a certainty of $\geqslant$ 95%. The data of one individual were excluded because of missing demographic data. Thus, the predicted haplotypes of 173 individuals were included in the subsequent association analysis.

The two SNPs E22E/R23K were found to be in complete linkage disequilibrium (Figure 3). This finding is in agreement with previous publications[15,16]. Furthermore, a strong but not complete linkage was found between the SNPs *rs6188* and *rs258750* (Figure 3).

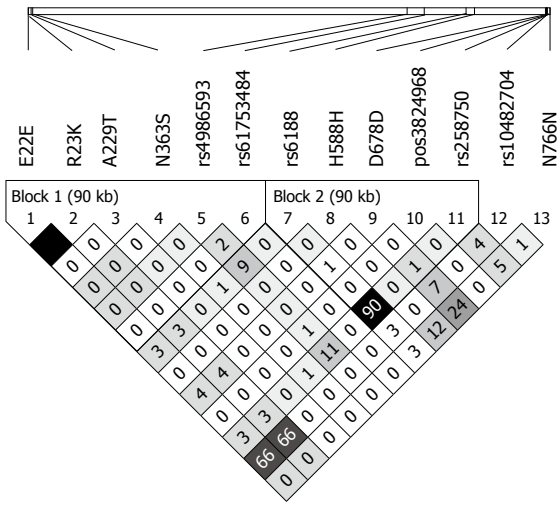

Figure 3 Linkage disequilibrium calculations of single nucleotide polymorphisms in the *NR3C1* gene. Linkage disequilibrium plot of r^2 values of observed variants in the *N3RC1* gene. Colour scheme: r^2 = 0%, white, 0% < r^2 < 100%, shades of grey, r^2 = 100%, black.

Table 4 Demographic data of 173 inflammatory bowel diseases patients included in the association analysis

Characteristics	Crohn's disease	Ulcerative colitis	All
Patients	84 (49%)	89 (51%)	173 (100%)
Age (documented for 171 individuals)	37.5 (± 15.3)	41.7 (± 14.2)	39.7 (± 14.9)
mean ± SD	35	42	39
Median	16	18	16
Minimum	72	82	82
Maximum			
Known GC treatment outcome in the past	50	50	100
No. of patients currently treated with GCs	83	60	143
Male/female	52 (58.4%)/ 37 (41.6%)	40 (47.6%)/ 44 (52.4%)	92 (53.2%)/ 81 (46.8%)
Wild-type carriers	15 (16.9%)	20 (23.8%)	35 (20.2%)
Carriers of one variant haplotype	48 (53.9%)	39 (46.4%)	87 (50.3%)
Carriers of two variant haplotypes	26 (29.2%)	25 (29.8%)	51 (29.5%)

GCs: Glucocorticoids.

Analysis of NR3C1 haplotypes in relation to steroid therapy outcome

An overview of the demographic data of the 173 subjects included in the association analysis is shown in Table 4 (further patient data on comedications and extraintestinal manifestations are given in Tables 5 and 6), and the haplotype combinations calculated for all patients are shown in Table 7. As the numbers of homozygous carriers of variant *NR3C1* haplotypes were low, the subjects were analyzed as carriers of one or two copies of a distinct variant haplotype, irrespective of whether the other allele was determined to be wild-type or variant in the case of heterozygotes (Table 8). Furthermore, haplotypes *GR_2* and *GR_3* were analyzed by testing the heterozygous allele combinations *GR_2* + *GR_1* (wt) together with the

Table 5 Past and current additional medication of 173 inflammatory bowel diseases patients included in the association analysis

Additional medication	*n*
5-Aminosalicylic acid	142
6-Mercaptopurine	33
Adalimumab	3
Antibiotics	64
Azathioprine	122
Bisphosphonates	8
Certulizumab	1
Cholestyramine	8
Cyclosporine	8
Infliximab	45
Methotrexate	27
Sulfasalazine	12
Ursodeoxycholic acid	3

Table 6 Extraintestinal manifestations

Extraintestinal manifestations	*n* (%)[1]
Peripheral arthritis	46 (27.2)
Uveitis/iritis	6 (3.6)
Pyoderma gangrenosum	4 (2.4)
Erythema nodosum	9 (5.3)
Aphthous oral ulcers	10 (5.9)
Ankylosing spondylitis	7 (4.1)
Primary sclerosing cholangitis	6 (3.6)

[1]Documented for 169 patients.

homozygous *GR_2* subjects and the allele combination *GR_3* + *GR_1* (wt) together with homozygous *GR_3* carriers against wild-type carriers. For all individuals, prior success of GC therapy was documented, and for patients under GC therapy at the point of study entry the applied dosage was also noted.

No significant associations were observed between haplotype *GR_2* and success of GC therapy (Figure 4). Upon stratification of the patient cohort according to gender or disease subgroup (UC or CD), no significant association between therapy success or haplotype *GR_2* was observed either. Similarly, when stratifying according to the subgroup of heterozygous *GR_2* + *GR_1* and homozygous *GR_2*, no statistically significant difference in therapy response compared to wild-type carriers could be observed.

No significant associations were observed between either haplotype *GR_3* (Figure 5) or *GR_4* (Table 8) and GC therapy outcome, or between individual SNPs and therapy success (Figure 6). Similarly, we observed no significant associations between the severity of disease (active or inactive state of UC or CD) or currently taken GC dose levels and *NR3C1* haplotypes (data not shown).

DISCUSSION

Glucocorticoid receptor (GR) plays an important role in many physiological and pathological processes and is the main target of glucocorticoids, widely used as therapeutic

Table 7 Predicted frequencies of haplotype combinations in 173 inflammatory bowel diseases patients

Haplotype combination	*n*	Frequency
GR_1 + *GR_2* or *GR_2* hom	51	0.295
GR_1 + *GR_3* or *GR_3* hom	41	0.237
GR_1 hom (wt)	36	0.208
GR_2 + *GR_3*	23	0.133
GR_1 + *GR_4* or *GR_4* hom	6	0.035
GR_1 + *GR_5*	3	0.017
GR_3 + *GR_6*	2	0.012
GR_1 + *GR_7* or *GR_7* hom	1	0.006
GR_1 + *GR_16* or *GR_16* hom	1	0.006
GR_2 + *GR_8*	1	0.006
GR_2 + *GR_6*	1	0.006
GR_2 + *GR_9*	1	0.006
GR_6 + *GR_13*	1	0.006
GR_7 + *GR_15*	1	0.006
GR_2 + *GR_4*	1	0.006
GR_2 + *GR_11*	1	0.006
GR_5 + *GR_9*	1	0.006
GR_9 + *GR_17*	1	0.006

Table 8 Association between glucocorticoids therapy outcome and the haplotype *GR_4*

Haplotype	Cohort composition	Therapy success rate in wt carriers (success/no success)	Therapy success rate in het/hom variant carriers (success/no success)	*P*-value	OR (CI)
GR_4 merged	All	0.682 (15/7)	0.4 (2/3)	0.326	3.214 (0.434-23.787)
	Male	0.733 (11/4)	NA (0/0)	NA	NA
	Female	0.571 (4/3)	0.4 (2/3)	1.000	2.000 (0.194-20.614)

NA: Not applicable (at least one cell box was counted as 0, OR and *P* not calculatable).

agents to treat a variety of autoimmune diseases[8,17]. Two GR isoforms, GRα and GRβ, generated by alternative mRNA splicing exist[18]. Only GRα can be activated by glucocorticoid ligands, while GRβ does not bind glucocorticoids and may in fact act as an inhibitor of glucocorticoid action[19]. Genetic variation in the *NR3C1* gene has been shown to affect both disease pathophysiology and response to glucocorticoid therapy[15,20-22], suggesting that SNPs might play a role in GR function and associated steroid therapy outcome also in IBD patients. GR is known to regulate the intestinal bile acid uptake transporter ASBT[23,24], the expression of which is altered in IBD patients[25]. While it has been reported that GR mRNA expression levels are not predictors of steroid response in IBD[26] and that the GR polymorphisms R23K and N363S are not associated with CD in a pediatric Caucasian population[27], no studies on the role of *NR3C1* gene variants in steroid therapy success were previously available. The aim of the current study was to analyze sequence variation and

Haplotype	Cohort composition	Therapy success rate in wt carriers (No. of success/no success)	Therapy success rate in het/hom variant carriers (No. of success/no success)	*P*-value	OR (CI)
GR_2 merged	All	0.682 (15/7)	0.837 (36/7)	0.204	0.417 (0.124-1.395)
	Male	0.733 (11/4)	0.905 (19/2)	0.210	0.289 (0.045-1.846)
	Female	0.571 (4/3)	0.773 (17/5)	0.357	0.392 (0.065-2.369)
	CD	0.615 (8/5)	0.900 (18/2)	0.084	0.178 (0.028-1.118)
	UC	0.778 (7/2)	0.783 (18/5)	1.000	0.972 (0.152-6.231)
GR_2 only	All	0.682 (15/7)	0.862 (25/4)	0.173	0.343 (0.086-1.370)
	Male	0.733 (11/4)	0.875 (14/2)	0.394	0.393 (0.060-2.554)
	Female	0.571 (4/3)	0.846 (11/2)	0.290	0.242 (0.029-2.027)

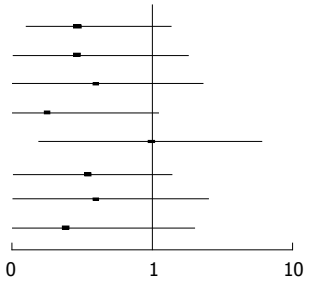

Figure 4 Haplotype *GR_2* and steroid therapy outcome. Odds ratios and confidence intervals for the number of *GR_2* carriers *vs* wild-type carriers in the responder group compared with non-responders to glucocorticoid therapy. No significant associations were found. Statistical analysis was performed with Fisher's exact test. OR: Odds ratio; CI: Confidence interval; CD: Crohn's disease; UC: Ulcerative colitis.

Haplotype	Cohort composition	Therapy success rate in wt carriers (No. of success/no success)	Therapy success rate in het/hom variant carriers (No. of success/no success)	*P*-value	OR (CI)
GR_2 merged	All	0.682 (15/7)	0.318 (28/5)	0.188	0.383 (0.103-1.415)
	Male	0.733 (11/4)	0.913 (4/1)	0.342	0.250 (0.024-2.609)
	Female	0.571 (4/3)	0.810 (17/4)	0.318	0.314 (0.049-1.998)
	CD	0.615 (8/5)	0.917 (11/1)	0.160	0.145 (0.014-1.489)
	UC	0.778 (7/2)	0.810 (17/4)	1.000	0.824 (0.122-5.573)
GR_2 only	All	0.682 (15/7)	0.842 (16/3)	0.292	0.402 (0.087-1.846)
	Male	0.733 (11/4)	1.000 (6/0)	0.281	NA
	Female	0.571 (4/3)	0.769 (10/3)	0.613	0.400 (0.055-2.886)

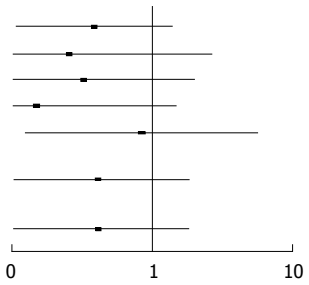

Figure 5 Haplotype *GR_3* and steroid therapy outcome. Odds ratios and confidence intervals for the number of *GR_3* carriers *vs* wild-type carriers in the responder group of responders compared with non-responders to glucocorticoid therapy. No significant associations were found. Statistical analysis was performed using Fisher's exact test. CD: Crohn's disease; UC: Ulcerative colitis; NA: Not applicable.

Mutation	Therapy success rate in wt carriers (success/no success)	Therapy success rate in het/hom mutated carriers (success/no success)	*P*-value	OR (CI)
E22E/R23K	0.774 (72/21)	0.571 (4/3)	0.354	2.571 (0.533-12.408)
N363S	0.777 (73/21)	0.500 (3/3)	0.148	3.476 (0.653-18.508)
rs4986593	0.746 (47/16)	0.784 (29/8)	0.670	0.810 (0.308-2.131)
rs6188	0.714 (35/14)	0.804 (41/10)	0.294	0.610 (0.241-1.543)
rs258750	0.694 (34/15)	0.824 (42/9)	0.129	0.486 (0.189-1.246)
rs10482704	0.760 (73/23)	0.750 (3/1)	1.000	1.058 (0.105-10.671)

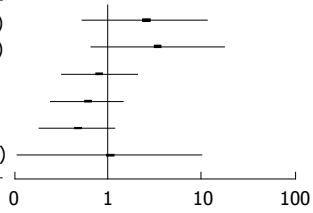

Figure 6 *NR3C1* variants and their influence on steroid therapy outcome. Odds ratios and confidence intervals for carriers of six single nucleotide polymorphisms against wild-type carriers in the group of glucocorticoid (GC) responders compared with GC non-responders. No significant associations were found. Statistical analysis was performed using chi-square or Fisher's exact test.

haplotype structures in the coding parts of the *NR3C1* gene in a cohort of 181 Swiss IBD patients. We investigated whether *NR3C1* genetic variants or haplotypes may influence steroid therapy outcome in IBD patients.

We identified 13 variants in this study, of which 12 had already been previously submitted to the NCBI SNP database. We calculated the corresponding haplotypes in the IBD patient cohort and studied the association of the most prevalent SNP combinations with steroid therapy outcome, disease activity, and age of disease onset. Several *NR3C1* SNPs have been previously associated with altered disease susceptibility or risk of disease progression in other autoimmune diseases, such as Guillain-Barré Syndrome or multiple sclerosis[15,20]. Most of these studies only analyzed the impact of a small number of pre-defined SNPs, such as the BclI polymorphism or the E22E/R23K polymorphisms[15,20,21]. Few reports have been published on the potential influence of *NR3C1* SNPs on sensitivity to endogenous or exogenously given GCs[17,28], and only one significant association between the polymorphism E22E/R23K and sensitivity to exogenously administered GCs in elderly Dutch people has been reported[22]. So far no large cohort studies have been reported in which the influence of *NR3C1* SNPs on GC therapy outcome in IBD patients

has been investigated. Here, we describe five *NR3C1* haplotypes occurring at a frequency > 1% and analyze the potential association of the three most common haplotypes *GR_2*, *GR_3* and *GR_4* with GC therapy outcome in IBD patients. While a large number of *NR3C1* variants are already registered in the NCBI SNP database, we observed only eight variants that occurred at a frequency > 1%, and these were responsible for the composition of a relatively small group of commonly occuring haplotypes. The overall risk for a certain UC and/or CD activity state or for a different steroid therapy outcome was not altered in *GR_2*, *GR_3* or *GR_4* carriers, in comparison with the wild-type carriers. Furthermore, no significant associations were observed between individual SNPs and GC therapy success. In the case of certain SNPs/haplotypes (e.g. *GR_4*, E22E/R23K), a larger cohort would have been preferable in order to obtain more reliable results, as these variants occurred quite rarely in our patient group. Similarly to our observations, Dekker *et al*[20] could not detect any associations between distinct haplotypes and SNPs in a Guillain-Barré Syndrome cohort treated with methylprednisolone, although the authors noted that their study group was too small to obtain statistically reliable results. It remains to be seen whether the rare GR variants present in our study cohort will show significant associations in larger cohorts of IBD patients.

In conclusion, we have performed a comprehensive study analyzing the role of genetic variants in the *NR3C1* gene in glucocorticoid sensitivity in a Swiss cohort of IBD patients. We show that *NR3C1* haplotypes are not a general modulating factor in glucocorticoid therapy outcome.

ACKNOWLEDGMENTS

We thank Christian Hiller for excellent technical assistance, members of our team for fruitful discussions, and the Swiss IBD Cohort Study Group: Michetti P, Vader JP, Burnand B, Pittet V, Ballabeni P, Mueller C, Rihs S, Braegger C, Mottet C, Juillerat P, Felley C, Beglinger C, Froehlich F, Vavricka S, Seibold F, Schoepfer A, von Kaenel R, Begré S, de Saussure P, Meyenberger C, Sagmeister M, Rogler G, Kullak-Ublick GA, Meier R, Straumann A, Criblez D, Engelmann M and Schenk M.

COMMENTS

Background

Crohn's disease and ulcerative colitis are two distinct types of inflammatory bowel disease (IBD), which is an increasingly prevalent disease condition worldwide. Wide variation is observed in clinical manifestation and therapy responses in IBD, partly due to individual genetic variation.

Research frontiers

Glucocorticoid therapy is commonly used in treatment of IBD, however the response to therapy varies between individuals. The authors hypothesized that genetic variation in the *NR3C1* gene encoding the glucocorticoid receptor (GR) may affect the response to glucocorticoids in IBD patients.

Innovations and breakthroughs

In this comprehensive genetic analysis, all coding exons and exon-intron junctions of the *NR3C1* gene were sequenced in 181 IBD patients, who had been treated with glucocorticoids and whose past responses to this treatment had been recorded. This is the first published study on the effects of genetic variation in GR on glucocorticoid therapy in IBD patients, in a modestly sized study cohort.

Applications

If significant associations between genetic GR variants and glucocorticoid therapy outcome had been observed, this could have allowed more considered design of the individual therapy options upon prior genotyping of the patients.

Terminology

The transcription factor of the steroid receptor family, GR, is proposed to be a major mediator of anti-inflammatory pathways elicited by therapeutically administered glucocorticoids.

Peer review

The genetic study investigates the predictive value of *NR3C1* gene variants towards the clinical outcome of patients with Crohn's disease and ulcerative colitis. Although the result of this study was negative, the study was meaningful in that abundant GR variants were determined and analyzed in IBD patients.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3897

World J Gastroenterol 2010 August 21; 16(31): 3897-3904
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Expression of protein S100A4 is a predictor of recurrence in colorectal cancer

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Received: February 17, 2010 Revised: March 25, 2010
Accepted: April 1, 2010
Published online: August 21, 2010

Abstract

AIM: To investigate the prognostic significance of S100A4 expression in colorectal cancer and its correlation with expression of E-cadherin and p53.

METHODS: A cohort of archival formalin-fixed paraffin-embedded specimens was selected from 127 patients with colorectal cancer who underwent surgical resection between April 2000 and March 2004 at the Department of Surgery, Korea University Guro Hospital. The expression of protein S100A4 was evaluated according to the proportion of positively stained cancer cells. In each case, three core biopsies with a diameter of 2 mm were punched out and positioned in a recipient paraffin array block. Four-μm sections of these tissue array blocks were used for immunohistochemical analysis of protein S100A4, E-cadherin, and p53. Clinicopathological data were based on the original histopathologic reports and clinical records of patients.

RESULTS: In normal colorectal mucosa, protein S100A4 immunoreactivity was clearly absent in both cytoplasm and nucleus. However, positive immunoreactivity of protein S100A4 was detected in 45 (35.4%) of the tumor cases. There was no significant association between positive immunoreactivity of protein S100A4 and clinicopathological parameters such as tumor differentiation or TNM stage, and also no correlation between the reactivity and E-cadherin or p53 expression. However, positive immunoreactivity of protein S100A4 was found to be associated with tumor recurrence (P = 0.004), and was also associated with significantly worse overall survival in the Kaplan-Meyer survival analysis (P = 0.044). After adjustment for tumor differentiation, tumor depth and nodal status, however, it failed to achieve statistical significance (P = 0.067).

CONCLUSION: The expression of protein S100A4 is associated with tumor recurrence and poor overall survival in patients with colorectal cancer.

Key words: S100A4; E-cadherin; p53; Prognostic factor; Colorectal cancer

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Kwak JM, Lee HJ, Kim SH, Kim HK, Mok YJ, Park YT, Choi JS, Moon HY. Expression of protein S100A4 is a predictor of recurrence in colorectal cancer. *World J Gastroenterol* 2010; 16(31): 3897-3904 Available from: URL: http://www.wjgnet.

com/1007-9327/full/v16/i31/3897.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3897

INTRODUCTION

Although the last decade has brought significant improvements in the disease-free and overall survival of colorectal cancer (CRC) patients; achieved largely by more accurate staging of disease, an improved and expanded role of surgery and increased number of available chemotherapeutic options, approximately 20% of advanced CRC patients still die of recurrence of the disease[1]. Invasion and metastasis, which are the most life-threatening properties of malignant tumors, result from the interaction between tumor cells and the surrounding tissues. The invasion and metastasis processes themselves consist of pathogenic sequential steps, such as proliferation and detachment of neoplastic cells, invasion to extracellular matrix, angiogenesis, vascular dissemination, lodging in a distant vascular bed, extravasation into the target organ, and proliferation. The activation of many genes and the expression of their products have been involved in this progression[2]. Current conventional staging has a significant impact on survival of CRC patients. However, there is marked variability in outcome that exists within each stage, and certain populations of patients with early recurrence, resistance to chemotherapy and decreased survival cannot be predicted using conventional histopathologic staging. Thus, the identification of molecular factors that have prognostic significance in CRC is essential to improve treatment and outcome[3-5].

Over the past few years, the S100 family of proteins has emerged as an important group with the capacity to promote invasiveness and metastasis of many human neoplasms. In particular, recent studies have established the mechanisms of action of protein S100A4, and indicate its possible prognostic role in human neoplasia[6-8]. However, studies regarding protein S100A4 have mainly been limited to research laboratories. Moreover, the mechanism of action of protein S100A4 in tumors is not fully understood. Therefore, it would be of great interest to find out whether the detection of protein S100A4 has any predictive value, and also whether it may help select patients who require more extensive diagnostic evaluation to rule out metastatic disease and/or more aggressive treatments.

The aims of this study were to investigate immunohistochemically the prognostic significance of protein S100A4 expression in CRC, compared with clinicopathologic parameters and overall survival, and to investigate the correlation between protein S100A4 expression and E-cadherin and p53, which have been suggested as possible targets of protein S100A4.

MATERIALS AND METHODS

Patients

Formalin-fixed paraffin-embedded specimens were selected from 127 patients with CRC who underwent curative or palliative surgical resection between April 2000 and March 2004 at the Department of Surgery, Korea University Guro Hospital. The 127 patients included 76 males (59.8%) and 51 females (40.2%) with a mean age of 59.3 years (range, 28-88). Clinicopathologic data were based on the histopathologic reports and the clinical records of the patients. Using the American Joint Committee on Cancer (AJCC) TNM system[9], tumors were classified as Stage I in 24 specimens (18.9%), Stage Ⅱ in 49 (38.6%), Stage Ⅲ in 49 (38.6%) and Stage Ⅳ in 5 (3.9%). The Korea University Medical Center Institutional Review Board granted permission for the study.

Preparation of tissue microarray

Paraffin blocks of formalin-fixed surgical specimens were obtained from the Department of Pathology, Korea University Guro Hospital. Pathological evaluation of all blocks was performed by two pathologists who did not know any information about the patients. In each case, three core biopsies were obtained from representative areas of the corresponding paraffin blocks with a precision instrument. These tissue cores from each specimen with a diameter of 2 mm were punched out and positioned in a recipient paraffin array block. Each case also included three internal controls consisting of non-neoplastic colorectal mucosa. Four-μm sections of these tissue array blocks were then cut and used for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical staining for protein S100A4, E-cadherin, and p53 was performed using a standard avidin-biotin complex (ABC) method. In brief, all sections were deparaffinized by using a series of xylene baths and then hydrated using a graded alcohol series. They were then placed in citric acid buffer (10 mmol/L) and heated in a microwave oven (700 W) for 12 min to retrieve the antigenicity. The sections were then immersed in methanol, containing 0.3% hydrogen peroxide, for 20 min to block endogenous peroxidase activity. The sections were then washed three times in phosphate-buffered saline (PBS) and incubated in 2.5% normal goat serum for 20 min to reduce nonspecific antibody binding. After washing with PBS, the sections were incubated with primary antibodies for 30 min at room temperature. Rabbit polyclonal antibodies against protein S100A4 (Ab-8, Neomarker, 1:100), monoclonal mouse anti-human E-cadherin (NCH-38, Dako, 1:100), and monoclonal mouse anti-human p53 (DO-7, Dako, 1:100) were used. The reaction products were visualized with diaminobenzidine as a chromogen, and counterstained with commercial hematoxylin.

Evaluation of immunohistochemical staining

Evaluation of immunohistochemical staining was performed by two independent pathologists. Any discrepancies in scoring were resolved by simultaneous reassess-

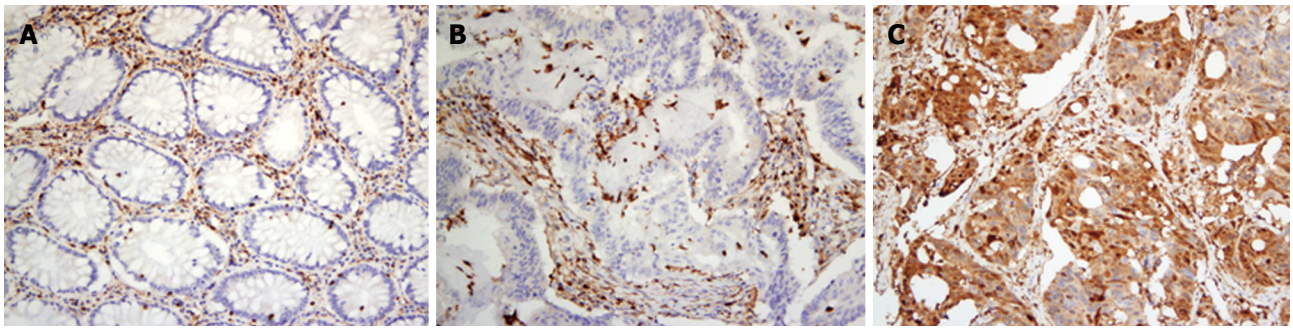

Figure 1 Immunohistochemical expression of protein S100A4. A: Protein S100A4 expression in normal colorectal epithelium. In all normal colonic epithelium, protein S100A4 immunoreactivity was clearly absent at both cytoplasm and nucleus; B: Negative expression of protein S100A4 in colorectal cancer (CRC); C: Positive expression of protein S100A4 in CRC. Cytoplasm of cancer cells was diffusely stained brown (all at × 200 magnification).

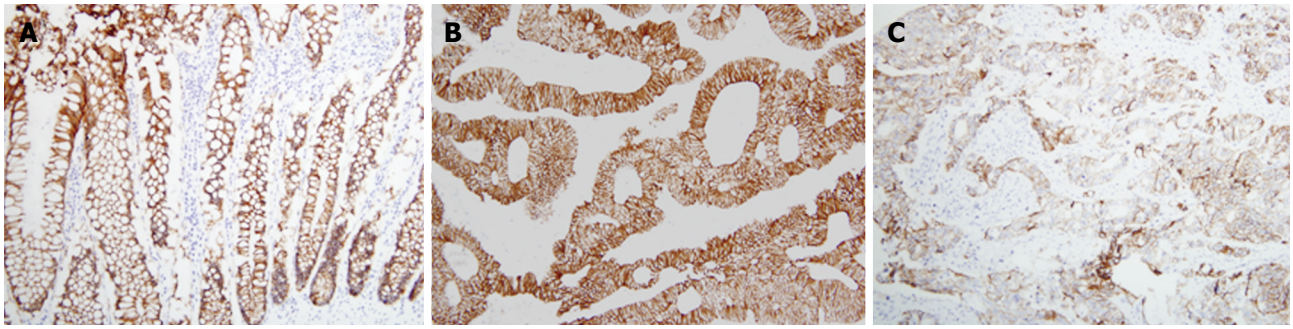

Figure 2 Immunohistochemical expression of E-cadherin. A: E-cadherin expression in normal colorectal epithelium. Normal epithelial cells strongly and homogeneously expressed E-cadherin at intercellular boundaries; B: Preserved expression of E-cadherin in colorectal cancer (CRC); C: Reduced expression of E-cadherin in CRC. Staining of cancer cell at intercellular border was weak and heterogeneous (all at × 200 magnification).

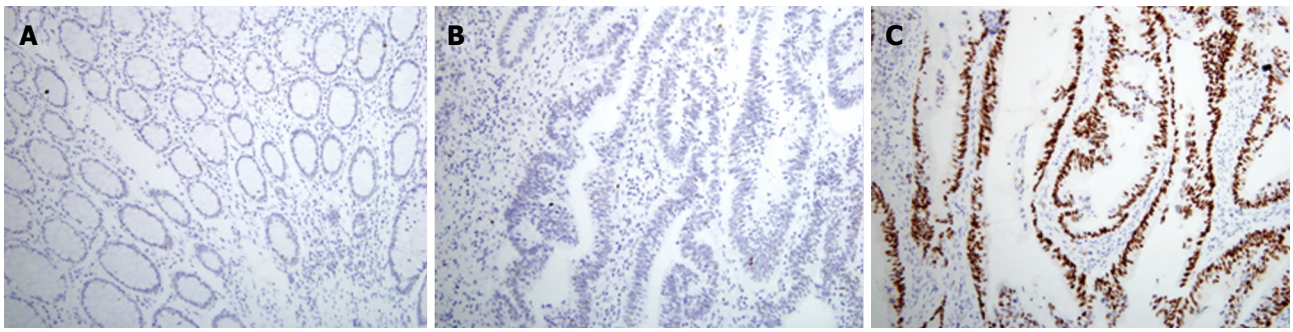

Figure 3 Immunohistochemical expression of p53. A: p53 expression in normal colorectal epithelium. In all normal colonic epithelium, p53 immunoreactivity was clearly absent at nucleus; B: Negative expression of p53 in colorectal cancer (CRC); C: Positive expression of p53 in CRC. More than 10% of cancer cells were stained strongly at their nuclei (all at × 200 magnification).

ment by both pathologists. The tumor cells whose cytoplasm was stained brown were classified as positive. The protein S100A4 expression of tumor cells was evaluated according to the proportion of positively stained tumor cells. When more than 10% of tumor cells were positively stained, the tumor was considered as "positive expression". On the other hand, the tumor was considered as "negative expression" when less than 10% of tumor cells were positively stained (Figure 1). In the case of E-cadherin, when more than 90% of tumor cells were positively stained, the tumor was considered as "preserved expression". On the other hand, the tumor was considered as "reduced expression" when less than 90% of tumor cells were positively stained (Figure 2). p53 expression was evaluated according to the proportion of tumor cells whose nuclei were positively stained. When more than 10% of tumor cells were positively stained, the tumor was considered as "positive expression". On the other hand, the tumor was considered as "negative expression" when less than 10% of tumor cells were positively stained (Figure 3).

Statistical analysis

Statistical analysis was performed using the SPSS for Windows software package (SPSS, Inc., Chicago, IL, Version 12.0). Correlation between the expression

Table 1 Relationship between expression of protein S100A4, E-cadherin, p53 and clinicopathologic parameters *n* (%)

Parameters	Expression of protein S100A4			Expression of E-cadherin			Expression of p53		
	Negative (*n* = 82)	Positive (*n* = 45)	*P* value	Reduced (*n* = 48)	Preserved (*n* = 79)	*P* value	Negative (*n* = 56)	Positive (*n* = 71)	*P* value
Gender			0.465			0.787			0.364
Male	51 (62.2)	25 (55.6)		28 (58.3)	48 (60.8)		36 (64.3)	40 (56.3)	
Female	31 (37.8)	20 (44.4)		20 (41.7)	31 (39.2)		20 (35.7)	31 (43.7)	
Age (yr)			0.797			0.963			0.344
mean ± SD	58.4 ± 11.1	60.9 ± 11.2		59.3 ± 13.1	59.4 ± 10.0		58.3 ± 9.6	60.2 ± 12.3	
Tumor location			0.352			0.120			0.528
Colon	38 (46.3)	17 (37.8)		25 (52.1)	30 (38.0)		26 (46.4)	29 (40.8)	
Rectum	44 (53.7)	28 (62.2)		23 (47.9)	49 (62.0)		30 (53.6)	42 (59.2)	
Differentiation			0.500[1]			0.001[1]			0.695
Differentiated	75 (91.5)	42 (93.3)		39 (81.3)	78 (98.7)		51 (91.1)	66 (93.0)	
Undifferentiated	7 (8.5)	3 (6.7)		9 (18.8)	1 (1.3)		5 (8.9)	5 (7.0)	
Depth of tumor			0.319			0.615			0.154
T1-2	25 (30.5)	10 (22.2)		12 (25.0)	23 (29.1)		19 (33.9)	16 (22.5)	
T3-4	57 (69.5)	35 (77.8)		36 (75.0)	56 (70.9)		37 (66.1)	55 (77.5)	
Lymph node metastasis			0.282			0.338			0.667
Absent	50 (61.0)	23 (51.1)		25 (52.1)	48 (60.8)		31 (55.4)	42 (59.2)	
Present	32 (39.0)	22 (48.9)		23 (47.9)	31 (39.2)		25 (44.6)	29 (40.8)	
Distant metastasis			0.053[1]			0.365[1]			0.654[1]
Absent	81 (98.8)	41 (91.1)		45 (93.8)	77 (97.5)		53 (94.6)	69 (97.2)	
Present	1 (1.2)	4 (8.9)		3 (6.3)	2 (2.5)		3 (5.4)	2 (2.8)	
pTNM stage			0.175			0.403			0.857
Ⅰ	17 (20.7)	7 (15.6)		6 (12.5)	18 (22.8)		11 (19.6)	13 (18.3)	
Ⅱ	33 (40.2)	16 (35.6)		19 (39.6)	30 (38.0)		20 (35.7)	29 (40.8)	
Ⅲ	31 (37.8)	18 (40.0)		20 (41.7)	29 (36.7)		22 (39.3)	27 (38.0)	
Ⅳ	1 (1.2)	4 (8.9)		3 (6.3)	2 (2.5)		3 (5.4)	2 (2.8)	
Recurrence[2]			0.004			0.269			0.845
Absent	67 (82.7)	24 (58.5)		31 (68.9)	60 (77.9)		40 (75.5)	51 (73.9)	
Present	14 (17.3)	17 (41.5)		14 (31.1)	17 (22.1)		13 (24.5)	18 (26.1)	

[1]Calculated by Fisher's exact test; [2]Stage Ⅳ patients were excluded.

of protein S100A4, E-cadherin, and p53 and various clinicopathologic parameters was evaluated using the chi-squared test or Fisher's exact test. Overall survival analysis was done by the Kaplan-Meier method. The difference between the survival curves was analyzed by the log-rank test. Significant variables identified on univariate analysis were subjected to multivariate analysis using the Cox regression model. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Expression of protein S100A4, E-cadherin and p53 in CRC

A wide range of cell types in normal colorectal tissues was stained with polyclonal antibody against protein S100A4. There was a high level of staining of smooth muscle, of the smooth muscle in the walls of vessels, and of infiltrating lymphocytes and macrophages in the stroma. However, in normal colorectal mucosa of all 127 cases, immunoreactivity of protein S100A4 was clearly absent in both cytoplasm and nucleus. Positive immunoreactivity of protein S100A4 was detected in 45 (35.4%) of the tumor specimens. E-cadherin was expressed in cell membranes of all normal colorectal mucosa, and reduced expression of E-cadherin was observed in 48 (37.8%) of the tumor specimens. All normal colorectal mucosa showed negative expression for p53; however, 71 (55.9%) tumors were stained for p53 in their nuclei.

Correlation of protein S100A4, E-cadherin, and p53 expression with clinicopathological parameters

Positive reactivity for protein S100A4 was found to be associated with tumor recurrence ($P = 0.004$). However, there was no significant association between the expression of protein S100A4 and other investigated clinicopathological parameters, including tumor location, differentiation or TNM stage. Reduced expression of E-cadherin was significantly correlated with tumor differentiation ($P = 0.001$). As for p53, there was no significant correlation between expression of p53 and clinicopathological parameters (Table 1).

Correlation between protein S100A4 and E-cadherin/p53 expression

There was no significant correlation in co-expression pattern between protein S100A4 and E-cadherin (Kendall's Tau-b correlation coefficient = 0.068, $P = 0.436$, Table 2). Also, there was no significant correlation between protein S100A4 expression and p53 expression (Kendall's Tau-b correlation coefficient = -0.105, $P = 0.239$, Table 3).

Table 2 Correlations of protein S100A4 and E-cadherin

S100A4	E-cadherin	*n* (%)
Co-expression pattern		
Negative	Negative	55 (39.9)
Negative	Positive	34 (24.6)
Positive	Negative	32 (23.2)
Positive	Positive	17 (12.3)

Kendall's Tau-b correlation coefficient = -0.035, $P = 0.681$.

Table 3 Correlations of protein S100A4 and p53

S100A4	p53	*n* (%)
Co-expression pattern		
Negative	Negative	35 (25.4)
Negative	Positive	54 (39.1)
Positive	Negative	26 (18.8)
Positive	Positive	23 (16.7)

Kendall's Tau-b correlation coefficient = -0.132, $P = 0.120$.

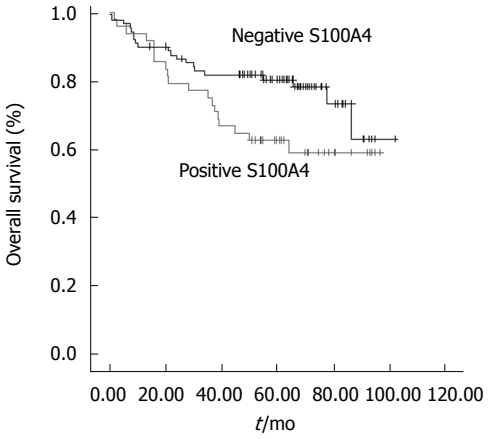

Figure 4 Kaplan-Meier survival curves demonstrating statistically significant differences according to the expression of protein S100A4 (log-rank test, *P* = 0.044). Censored observations are shown as tick marks.

Survival analysis

The median follow-up period for all patients was 58.7 mo (range, 1.1-101.8). The 5-year overall survival rate for the 127 patients was 79.7%. Kaplan-Meier survival analysis showed that tumor differentiation (5-year survival rate 82.3% *vs* 50.0%, $P = 0.001$), depth of tumor (97.1% *vs* 72.9%, $P = 0.001$), lymph node metastasis (88.5% *vs* 68.0%, $P = 0.001$) and positive immunoreactivity of protein S100A4 (86.1% *vs* 68.3%, $P = 0.044$) were associated with poor overall survival (Table 4 and Figure 4).

In a multivariate analysis, however, the positive immunoreactivity of protein S100A4 failed to have association with worse overall survival after adjustment for tumor differentiation, tumor depth and nodal status, which were significant parameters in a univariate analysis (hazard ratio, 1.985; 95% confidence interval: 0.953-4.134; $P = 0.067$, Table 5).

Table 4 Univariate overall survival analysis for seven clinicopathologic parameters

Parameters	*n*	5-yr overall survival rate (%)	*P* value
Gender			0.817
Male	76	80.9	
Female	51	78.0	
Differentiation			0.001
Differentiated	117	82.3	
Undifferentiated	10	50.0	
Depth of tumor			0.001
T1-2	35	97.1	
T3-4	92	72.9	
Lymph node metastasis			0.001
Absent	73	88.5	
Present	54	68.0	
S100A4 expression			0.044
Negative	82	86.1	
Positive	45	68.3	
E-cadherin			0.105
Preserved	79	83.1	
Reduced	48	74.3	
p53			0.218
Negative	56	83.6	
Positive	71	76.6	

Table 5 Cox regression analysis on those parameters shown to significantly influence overall survival in a univariate analysis

Parameters	Hazard ratio	95% CI	*P* value
Lymph node metastasis	2.283	1.014-5.141	0.046
Depth of tumor	10.374	1.391-77.352	0.022
Differentiation	2.748	1.059-7.133	0.038
S100A4 expression	1.985	0.953-4.134	0.067

DISCUSSION

Calcium binding proteins form a large family involved in numerous functions ranging from the control of cell-cycle progression and cell differentiation to enzyme activation and regulation of muscle contraction[10,11]. The S100 proteins represent one of the largest subfamilies of the calcium binding proteins with at least 19 different members; the degree of homology ranges from 25% to 65%. They were initially characterized as low-molecular weight acidic proteins and named by their solubility in 100% ammonium sulfate ("S100"). S100A4, also known as p9Ka, CAPL, or calvasculin, is a member of the S100 family consisting of 101 amino acids and with a molecular weight of about 11.6 kDa. The corresponding gene, cloned by different groups, is known as *mts1* (metastasin), *pEL98, 18A2, 42A*, and *fsp* (fibroblast-specific protein)[10-13].

The biologic functions of several S100 proteins in carcinogenesis have not been fully elucidated to date. Recently, however, much interest has focused on S100A4 and some other S100 family members, such as S100A2, S100A6, and S100B, for their potential roles in invasive growth and metastasis of neoplastic diseases. S100A4 or its corresponding mRNA are found at higher levels in

metastatic relative to non-metastatic rat[14] and mouse[15] tumor cell lines. Transfection experiments further showed that rodent or human S100A4 can induce a metastatic phenotype in previously non-metastatic rat mammary cells[16,17]. Conversely, antisense S100A4 RNA or anti-S100A4 ribozyme suppressed the metastatic potential of highly metastatic cell lines[18,19]. Moreover, in pilot studies of human colorectal adenocarcinoma specimens, elevated levels of immunohistochemically detected S100A4 are associated with the more malignant carcinomatous regions of the primary tumors and with liver metastases[20]. The tight association between S100A4 expression and metastasis observed in these laboratory analyses has led to a number of studies examining the utility of S100A4 expression as a prognostic marker in human cancers. Protein S100A4 has been shown to be a prognostic marker in a number of human cancers, including breast cancer[21], esophageal-squamous cancer[22], non-small cell lung cancer[23], gastric cancer[24], malignant melanoma[25], prostate cancer[26], and pancreatic cancer[27]. The universality of S100A4 expression in a variety of cancers illustrates the potential use of S100A4 as a marker for tumor metastasis and disease progression.

The purpose of this investigation was to establish clinical significance of the calcium-binding protein, S100A4, in CRC. It was found that 35.4% of CRC specimens were stained strongly by the polyclonal antibodies against protein S100A4, in concordance with earlier reports[28,29]. The staining in specimens is not restricted to only carcinoma cells, because highly expressed levels are also detected in normal tissues, in particular, smooth muscle cells, endothelial cells of both arteries and veins, and some reactive fibroblast-like cells and lymphocytes[30]. However, the present study was undertaken on only carcinoma cells.

In this study, positive expression of protein S100A4 was associated with tumor recurrence, in accordance with previous study[28]. This result suggests that the protein S100A4 may play a role in predicting a patient subgroup which would show more unfavorable outcome, thus leading us to substage-oriented tailored therapy with more intensive treatment and more strict follow-up surveillance.

This study showed that the overall survival of CRC patients who had immunohistochemically detectable levels of protein S100A4 was significantly worse than those CRC patients with negative expression of protein S100A4 according to univariate analysis. Because S100A4 was first discovered as a metastasis-inducing protein in experimental models[14-19], and metastasis is the major event responsible for death in patients of CRC, it is quite possible that protein S100A4 causes earlier deaths by its ability to induce metastasis in human CRC. Although it failed to achieve statistical significance in multivariate analysis, the present result suggests a need for further and larger studies to investigate the role of protein S100A4 expression in CRC.

As a typical member of the S100 family, S100A4 exerts dual functions, both intracellular and extracellular. Intracellularly, it interacts with and functionally modifies the tumor suppressor protein p53, non-muscle myosin II, and liprin β1[12,13]. S100A4 interacts with the C terminus of p53 and inhibits protein kinase C (PKC) phosphorylation of the tumor suppressor *in vitro*. Likewise, the interaction between p53 and S100A4 inhibits p53 from binding to its consensus DNA-binding sequence[31]; thus it was expected that S100A4 would be a general inhibitor of p53 function. It has been suggested that a complex of S100A4 with p53 and the sequestration of p53 may result in stimulation of the cells to enter the S phase by abrogating the control functions of p53 at the G1-S checkpoint[8,31,32]. However, as shown in this study, this possibility was difficult to prove by immunohistochemical analysis of these two proteins in CRC. An examination of p53-regulated genes in S100A4-expressing cells indicates that the expression of several genes are up-regulated (e.g. *bax*); other genes are down-regulated initially and then later up-regulated (e.g. *mdm2*), and some genes are inhibited (e.g. *p21*, *thrombospondin-1*)[31]. These opposite effects of S100A4 expression on p53-regulated genes could explain why there was no correlation between S100A4 and p53, notwithstanding the potential interaction of these two proteins.

Another possible mechanism of action of S100A4 in carcinogenesis is cytoskeletal dysregulation by down-regulation of E-cadherin induced by protein S100A4. E-cadherin is a member of the large cadherin superfamily. It is the predominant intercellular adhesion molecule expressed by intestinal epithelial cells, and functions to mediate epithelial cell-cell adhesion and maintain the integrity of the epithelium[33-35]. The expressions of E-cadherin and protein S100A4 in two mouse tumor cell lines were found to be inversely regulated, and transfection experiments showed a reciprocal down-regulation of both molecules, suggesting that the invasiveness of tumors expressing protein S100A4 may be at least partially induced by the abrogation of E-cadherin expression[36]. A similar mechanism has also been postulated in humans, on the basis of immunohistochemical analysis of both proteins in a series of non-small cell lung cancer[23] and gastric cancer[24]; an inverse correlation of E-cadherin and protein S100A4 expression was demonstrated. In this study, we attempted to immunohistochemically establish an inverse correlation between the expression of protein S100A4 and E-cadherin in CRC; however, data failed to prove the relationship (Kendall's Tau-b correlation coefficient = -0.035, P = 0.681). Nevertheless, it is quite possible that different antibodies against protein S100A4, different cancer tissue, and small numbers enrolled in this study might have contributed to this difference.

In conclusion, in the present retrospective study, positive immunoreactivity of protein S100A4 is closely associated with cancer recurrence. However, there is no correlation between the expression of protein S100A4 and E-cadherin or p53. The overall survival for patients with CRC expressing immunohistochemically detectable levels of protein S100A4 is significantly worse than for those patients with CRC considered negative for S100A4. Furthermore, protein S100A4 shows borderline tendency toward being a prognostic marker in multivariate regres-

sion analysis. Although these results suggest that protein S100A4 could be a useful biologic predictor of cancer recurrence and poor outcome, subsequent prospective, large-scale studies are required to confirm its importance and relevance in the invasive potential of human CRC.

COMMENTS

Background

Although current conventional staging has a significant impact on survival of colorectal cancer patients, there is marked variability in outcome within each stage. As the protein S100A4 has been known to promote invasiveness and metastasis of many human neoplasms, the question is raised as to whether this protein represents a useful prognostic marker in clinical practice.

Research frontiers

Studies regarding the protein S100A4 have mainly been limited to research laboratories and clinical data are extremely limited. Therefore, it would be of great interest to find out whether the expression of protein S100A4 has any predictive value and may help select patients who require more extensive diagnostic evaluation to rule out metastasis and/or more aggressive treatments.

Innovations and breakthroughs

The results of this study showed that positive immunoreactivity of protein S100A4 was associated with tumor recurrence and worse overall survival.

Applications

Since there is an association between protein S100A4 expression, tumor recurrence and poor overall survival, this can lead to substage-oriented tailored therapy with more intensive treatment and more strict follow-up surveillance in colorectal cancer patients.

Terminology

The protein S100A4 was first discovered as a metastasis-inducing protein in experimental models. It is a polypeptide of 101 amino acids with a molecular mass of 11.5 kDa. The evidence gathered throughout the past few years demonstrates that protein S100A4 is involved in the regulation of invasiveness and metastasis in many human cancers.

Peer review

This paper is very well written and has a strong message about expression of protein S100A4 in 127 cases of colorectal cancer, showing a statistically significant association with tumor recurrence and overall survival.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3905

World J Gastroenterol 2010 August 21; 16(31): 3905-3910
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Prospective randomized controlled trial evaluating cap-assisted colonoscopy *vs* standard colonoscopy

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Supported by Own Departmental Fund
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Received: December 19, 2009 Revised: February 22, 2010
Accepted: February 29, 2010
Published online: August 21, 2010

Abstract

AIM: To study the significance of cap-fitted colonoscopy in improving cecal intubation time and polyp detection rate.

METHODS: This study was a prospective randomized controlled trial conducted from March 2008 to February 2009 in a tertiary referral hospital at Sydney. The primary end point was cecal intubation time and the secondary endpoint was polyp detection rate. Consecutive cases of total colonoscopy over a 1-year period were recruited. Randomization into either standard colonoscopy (SC) or cap-assisted colonoscopy (CAC) was performed after consent was obtained. For cases randomized to CAC, one of the three sizes of cap was used: D-201-15004 (with a diameter of 15.3 mm), D-201-14304 (14.6 mm) and D-201-12704 (13.0 mm). All of these caps were produced by Olympus Medical Systems, Japan. Independent predictors for faster cecal time and better polyp detection rate were also determined from this study.

RESULTS: There were 200 cases in each group. There was no significant difference in terms of demographic characteristics between the two groups. CAC, when compared to the SC group, had no significant difference in terms of cecal intubation rate (96.0% *vs* 97.0%, P = 0.40) and time (9.94 ± 7.05 min *vs* 10.34 ± 6.82 min, P = 0.21), or polyp detection rate (32.8% *vs* 31.3%, P = 0.75). On the subgroup analysis, there was no significant difference in terms of cecal intubation time by trainees (88.1% *vs* 84.8%, P = 0.40), ileal intubation rate (82.5% *vs* 79.0%, P = 0.38) or total colonoscopy time (23.24 ± 13.95 min *vs* 22.56 ± 9.94 min, P = 0.88). On multivariate analysis, the independent determinants of faster cecal time were consultant-performed procedures (P < 0.001), male patients (P < 0.001), non-usage of hyoscine (P < 0.001) and better bowel preparation (P = 0.01). The determinants of better polyp detection rate were older age (P < 0.001), no history of previous abdominal surgery (P = 0.04), patients not having esophagogastroduodenoscopy in the same setting (P = 0.003), trainee-performed procedures (P = 0.01), usage of hyoscine (P = 0.01) and procedures performed for polyp follow-up (P = 0.01). The limitations of the study were that it was a single-center experience, no blinding was possible, and there were a large number of endoscopists.

CONCLUSION: CAC did not significantly different from SC in term of cecal intubation time and polyp detection rate.

Key words: Cap; Hood; Cecum; Colonoscopy; Cecal intubation; Colonic polyps

Peer reviewers: Dr. Vui Heng Chong, Gastroenterology and Hepatology Unit, Department of Medicine, Raja Isteri Pengiran Anak Saleha Hospital, Bandar Seri Begawan BA 1710, Brunei Darussalam; Keiji Hirata, MD, Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan; Ian C Roberts-Thomson, Professor, Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Tee HP, Corte C, Al-Ghamdi H, Prakoso E, Darke J, Chettiar R, Rahman W, Davison S, Griffin SP, Selby WS, Kaffes AJ. Prospective randomized controlled trial evaluating cap-assisted colonoscopy *vs* standard colonoscopy. *World J Gastroenterol* 2010; 16(31): 3905-3910 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3905.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3905

INTRODUCTION

Colonoscopic examination has been used in clinical practice for approximately 40 years. Despite the fact that colonoscopy is widely available and is performed by many experienced colonoscopists, there are concerns about the quality of colonoscopy as measured by several technical endpoints such as rate of failed cecal intubation and polyp miss rate. A large population-based study[1] has revealed that 13.1% of colonoscopies failed to reach the cecum. In addition, one large review of back-to-back colonoscopies has shown polyp miss rates of 24%[2] for adenoma. Numerous innovations, as described by Rex[3], have been studied to improve these two key issues, with some showing promise.

One potentially promising technique is cap-assisted colonoscopy (CAC). A transparent cap (or hood) is a simple plastic device that can be attached to the tip of a colonoscope before performing the colonoscopy. Several randomized trials from Japan[4-8] have reported mixed results regarding improved cecal intubation times and polyp detection rates. A recent large study from Hong Kong[9] has shown improved time to cecum intubation but a reduced polyp detection rate. To date, as far as we are aware, there is no large randomized study that has used the cap in a western population, in whom the colorectal cancer (CRC) incidence is known to be higher than Asian population[10]. We conducted a randomized controlled trial to investigate the usefulness of CAC in a western population at Royal Prince Alfred Hospital, Sydney.

MATERIALS AND METHODS

This was a prospective randomized controlled trial conducted in a tertiary referral hospital from March 2008 to February 2009. All patients who were referred to our endoscopy service for colonoscopy were invited to participate in the study. All were aged 18 years or older. Exclusion criteria included prior colonic resection, pregnancy, severe comorbidity and acute surgical conditions such as severe colitis, toxic megacolon, ischemic colitis, tertiary referral for endomucosal resection, acute gastrointestinal bleeding, or inability to provide consent, such as dementia. This prospective study was approved by Sydney South West Area Health Service Ethics Review Committee. The trial was registered with ClinicalTrial.gov with the registration identification number NCT00930462.

The preparation for the procedure included a clear fluid diet the day before the procedure, routine bowel preparation with either a sodium picosulfate-based (Picoprep, Pharmatel Fresenius Kabi Pty Ltd., Hornby, Australia), or sodium-phosphate-based (Fleet, Ferring Pharmaceuticals, Gordon, Australia) preparation, and a fasting period of 8 h. Colonoscopy was performed in either the hospital endoscopy unit or a private outpatient endoscopy center associated with our hospital. Procedures were performed by a team of five consultant gastroenterologists and 10 trainees. All procedures were performed under conscious or deep sedation with a combination of intravenous midazolam (Pfizer, Bentley, Australia), fentanyl (Mayne Pharma Ltd, Mulgrave, Australia), and propofol (Fresofol 1%; Pharmatel Fresenius Kabi Pty Ltd.) administered by the assistant or attending anesthetist. As an antispasmodic, hyoscine butylbromide was administered as appropriate. Colonoscopes used were CF-Q160AL, CF-Q180AL, PCF-160AL and PCF-180AL (Olympus Optical Co., Tokyo, Japan).

Three sizes of cap were used: D-201-15004 (with a diameter of 15.3 mm, used for CF-Q180AL), D-201-14304 (14.6 mm, used for CF-Q160AL) and D-201-12704 (13.0 mm, used for both PCF-160AL and PCF-180AL). All of these caps were produced by Olympus Medical Systems (Tokyo, Japan). The cap was placed so that 4 mm was beyond the tip of the colonoscope (Figure 1). Even though the rim of the cap was visible on the monitor, the visual field was not limited as the endoscopist was able to see through the transparent cap.

Once informed consent was obtained, patients were randomized according to a computer-generated randomization protocol to standard colonoscopy without the cap (SC) or CAC. Patients were blinded to the allocation. The colonoscopes were assigned in no specific order and were used according to availability after cleaning. Data were collected during and after the colonoscopic examinations on procedure times, polyps, complications and other parameters.

The procedure was defined as successful if the colonoscope reached the cecum, confirmed by either visualization of the appendicular orifice or the ileocecal valve. Trainee success was only recorded if the cecum was reached without help from a consultant. Terminal ileal intubation was attempted in all cases. The quality of bowel preparation was graded either as good (no or small volume of clear liquid, easily removed), satisfactory (moderate to large volume of liquid stool, removable with suction), or poor (presence of semi-solid stool that could not be cleared or washed away).

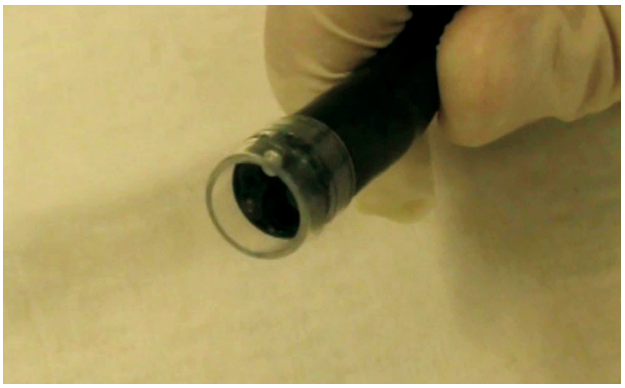

Figure 1 A cap attached to the tip of the colonoscope, with approximately 4 mm of the cap beyond the tip.

Polyps were removed in standard fashion and sent for pathology. The size of the polyps was determined by the endoscopist performing the polypectomy. Advanced lesions were defined as lesions > 10 mm, with high grade dysplasia, or villous in nature. If a diminutive polyp was encountered on insertion and the endoscopist wished to remove this during the insertion phase of the procedure, the time taken for this was subtracted from the insertion time but included in the total procedure time.

Outcome measurement

The primary endpoint of this study was cecal intubation time. The secondary endpoint was polyp detection rate. Other endpoints included ileal intubation rate, total colonoscopy time, trainee success rate and complication rate.

The study also determined the independent predictors for faster cecal time and better polyp detection rate.

Statistical analysis

The study sample size was calculated to be 200 in each arm to detect a difference with a power of 0.8 and an α of 0.05 by two-tailed testing based on historical data[5], for the primary endpoint of cecal intubation time available at the time the study was designed. All statistical analyses were performed using SPSS for Windows version 12.0 (Chicago, IL, USA). The Mann-Whitney test was used for continuous variables that appeared to have a skewed distribution, and the χ^2 or Fisher's exact test for categorical variables. Statistical significance was defined as $P < 0.05$ (two-tailed).

Cecal intubation time and polyp detection rate were log transformed to approximate normality prior to fitting a multiple linear regression model using stepwise variable selection. This model was used to identify only the independent predictors of these parameters.

RESULTS

Baseline demographics

Between March 2008 and February 2009, a total of 400 patients were recruited, with 200 each in the CAC and SC groups. A flow diagram of the enrollment is shown in Figure 2. Mean age was 52 years, and 49% of the subjects were male. The characteristics of the two groups are shown in Table 1, and no significant differences were found between them. Trainees performed a total of 310 (77.5%) procedures in this study.

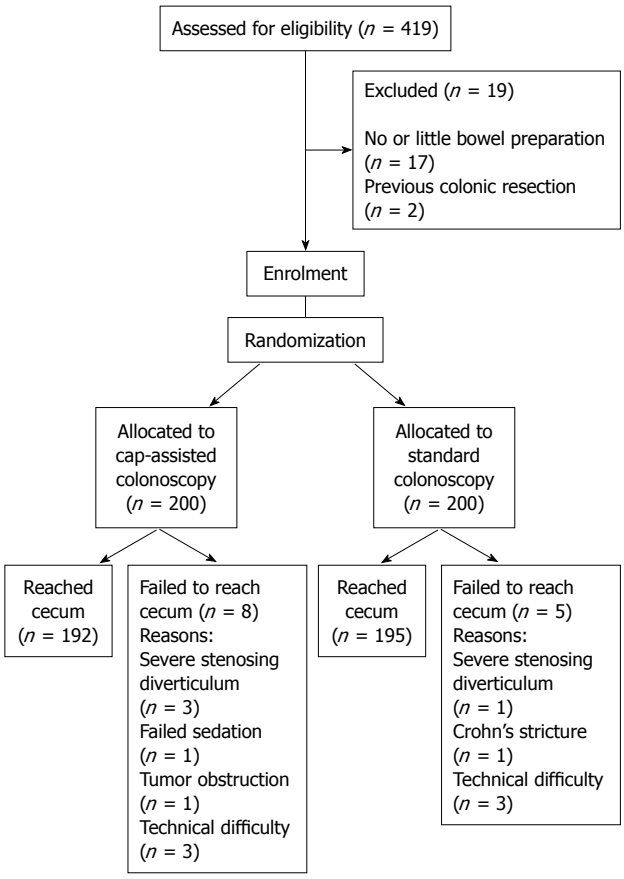

Figure 2 Flow diagram showing enrolment of patients in the study.

Cecal intubation rate

Cecal intubation was achieved in 387 (96.8%) of the 400 colonoscopic examinations. The success rate of cecal intubation was 192/200 (96.0%) in the CAC group and 195/200 (97.5%) in the SC group ($P = 0.40$). When analyzing the data on procedures by trainees only, the unassisted cecal intubation rate was 268/310 (86.5%), with no significant difference between CAC (140/159, 88.1%) and SC (128/151, 84.8%), and P value was 0.40 (Table 2). Forty-two procedures performed by trainees required assistance from consultants, and among these, 32 (76.2%) were successful in reaching the cecum.

Of the 13 cases of failed cecal intubation, four were due to severe sigmoid stenosing diverticular disease (three CAC, and one SC); one each for failed sedation (CAC), Crohn's stricture (SC) and tumor obstruction (CAC); and the other six cases were due to technical difficulties that included irreducible colonic loops (three CAC, and three SC).

Overall ileal intubation rate was 323/400 (80.8%). There was no significant difference between CAC and SC with regards to ileal intubation rate, either overall

Table 1 Baseline characteristics of all patients *n* (%)

	CAC (*n* = 200)	SC (*n* = 200)	*P* value
Sex (male)	102 (51.0)	88 (44.0)	0.16
Age (yr) (mean ± SD)	53.8 ± 15.1	53.6 ± 14.8	0.89
Previous difficult colonoscopy	19 (9.5)	15 (7.5)	0.65
Known diverticular disease	8 (0.04)	11 (0.06)	0.74
Previous pelvic surgery	25 (12.5)	26 (13.0)	0.80
Gastroscopy at the same time	74 (37.0)	89 (44.5)	0.17
Indications			
CRC screening	38 (19.0)	35 (17.5)	
Rectal bleeding	37 (18.5)	32 (16.0)	
Abdominal pain	32 (16.0)	33 (16.5)	
Polyp follow up	30 (15.0)	27 (13.5)	
Change in bowel habit	18 (9.0)	28 (14.0)	
Others	45 (22.5)	45 (22.5)	0.58
Endoscopist			
Specialist	41 (20.5)	49 (24.5)	
Trainee	159 (79.5)	151 (75.5)	0.34
Bowel preparation			
Good	107 (54.0)	130 (65.7)	
Satisfactory	71 (35.9)	53 (26.8)	
Poor	20 (10.1)	15 (7.6)	0.10
Scopes used			
P160/P180	137 (66.5)	143 (70.5)	
A160/A180	63 (32.0)	57 (28.5)	0.61

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; CRC: Colorectal cancer.

Table 2 Primary outcomes: performance of colonoscopy *n* (%)

	CAC	SC	*P* value
Cecal intubation rate	192/200 (96.0)	195/200 (97.0)	0.40[1]
Cecal intubation rate by trainees	140/159 (88.1)	128/151 (84.8)	0.40[1]
Ileal intubation rate	165/200 (82.5)	158/200 (79.0)	0.38[1]
Ileal intubation rate by trainees	126/159 (79.2)	115/151 (76.2)	0.51[1]
Cecal time (min) (mean ± SD) (*n* = 387)	9.94 ± 7.05	10.34 ± 6.82	0.21[2]
Trainee cecal time (min) (mean ± SD) (*n* = 268)	10.72 ± 6.75	9.66 ± 4.86	0.64[2]
Total colonoscopy time (min) (mean ± SD) (*n* = 387)	23.24 ± 13.95	22.56 ± 9.94	0.86[2]
Trainee total colonoscopy time (min) (mean ± SD) (*n* = 268)	25.97 ± 1.22	23.70 ± 0.72	0.47[2]

[1]χ^2 test; [2]Mann-Whitney test. CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy.

(CAC 82.5% *vs* SC 79.0%, *P* = 0.38), or for procedures performed by trainees only (CAC 79.2% *vs* SC 76.2%, *P* = 0.51) (Table 2).

Cecal intubation time

The mean (± SD) time to reach the cecum was 9.9 ± 7.1 min in the CAC group and 10.4 ± 6.8 min in the SC group (*P* = 0.19). Mean total colonoscopy times were 23.3 ± 14.0 min in the CAC group and 22.6 ± 10.0 in the SC group (*P* = 0.86). When confined to procedures performed by trainees unassisted by consultants, the mean time to reach the cecum was 10.7 ± 6.75 min in the CAC group and 9.66 ± 4.86 min in the SC group (*P* = 0.64) (Table 2).

Table 3 Multivariate analysis of determinants of faster cecal intubation time

Independent predictors	B[1]	95% CI	*P* value
Consultant	0.55	0.47-0.63	< 0.001
Female sex	1.38	1.22-1.57	< 0.001
Use of hyoscine	1.48	1.21-1.81	< 0.001
Poor preparation	1.33	1.07-1.66	0.01

[1]Average increase in cecal time per unit increase in the explanatory variable. e.g. the cecal time of consultants was 0.55 (95% CI: 0.47-0.63) times that of trainees.

Table 4 Secondary outcomes: polyp detection rate *n* (%)

	CAC	SC	*P* value
Subjects with polyps (*n* = 387)	63/192 (32.8)	61/195 (31.3)	0.75[1]
Subjects aged ⩾ 50 yr with polyps (*n* = 246)	51/118 (43.2)	46/128 (35.9)	0.24[1]
Total number of polyps	147	107	0.59[2]
Total number of polyps with size ⩽ 5 mm	121	84	0.45[2]
Total number of adenomas	75	55	0.26[2]
Total number of advanced adenomas	23	14	0.52[2]

[1]χ^2 test; [2]Mann-Whitney test. CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy.

On univariate analysis, factors found to be significantly associated with faster time to the cecum were: male sex, younger age, colonoscopy by a consultant, good or satisfactory bowel preparation, and non-use of hyoscine. Multiple linear regression confirmed each of these as independent predictors, apart from young age (Table 3).

Polyp detection rate

Among the 387 subjects who had successful cecal intubation, colorectal polyps were detected in 123 (31.8%) of them (63 in the CAC and 61 in the SC group, *P* = 0.75). CAC and SC detected polyps in 32.8% and 31.3% of their subjects, respectively. When confined to subjects aged 50 years and above, CAC detected polyps in 43.2% and SC in 35.9% (*P* = 0.24) (Table 4). The total number of polyps detected was 254, and this also did not differ between the two groups (147 in CAC and 107 in SC, *P* = 0.59). There was no difference between the two groups in terms of the number of polyps of 5 mm or less (*P* = 0.45), adenoma number (*P* = 0.26), or advanced adenoma (*P* = 0.52) (Table 4). On univariate analysis, factors found to be significantly associated with detection of polyp were: male sex, older age, polyp follow-up as the indication, patients who had colonoscopy alone (*vs* patients having combined gastroscopy and colonoscopy), non-use of hyoscine, and colonoscopy performed by a trainee. On multivariate analysis, the independent predic-

Table 5 Multivariate analysis of determinants of higher polyp detection rate

Independent predictors	OR	95% CI	*P* value
Age	1.450	1.213-1.734	< 0.001
Previous abdominal surgery	0.559	0.322-0.971	0.039
Having EGD on the same setting	0.451	0.266-0.765	0.003
Consultant	0.460	0.250-0.845	0.012
Use of hyoscine	2.877	1.374-6.026	0.005
Indication: polyp follow-up	2.722	1.342-5.523	0.006

EGD: Esophago-gastro-duodenoscopy.

tors were older age, polyp follow-up as the indication, no history of prior abdominal surgery, patients having colonoscopy alone, trainee-performed colonoscopy, and the use of hyoscine (Table 5).

Complications

There was no complication associated with the use of the cap. There were two cases of post-polypectomy bleeding, one each in the SC and CAC groups, although these cases were minor and did not require transfusion.

DISCUSSION

Despite the fact that polypectomy prevents 76%-90% of CRC when compared to the expected incidence[11], data from interval CRCs (CRCs diagnosed between the time of a negative screening colonoscopy to that of next recommended colonoscopy) raise important questions about how effective colonoscopy is as a screening practice[12]. Possible reasons for these interval cancers include failed colonoscopy and missed lesions. A large population-based study[1] has revealed that 13.1% of colonoscopy failed to reach the cecum. Rex *et al*[2] have published data on adenoma miss rates based on a large back-to-back colonoscopy series, and estimated that the rate was 17%-48%. Important advances in colonoscopy have been suggested to improve the outcome of colonoscopy. These include improving colonoscopy techniques (optimizing withdrawal time[13-15], chromoendoscopy and bowel preparation quality[16,17]) and technologies (wide-angle colonoscopy in 2003[18], narrow band imaging in 2004[19], and fluorescence confocal endomicroscopy[20].

Caps have been used previously in endoscopic procedures, for instance, in mucosal resection and double balloon enteroscopy. Their use in colonoscopy has been studied in the past with regards to cecal intubation and polyp detection. Our study did not show any benefit of CAC for our primary endpoint of time to cecal intubation Even though all six previous randomized trials of CAC have been faster with the cap, only four were significantly faster. In our study, CAC had a shorter cecal intubation time although this was not statistically significant. One possible explanation is that studies with cecal intubation times below 10 min are inclined to have no significance between the two study arms, with the exception of the study of Lee *et al*[9]. This illustrates a point whereby the benefit of the cap is small in those procedures where cecal intubation times are already short. The short cecal intubation time and high cecal success rate in our study probably left little room for improvement with the cap. The overall cecal intubation rate was 96.8%, this high success rate was probably due to the fact that our center is a tertiary teaching hospital.

Factors that predicted faster colonoscopy in our multivariate analysis (Table 3) were as expected and well-described in the literature[21-23], except for the use of hyoscine. We reserved hyoscine for difficult insertions, which suggests that we selected patients with difficult colonoscopy, hence the prolonged cecal intubation time in that group.

We also showed no significant improvement of the polyp detection rate in the CAC group. This was almost certainly due to a small sample size because this study was powered to look at cecal intubation time rather than polyp detection rate. Furthermore, our study cohort had a mean age of 52 years (*vs* 62.0-66.4 in Asian studies[5-8]), older only than the patients in the study of Lee *et al*[9]. Our low polyp detection rate at 31.8%, despite the fact that the study was carried out on a western population, was likely a consequence of this younger patient population. The youngest population studied to date was in Hong Kong[9], which demonstrated significantly lower polyp detection in the CAC group. Our study does not support the use of a cap for polyp detection, however, the question of any benefit with the cap has not been sufficiently answered. Further studies, particularly in a western population, are required. With the known polyp miss rates at about 24%[2], it is likely that large numbers will need to be recruited in prospective randomized studies to show any benefit of this intervention.

In conclusion, there was no statistically significant difference between CAC and SC with regards to cecal intubation success, time and polyp detection rate.

ACKNOWLEDGMENTS

The authors would like to thank the endoscopy nurses headed by Kaye Ireson at Royal Prince Alfred Hospital, Sydney for their tireless effort in assisting this research.

COMMENTS

Background

Despite 40 years of advances in colonoscopic practices, there are a significant number of incomplete procedures and polyp miss rate. This study looked at the use of cap-assisted colonoscopy (CAC) in improving these endpoints.

Research frontiers

Colonoscopy is the most important screening tool for cancer of the colon. Many new developments are aimed at improving the quality of colonoscopy. These include improving colonoscopy techniques (optimizing withdrawal time, chromoendoscopy and bowel preparation quality) and improved technologies (wide-angle colonoscopy in 2003, narrow band imaging in 2004, and fluorescence confocal endomicroscope in 2005).

Innovations and breakthroughs

CAC is a relatively simple and inexpensive tool that is used to improve the usefulness of colonoscopy. Several Asian centers have performed trials on CAC, with conflicting results, and we aim to add more information on the usefulness of this device.

Applications

This article provides important data on the usefulness of CAC especially in a Western population.

Peer review

This is an interesting study that investigated the use of CAC in a day-to-day setting of a busy tertiary endoscopy unit.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3911

World J Gastroenterol 2010 August 21; 16(31): 3911-3918
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Anti-proliferative and pro-apoptotic effects of tectorigenin on hepatic stellate cells

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Supported by The National Natural Science Foundation of China, No. NSFC30801417; Natural Science Foundation of Jiangsu Province, No. BK2008267; and Doctoral Fund of Ministry of Education of China, No. RFDP200802841004

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Received: February 27, 2010 Revised: April 20, 2010
Accepted: April 27, 2010
Published online: August 21, 2010

Abstract

AIM: To investigate the effect of tectorigenin on proliferation and apoptosis of hepatic stellate cells (HSC)-T6 cells.

METHODS: HSC-T6 cells were incubated with tectorigenin at different concentrations, and their proliferation was assessed by bromodeoxyuridine incorporation assay. Apoptosis was detected by flow cytometry assay with Hoechst 33342 staining. Also, generation of reactive oxygen species (ROS), intracellular $[Ca^{2+}]_i$, potential of mitochondrial membrane, activities of cytochrome c and caspase-9 and -3 were investigated to explore a conceivable apoptotic pathway.

RESULTS: Tectorigenin suppressed the proliferation of HSC-T6 cells and induced apoptosis of HSC-T6 cells in a time- and dose-dependent manner. Tectorigenin at the concentration of 100 μg/mL greatly inhibited the viability of HSC-T6 cells and induced the condensation of chromatin and fragmentation of nuclei. When treated for 48 h, the percentage of cell growth and apoptosis reached 46.3% ± 2.37% (P = 0.004) and 50.67% ± 3.24% (P = 0.003), respectively. Furthermore, tectorigenin-induced apoptosis of HSC-T6 cells was associated with the generation of ROS, increased intracellular $[Ca^{2+}]_i$, loss of mitochondrial membrane potential, translocation of cytochrome c, and activation of caspase-9 and -3.

CONCLUSION: Tectorigenin inhibits proliferation of HSC-T6 cells and induces apoptosis of HSC-T6 cells.

Key words: Tectorigenin; Apoptosis; Hepatic stellate cells; Hepatic fibrosis; Mitochondria; Proliferation

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Wu JH, Wang YR, Huang WY, Tan RX. Anti-proliferative and pro-apoptotic effects of tectorigenin on hepatic stellate cells. *World J Gastroenterol* 2010; 16(31): 3911-3918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3911.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3911

INTRODUCTION

Hepatic fibrosis, a common wound-healing response to diseases such as chronic hepatitis and alcoholic liver damage, is a result of destruction in architecture of the liver parenchyma and an imbalance between fibrogenesis

and fibrolysis forming scars or fibrous tissues. Hepatic stellate cells (HSC), the major cells in hepatic fibrosis, are responsible for the development of fibrosis[1,2]. Activation and proliferation of HSC are the key to fibrogenesis while apoptosis of HSC is associated with the resolution of fibrosis. So, HSC have attracted increasing attention due to their essential role in liver fibrosis. With a better understanding of their biological properties, inhibiting the activation and proliferation of HSC and inducing apoptosis of activated HSC have been proposed as potential anti-fibrosis strategies.

In traditional Chinese medicine, *Iris tectorum* (*I. tectorum*) has been used in treatment of liver injury for a long time. It has been shown that tectorigenin, an important bioactive compound isolated from *I. tectorum*, has antioxidant, anti-inflammatory, and anticancer activities[3-5]. Experiments on animal models have also demonstrated that tectorigenin exhibits a hepatoprotective effect on CCl_4-induced or *t*-BHP-induced hepatic injury in rats[6,7]. Since hepatic fibrosis is a common wound-healing response to liver injury, we studied whether tectorigenin has anti-fibrosis potentials and exhibits its hepatoprotective effect by playing a role in liver fibrosis.

The present study was therefore performed to investigate the effects of tectorigenin on proliferation and apoptosis-related events of activated HSC-T6 cells and disclose its possible mechanism underlying apoptosis of HSC-T6 cells.

MATERIALS AND METHODS

Materials

Tectorigenin was isolated from *I. tectorum* with a purity of over 98% as confirmed by high-performance liquid chromatography (HPLC) analysis.

Cell culture

HSC-T6 cells, an immortalized rat hepatic stellate cell line, exhibit an activated HSC phenotype[8]. The cells, purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China), were cultured in Dulbecco's-modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 12% new bovine serum (Hangzhou Sijiqing Co., Ltd., Hangzhou, China), in a humidified atmosphere containing 5% (v/v) CO_2 at 37℃. L02 cells (a human hepatocyte cell line), purchased from Xiangya Central Experiment Laboratory, Central South University, China, were cultured in DMEM medium (Gibco, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (Hangzhou Sijiqing Co., Ltd., Hangzhou, China), in a humidified atmosphere containing 5% (v/v) CO_2 at 37℃.

Cell viability assay

Cells were plated in 96-well plates at a density of 5×10^5 cells/well and grown for 24 h. Tectorigenin at different concentrations was added to the cells while only DMSO (solvent) was added as a negative control. After growing for 12, 24, and 48 h, cell viability was evaluated by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Amresco, OH, USA)[9].

Bromodeoxyuridine uptake

HSC-T6 cells were incubated for 48 h with 20, 40, 60 and 100 μg/mL tectorigenin, respectively. Two hours before the cells were harvested, bromodeoxyuridine (BrdU; GenMed Scienfics Inc., USA) was added. The cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342 following the manufacturer's protocol[10].

Morphological observation of nuclear change

HSC-T6 cells were incubated for 48 h with tectorigenin at 20, 40, 60 and 100 μg/mL, respectively. Nuclear morphological change was assessed using Hoechst 33342 staining[11]. In brief, cells were fixed in 4% paraformaldehyde for 10 min, washed three times with pre-chilled PBS and exposed to 5 μg/mL of Hoechst 33342 at 37℃ in dark for 15 min. Samples were observed under a fluorescent microscope (Nikon UFX-Ⅱ, Japan). Cells showing cytoplasmic and nuclear shrinkage, chromatin condensation or fragmentation, were defined as apoptotic cells.

Flow cytometry analysis

To quantify apoptotic cells, HSC-T6 cells were harvested after exposed to tectorigenin for 24 and 48 h, respectively, washed twice with cold PBS, resuspended in PBS containing fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for 10 min, and measured using a FACScan flow cytometer[12,13] (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of ROS generation

Intracellular ROS was quantified with a fluorescence plate reader using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma)[14]. The cells on black 96-well plates were treated with tectorigenin at with tectorigenin at 20, 40, 60 and 100 μg/mL for 1, 3, 6 and 24 h, respectively, and incubated with DCFH-DA at 37℃ for 30 min. After DCFH-DA was removed, the cells were washed with phosphate buffered saline (PBS). DCFH-DA-loaded cells were read on a Safire fluorescence plate reader (Tecan, Crailsheim, Germany).

Measurement of intracellular $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was monitored using fluorescent Ca^{2+}-sensitive dye, a Fura 2-acetoxymethyl ester (Fura 2-AM)[15]. Cells were cultured and treated with tectorigenin for 1, 3, 6 and 24 h, respectively, and preloaded with 1 μmol/L Fura2-AM for 30 min in dark at 37℃ in a humidified incubator. After loading with Fura2-AM, cells were collected, gently rinsed three times with D-Hanks' solution, and resuspended in D-Hanks' solution containing 0.2% BSA at 10^6cells/mL. Intracellular $[Ca^{2+}]_i$ was measured at an emission wavelength of 510 nm and an excitation wavelength of 340 and 380 nm on a Safire fluorescence

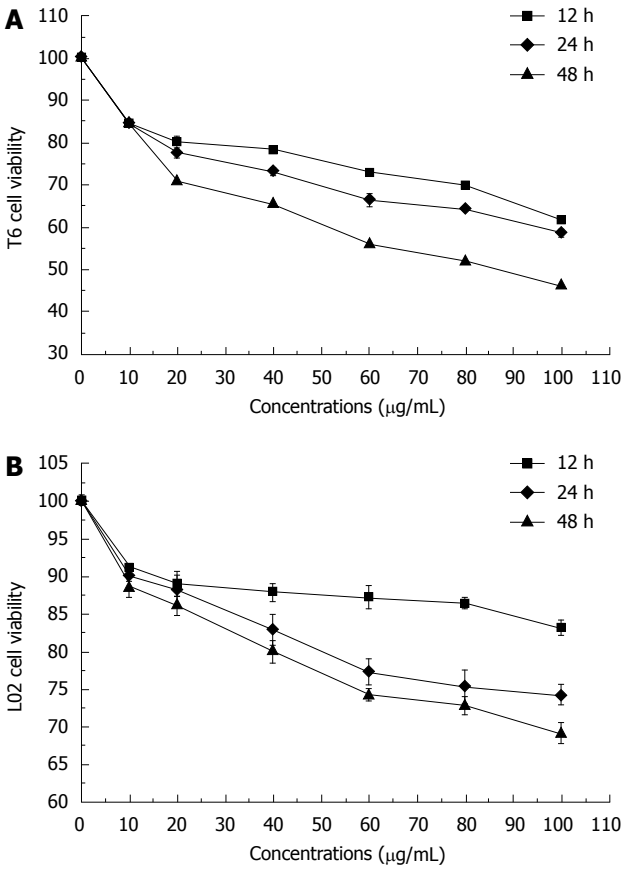

Figure 1 Effects of tectorigenin on the viability of hepatic stellate cell -T6 (A) and -L02 cells (B). Cells were treated for 12, 24 and 48 h with tectorigenin at 10, 20, 40, 60, 80 and 100 μg/mL, respectively, followed by assessing the cell viability relative to that of untreated cells (= control). Bars represent mean ± SD.

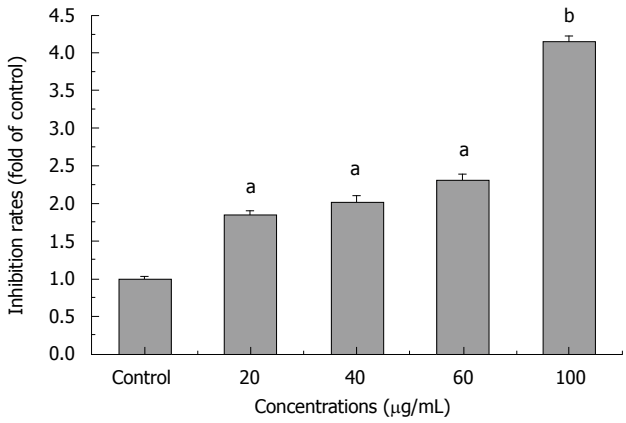

Figure 2 Proliferative inhibition of tectorigenin on hepatic stellate cells-T6 cells. Cells were treated for 48 h with tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively. Data are expressed as fold increase over that of untreated cells. Bars represent mean ± SD ($^aP < 0.05$, $^bP < 0.01$).

plate reader (Tecan, Crailsheim, Germany). The ratio of fluorescence intensity at 340 to 380 nm (F340/F380) was used to estimate intracellular free calcium.

Measurement of mitochondrial membrane potential

Change in mitochondrial membrane potential (MMP) was monitored using Rhodamine 123 (Rh-123)[16]. In brief, Rh-123 was added to cells to attain a final concentration of 3 μg/mL. After incubated at 37℃ for 30 min, cells were collected, washed twice with PBS, and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting analysis

HSC-T6 cells were seeded into 60-mm dishes (1×10^6 cells/dish). After treated on the next day for 48 h with tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively, HSC-T6 cells were harvested, resuspended in an ice-cold lysis buffer consisting of 50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 5 mmol/L DTT, 1 mmol/L EDTA, 0.1% SDS, 0.5% Triton X-100, and protease inhibitor cocktail tablets (Roche, IN), incubated for 10 min on ice, disrupted in a micro ultrasonic cell disrupter for 10 s and centrifuged at 750 *g* for 15 min at 4℃. The supernatant (cytosolic fraction) was removed and maintained at -80℃. The pellet containing mitochondria was resolved in a lysis buffer. Protein level was measured using a standard colorimetric assay kit (BCA kit). Proteins were separated by polyacrylamide/SDS gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, IN). The membranes were probed with antibodies (cytochrome c and caspase-9 diluted at 1:1000, Cell Signaling Technology, MA, USA) overnight at 4℃, and incubated with a HRP coupled secondary antibody (HRP; 1:5000, Cell Signaling Technology, MA, USA). Detection was performed using a LumiGLO chemiluminescent subtract system (KPL, Guildford, UK). β-actin (1:200, Boster, Wuhan, China) as a loading control. Results were quantified with a scanning densitometer (Bio-Rad, USA).

Measurement of caspase-3 activity

Activity of caspase-3, the main execution caspase, was detected with a caspase-3 colorimetric assay kit (KenGen Biotech, Nanjing, China) according to its manufacturer's instructions. Cultured HSC-T6 cells were washed twice with cold PBS, resuspended in a lysis buffer and left on ice for 20 min. Lysate was centrifuged at 10000 r/min for 3 min at 4℃. Supernatants were collected and protein concentrations were measured with a BCA kit. Proteins (100 μg) were incubated for 4 h at 37℃ with a reaction buffer in a total volume of 105 μL containing 5 μL caspase-3 substrate, and detected with a fluorescence microplate reader (Tecan, Crailsheim, Germany) at λ 405 nm.

Statistical analysis

All data were expressed as mean ± SD. Origin Pro 7.0 statistical package was used to determine statistical significance. Difference between two groups was analyzed by two-tailed Student's *t*-test, and difference among three or more groups was analyzed by one-way ANOVA multiple comparisons. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

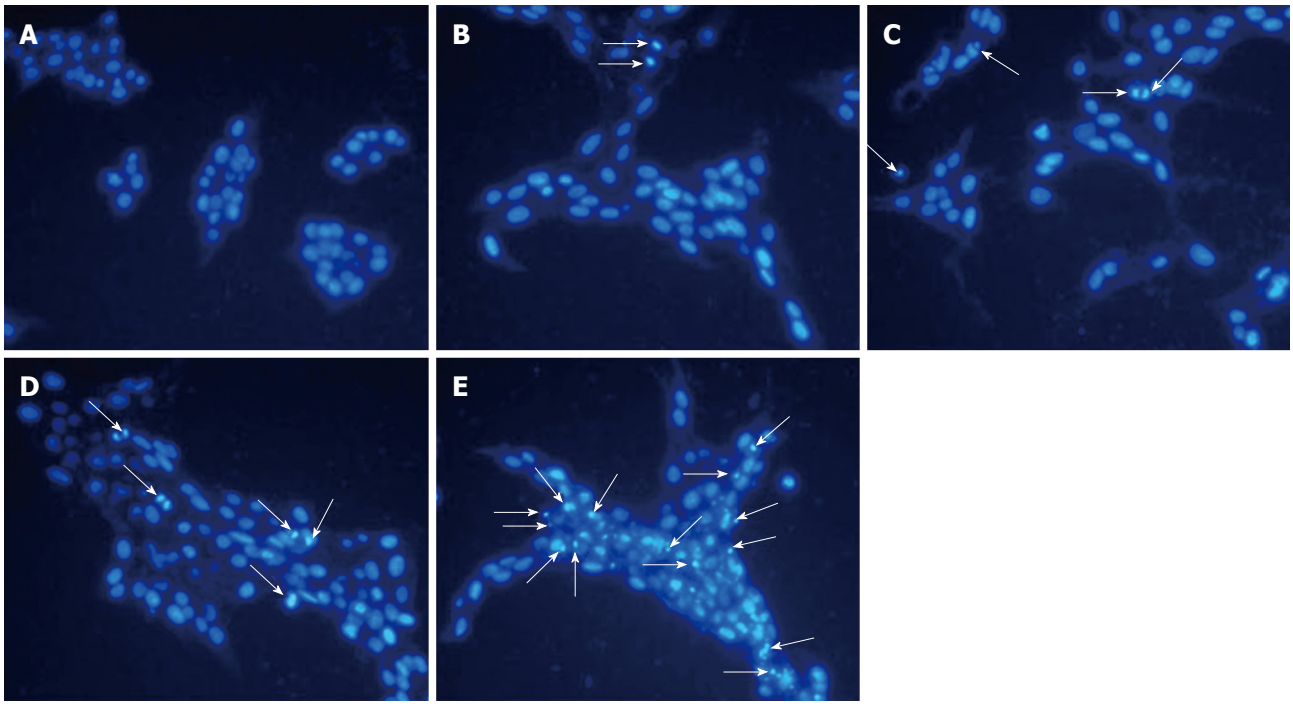

Figure 3 Fluorescent staining of nuclei in hepatic stellate cells-T6 cells (200 ×). Cells were treated with tectorigenin for 48 h at 0 μg/mL (A), 20 μg/mL (B), 40 μg/mL (C), 60 μg/mL (D) and 100 μg/mL (E), respectively. The arrows in B-E indicate the apoptotic cells.

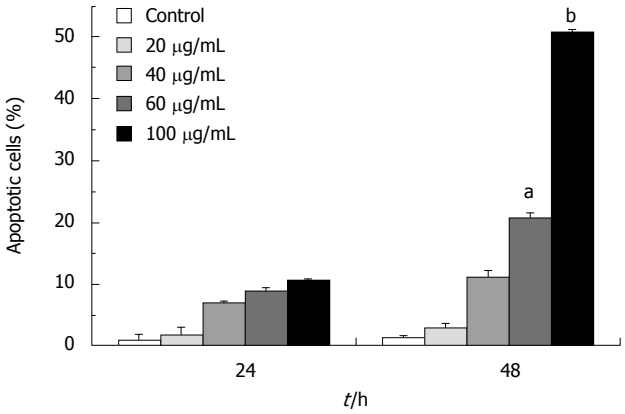

Figure 4 Flow cytometry-evidenced apoptosis of hepatic stellate cells-T6 cells upon exposure to tectorigenin. Cells were incubated for 24 and 48 h with tectorigenin at 20, 40, 60 and 100 μg/mL, respectively, followed by being stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide. Bars represent mean ± SD ($^aP < 0.05$, $^bP < 0.01$).

RESULTS

Effect of tectorigenin on viability of HSC-T6 and L02 cells

The percentage of cell growth was significantly different between tectorigenin-treated and untreated groups. Tectorigenin inhibited the growth of HSC-T6 cells in a dose-dependent manner (Figure 1A). Increased incubation time of HSC-T6 cells decreased the percentage of cell growth, indicating that tectorigenin also inhibits the growth of HSC-T6 cells in a time-dependent manner. Treatment with tectorigenin at 100 μg/mL resulted in a moderate cytotoxicity to L02 cells after incubated for 48 h (Figure 1B). To further investigate the inhibitory effect of tectorigenin on proliferation of HSC-T6 cells, BrdU incorporation, another indicator of cell proliferation, was detected. Tectorigenin at 20-100 μg/mL could significantly inhibit the proliferation of HSC-T6 cells (Figure 2).

Tectorigenin induced apoptosis of HSC-T6 cells

We studied if tectorigenin can induce apoptosis of HSC-T6 cells. In brief, apoptotic cells were visualized using DNA-binding Hoechst 33342. Regular and round-shaped nuclei were observed in control HSC-T6 cells (Figure 3). After treated for 48 h with tectorigenin at 40 and 60 μg/mL, condensed nuclei were found in HSC-T6 cells, which became smaller in size, and eventually fragmented into apoptotic bodies. Moreover, after treated with 100 μg/mL tectorigenin, the nuclei of HSC-T6 cells were further condensed with the number of apoptotic bodies sharply increased. These data suggest that tectorigenin greatly induces the condensation of chromatin and fragmentation of nuclei. The apoptosis rate of HSC-T6 cells was determined by flow cytometry analysis with annexin V-FITC and PI staining. In the control group, most cells were viable. When HSC-T6 cells were exposed for 48 h to tectorigenin at 60 and 100 μg/mL, the percentage of apoptotic cells increased to 20.69% ± 2.57% and 50.67% ± 3.24% ($P = 0.003$), respectively (Figure 4), which was significantly higher than that of those not exposed to tectorigenin. In addition, tectorigenin induced apoptosis of HSC-T6 cells in a dose- and time-dependent manner.

Tectorigenin induced ROS generation in HSC-T6 cells

To determine whether tectorigenin is able to induce ROS generation in HSC-T6 cells, the level of ROS, measured using the fluorescence probe DCFH-DA, was significantly

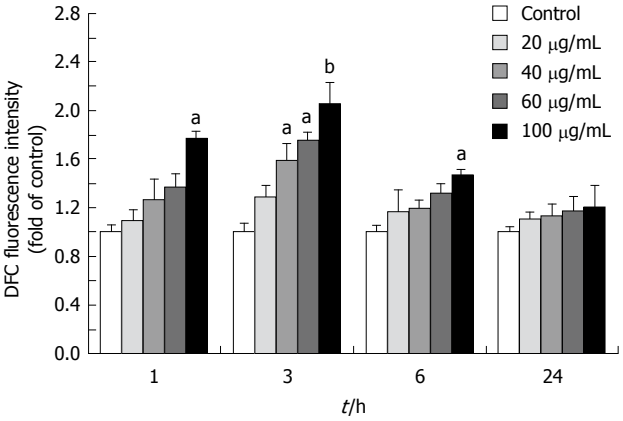

Figure 5 Tectorigenin produces reactive oxygen species in hepatic stellate cells-T6 cells. Cells were treated with tectorigenin for 1, 3, 6 and 24 h, followed by a 30-min incubation at 37℃ with reactive oxygen species detected by dichlorodihydrofluorescein diacetate. Data are expressed as fold increase over that of untreated cells. Bars represent mean ± SD ($^aP < 0.05$, $^bP < 0.01$).

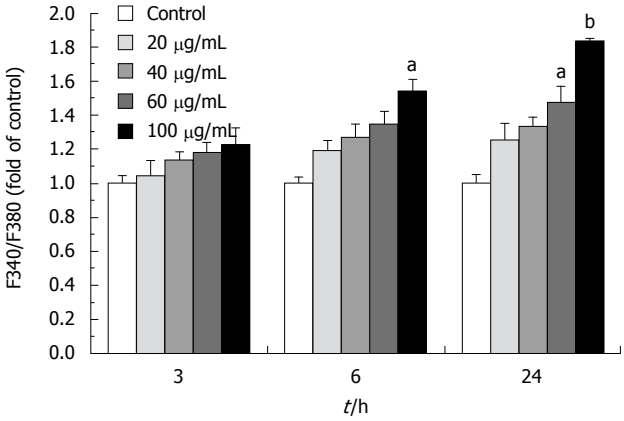

Figure 6 Tectorigenin increases $[Ca^{2+}]_i$ in hepatic stellate cells-T6 cells. Cells were treated with tectorigenin for 3, 6 and 24 h. $[Ca^{2+}]_i$ was detected by Fura-2/AM 380nm/340nm fluorescence ratio (F340/F380). Data are expressed as fold increase over that of untreated cells. Bars represent mean ± SD ($^aP < 0.05$, $^bP < 0.01$).

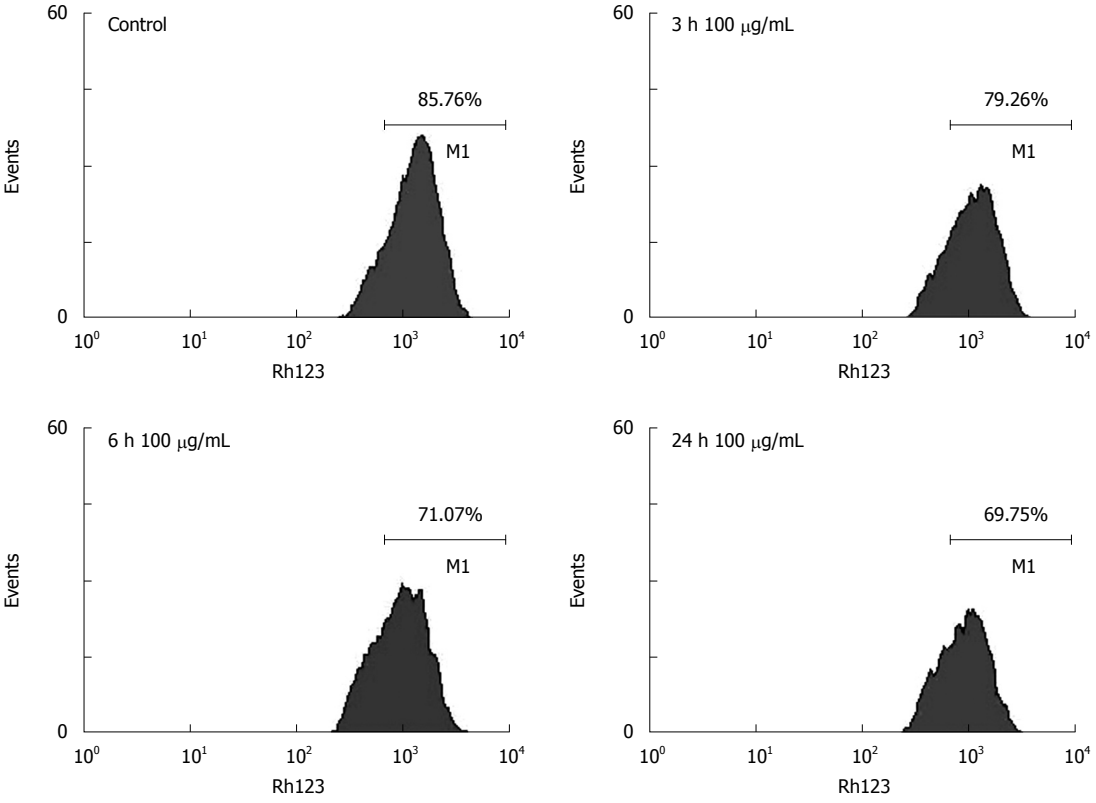

Figure 7 Tectorigenin reduces mitochondrial membrane potential in hepatic stellate cells-T6 cells. After treated with tectorigenin at 100 μg/mL for 3, 6 and 24 h, respectively, cells were harvested and stained with Rhodamine 123 to determine mitochondrial membrane potential by flow cytometry.

higher in HSC-T6 cells than in control cells after treated with 100 μg/mL tectorigenin for 1, 3 (peaked) and 6 h (Figure 5).

Tectorigenin increased $[Ca^{2+}]_i$ in HSC-T6 cells

To determine whether tectorigenin influences the level of intracellular Ca^{2+}, the level of intracellular Ca^{2+} was measured with Fura 2-AM staining. After treated with 100 μg/mL tectorigenin for 3 h, the fluorescence ratio (F340/F380) increased to 123.1% ± 7.18% compared to that not treated with tectorigenin (Figure 6). In addition, tectorigenin increased cytoplasmic Ca^{2+} in a time-dependent manner. When the incubation time was prolonged to 24 h, the F340/F380 value increased from 123.1% ± 7.18% to 183.3% ± 8.64% ($P = 0.002$).

Tectorigenin decreased mitochondrial membrane potential in HSC-T6 cells

To determine whether tectorigenin decreases mitochondrial membrane potential in HSC-T6 cells, flow cytometry

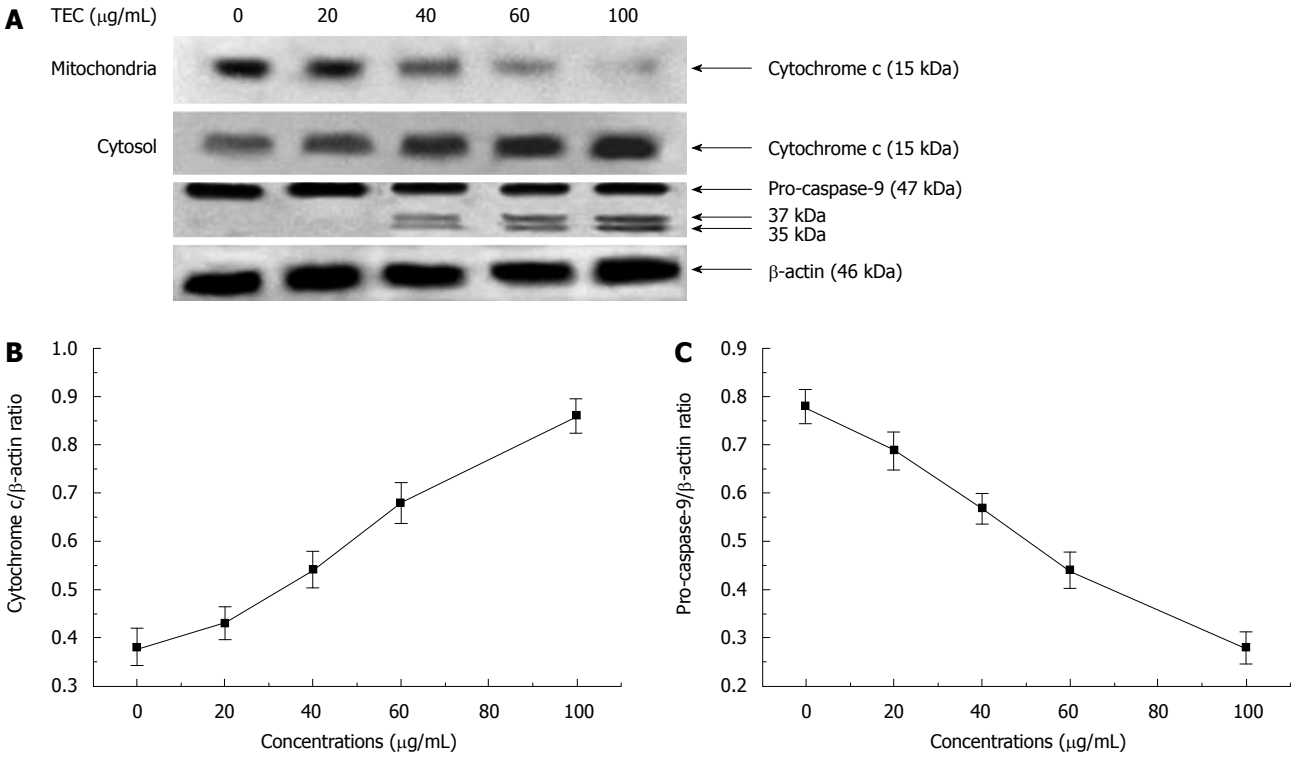

Figure 8 Western blotting analysis. After 48-h exposure to tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively, levels of cytochrome c and caspase-9 in hepatic stellate cells-T6 cells (A), along with those of cytochrome c (B) and pro-caspase-9 (C) in cytosol, were evaluated. Protein (30 μg) from each sample was resolved on 12% SDS-PAGE and β-actin was used as a loading control.

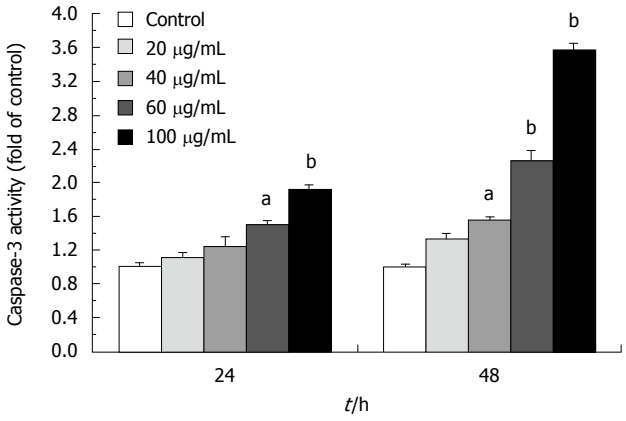

Figure 9 Tectorigenin activates pro-caspase-3 in hepatic stellate cells-T6 cells. Cells were treated for 24 and 48 h with tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively. Data are expressed as fold increase over that of untreated cells. Bars represent mean ± SD ($^aP < 0.05$, $^bP < 0.01$).

analysis was carried out using Rhodamine 123. Compared to control cells not treated with tectorigenin, HSC-T6 cells treated with 100 μg/mL tectorigenin for 24 h decreased the mitochondrial membrane potential (MMP) from 85.76% ± 6.39% to 69.75% ± 5.28% ($P = 0.03$) (Figure 7).

Effect of tectorigenin on release of cytochrome c and activities of caspase-9 and -3

To assess whether tectorigenin-treated cells accompany increased cytosolic translocation of cytochrome c, the activated caspases-9 and -3 were detected, and the cytosolic and mitochondrial levels in cytochrome c and intracellular caspases-9 and -3 were measured. The results reveal that tectorigenin releases mitochondrial cytochrome c into cytosol in a dose-dependent manner (Figure 8B). Western blotting also showed that tectorigenin induced proteolytic cleavage of pro-caspase-9 into the active form of HSC-T6 cells and tectorigenin activated the caspase-9 in a dose-dependent manner (Figure 8C). Tectorigenin (100 μg/mL) significantly increased the activity of caspase-3 in HSC-T6 cells (Figure 9).

DISCUSSION

In the present study, tectorigenin, a bioactive compound isolated from *I. tectorum* used traditionally for severe liver disorder, suppressed the proliferation of HSC and induced apoptosis of HSC in a time- and dose-dependent manner.

As an immortalized rat liver stellate cell line, HSC-T6 cell line exhibits an activated phenotype of HSC and a fibroblast-like morphology, which presents as a useful tool in exploring hepatic fibrosis[17]. MTT and BrdU incorporation assay demonstrated that tectorigenin could inhibit the proliferation of HSC-T6 cells, with a lower cytotoxicity to human hepatocytes (L02). Furthermore, flow cytometry analysis indicated that tectorigenin could induce apoptosis of HSC-T6 cells in a dose- and time-dependent manner.

Clarification of the molecular mechanism of tectorigenin underlying the discerned apoptosis is of great importance. It has been shown that ROS generation

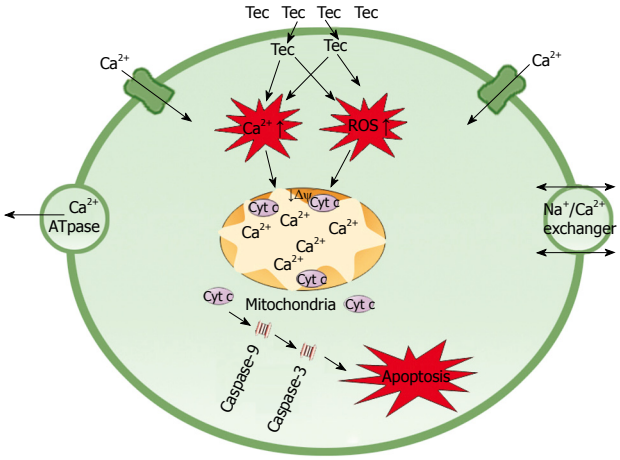

Figure 10 Tectorigenin produces reactive oxygen species and increases Ca^{2+} concentration in cytoplasm of hepatic stellate cells-T6 cells, leading to the depletion of mitochondrial membrane potential and release of cytochrome c from mitochondria. Cytochrome c facilitates apoptosis of hepatic stellate cells-T6 cells by activating caspase cascade. ROS: Reactive oxygen species.

increases membrane permeability of mitochondria, triggers abrupt mitochondrial depolarization and release of cytochrome c from inner mitochondrial membrane[18]. Calcium homeostasis is an essential mechanism underlying physiological process. Since increased cytosolic calcium may induce mitochondria to take up intracellular overloaded Ca^{2+}, a much larger amount of Ca^{2+} will accumulate in mitochondria, which can potentially damage the electron transfer chain, leading to failure in maintaining the mitochondrial membrane potential[19-21]. Damages to mitochondrion result in loss of its function. For example, release of cytochrome c from mitochondria leads to cell apoptosis[22]. In this study, tectorigenin increased the ROS production and Ca^{2+} concentration in cytoplasm of HSC-T6 cells, the intracellular accumulation of ROS and Ca^{2+} further induced the loss of MMP. The disruption of MMP caused release of cytochrome c from mitochondria to cytosol. Cytosolic cytochrome c activated the pro-caspase-9 and subsequently, caspase-9 activated the downstream effector caspases-3, eventually triggered apoptosis of HSC-T6 cells (Figure 10).

In conclusion, tectorigenin suppresses the proliferation of HSC-T6 cells in a dose- and time-dependent manner, and produces a slight cytotoxicity to L02 cells. More importantly, tectorigenin induces apoptosis of HSC-T6 cells and may have anti-fibrosis potentials.

COMMENTS

Background

Liver fibrosis represents a significant health problem worldwide without any currently available and effective therapeutic approach. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension, and often requires liver transplantation. The most characteristic feature of liver fibrosis is excess deposition of type I collagen. A great number of researches have been performed to understand the molecular mechanism underlying liver fibrosis. Activated hepatic stellate cells (HSC) are the primary cell type responsible for the excess production of collagen.

Research frontiers

HSC are the major cells responsible for the development of liver fibrosis and cirrhosis. Activated HSC are proliferative and fibrogenic, with accumulation of extra cellular matrix, including α-smooth muscle actin (α-SMA) and type I collagen. Suppression of activation and proliferation, and induction of apoptosis in HSC have been reported as the therapeutic strategies against liver fibrosis. Tectorigenin, a bioactive compound of *Iris tectorum* (*I. tectorum*), shows antioxidant, anti-inflammatory, anticancer, and hepatoprotective activities, and has been used in therapy for liver injury. However, how tectorigenin works in live fibrosis has not been unequivocally addressed. In this study, tectorigenin suppressed proliferation of HSC and induced apoptotis of HSC.

Innovations and breakthroughs

Currently used drugs such as corticosteroids and colchicine usually show various side effects such as immunosuppression or cytotoxicity. Tectorigenin, an antioxidant, anti-inflammatory, and hepatoprotective compound isolated from *I. tectorum*, shows its inhibitory effect on proliferation of HSC and produces a low toxicity. Furthermore, *in vitro* studies suggest that tectorigenin produces its pro-apoptotic effect on HSC is probably *via* the mitochondrial pathway to some extent.

Applications

By showing how tectorigenin works in HSC, this study may represent a new and future strategy for managing hepatic fibrosis.

Peer review

This study showed that tectorigenin inhibited the growth and viability of rat hepatic HSC-T6 cells in a dose-dependent manner. The authors conclude that the anti-proliferative and pro-apoptotic effects of this component on HSC might explain its anti-fibrotic properties observed *in vivo*. This is an interesting study. However, the mechanism of tectorigenin underlying the growth and viability of HSC-T6 cells needs to be further studied.

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wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3919

World J Gastroenterol 2010 August 21; 16(31): 3919-3927
ISSN 1007-9327 (print)

BRIEF ARTICLE

Mapping of liver-enriched transcription factors in the human intestine

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Author contributions: Lehner F and Kulik U performed the majority of the experiments; Klempnauer J was involved in editing the manuscript and provided the collection of the human material; Borlak J designed the study; Borlak J and Lehner F wrote the manuscript.
Supported by (in part) Novartis Pharma GmbH, Germany, BU Transplantation and Immunology (to Lehner F) and by the Lower Saxony Ministry of Culture and Sciences and the Volkswagen foundation, Germany, Grant No. 25A.5-7251-99-3/00
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Received: February 23, 2010 Revised: April 13, 2010
Accepted: April 20, 2010
Published online: August 21, 2010

Abstract

AIM: To investigate the gene expression pattern of hepatocyte nuclear factor 6 (HNF6) and other liver-enriched transcription factors in various segments of the human intestine to better understand the differentiation of the gut epithelium.

METHODS: Samples of healthy duodenum and jejunum were obtained from patients with pancreatic cancer whereas ileum and colon was obtained from patients undergoing right or left hemicolectomy or (recto)sigmoid or rectal resection. All surgical specimens were subjected to histopathology. Excised tissue was shock-frozen and analyzed for gene expression of liver-enriched transcription factors by semiquantitative reverse transcription polymerase chain and compared to the human colon carcinoma cell line Caco-2. Protein expression of major liver-enriched transcription factors was determined by Western blotting while the DNA binding of HNF6 was investigated by electromobility shift assays.

RESULTS: The gene expression patterning of liver-enriched transcription factors differed in the various segments of the human intestine with HNF6 gene expression being most abundant in the duodenum ($P < 0.05$) whereas expression of the zinc finger protein GATA4 and of the HNF6 target gene ALDH3A1 was most abundant in the jejunum ($P < 0.05$). Likewise, expression of FOXA2 and the splice variants 2 and 4 of HNF4α were most abundantly expressed in the jejunum ($P < 0.05$). Essentially, expression of transcription factors declined from the duodenum towards the colon with the most abundant expression in the jejunum and less in the ileum. The expression of HNF6 and of genes targeted by this factor, i.e. neurogenin 3 (NGN3) was most abundant in the jejunum followed by the ileum and the colon while DNA binding activity of HNF4α and of NGN3 was confirmed by electromobility shift assays to an optimized probe. Furthermore, Western blotting provided evidence of the expression of several liver-enriched transcription factors in cultures of colon epithelial cells, albeit at different levels.

CONCLUSION: We describe significant local and segmental differences in the expression of liver-enriched transcription factors in the human intestine which impact epithelial cell biology of the gut.

Key words: Liver-enriched transcription factors; Human intestine; Caco-2; Gene expression

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Lehner F, Kulik U, Klempnauer J, Borlak J. Mapping of liver-enriched transcription factors in the human intestine. *World J Gastroenterol* 2010; 16(31): 3919-3927 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3919.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3919

INTRODUCTION

Numerous studies established the pivotal role of liver-enriched transcription factors in organ development and cellular function. These nuclear proteins are known to work in a hierarchical and cooperative network. The timely expression of specific transcription factors is necessary for cellular differentiation[1,2] and *in situ* hybridization studies of staged embryos demonstrate that hepatocyte nuclear factor 6 (HNF6) and its target gene FOXA2 are expressed in the hepatic diverticulum. More detailed analysis of the developmental expression patterns of HNF6 and FOXA2 provides evidence for their colocalization in intestinal epithelium. The expression patterns of these 2 transcription factors do not overlap in other endoderm-derived tissues[3].

There is growing evidence that the liver-enriched transcription factor plays a role in cancerous diseases of the digestive tract[4], and recent studies from our own laboratory provide evidence for HNF6 and FOXA2 as key regulators in colorectal liver metastases[5].

To better understand the molecular pathology of colorectal liver metastases we, and others, carried out a genome-wide expression analysis[6,7]. Essentially, the genes coding for the liver-enriched transcription factors HNF6, HNF1β and CCAAT enhancer binding protein γ (C/EBPγ) were selectively regulated but protein expression of regulated transcription factors identified unacetylated HNF6 to be a hallmark of colorectal liver metastases[5]. For its proposed interaction with HNF6, expression of FOXA2 and HNF6 was investigated. Notably, FOXA2 was significantly induced in colorectal liver metastases[5]. From the electromobility shift assay, evidence was obtained for HNF6 DNA binding activity to be specifically repressed in nuclear extracts of colorectal liver metastases. Taken collectively, we found HNF6 expression in colorectal liver metastases to be driven by the hepatic environment. Its expression is not observed in healthy colon nor in primary colonic cancer. Thus, HNF6 DNA binding is selectively prevented through lack of posttranslational modification and interaction with FOXA2.

As HNF6 is only expressed in colorectal liver metastases but not in primary colonic cancer or healthy colonic tissue we wished to investigate the regulation of HNF6 and of other liver-enriched transcription factors in different segments of the human intestine thereby providing information on their expression patterning. We therefore mapped HNF6 and other liver-enriched transcription factors in the human duodenum, jejunum, ileum and colon of patients undergoing large intestinal surgery. Regional differences in the expression and regulation of transcription factors might participate in growth of intestinal tumors and may influence metastatic spread. We also investigated regulation of liver-enriched transcription factors in the human colon carcinoma cell line Caco-2 that is considered to be valuable for the study of gut epithelial biology. The expression patterning of liver-enriched transcription factors was investigated as a function of time and confluency. Note, upon confluency the Caco-2 colon carcinoma cell line acquires many of the features of the enterocyte as detailed elsewhere[8,9]. We additionally investigated the DNA binding of HNF6, HNF4α and the HNF6 target neurogenin 3 (NGN3) to well known regulatory DNA sequences to link the DNA binding activity of these transcription factors to gene expression data.

Overall, we report the gene expression pattern of liver-enriched transcription factors in the human intestine and compare these findings with results obtained from the human colon carcinoma cell line, to facilitate the construction of a gene expression map for a better understanding of their regulation in gut biology.

MATERIALS AND METHODS

Ethical approval and patient's characteristics

Approval for the use of surplus tissue material from elective surgery was obtained from the ethics committee of the Medical School of Hanover, Germany. All patients participating in this study gave written informed consent and were fully aware of the aims of the study. A summary of the patients' characteristics is given in Table 1. Duodenum and jejunum were obtained from patients mainly with pancreatic cancer whereas ileum and colon was obtained from patients undergoing right or left hemicolectomy, (recto)sigmoid resection or rectal resection. All surgical specimens were subjected to histopathology. Excised tissue was shock-frozen in liquid nitrogen and stored at -80℃ until analyzed.

Cell culture

Expression of liver-enriched transcription factors was compared between colonic epithelium obtained from tissue resection material and Caco-2 cells, which were derived from a Caucasian patient with colonic adenocarcinoma. The Caco-2 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK) and were cultured as described previously[10].

RNA isolation and cDNA synthesis

RNA was isolated form tissue samples using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quality and quantity of isolated RNA were checked by capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) following the manufacturer's instructions or by gel electrophoresis. Reverse transcription (RT) used 2 μg total RNA from each sample. RNA and random

Table 1 Patient characteristics

Patient	Sex	Age (yr)	Tumor localization	Tumor type	TNM classification	UICC classification
CN2/CP2	M	64	Rectum	Colorectal cancer	pT2 pN0 M0-G2	I
CN3/CP3	F	74	Colon transversum	Colorectal cancer	pT2 pN1 pM1-G2	IV
CN7/CP7	F	51	Colon ascendens	Colorectal cancer	pT3 pN2 M0-G2	III
CN8/CP8	M	81	Colon sigmoideum	Colorectal cancer	pT3 pN0 M0-G3	II
CN9/CP9	F	63	Colon sigmoideum	Colorectal cancer	pT2 pN1 M0-G2	III
CN10/CP10	M	81	Rectum	Colorectal cancer	pT3 pN1 M0-G2	III
CN11/CP11	F	49	Colon ascendens	Colorectal cancer	pT3 pN2 M1-G2	IV
CN15/CP15	M	73	Colon sigmoideum	Colorectal cancer	pT4 pN0 M0-G3	II
CN16/CP16	M	72	Rectum	Colorectal cancer	pT2 pN0 M0-G2	I
CN17/CP17	M	44	Rectum	Colorectal cancer	pT2 pN1 M1-G2	IV
CN18/CP18	F	67	Rectum	Colorectal cancer	pT3 pN1 M1-G2	IV
CN19/CP19	M	61	Rectum	Colorectal cancer	pT2 pN2 M1-G2	IV
CN20/CP20	M	56	Rectum	Colorectal cancer	pT3 pN0 M0-G2	II
CN21/CP21	M	61	Colon sigmoideum	Colorectal cancer	pT3 pN2 M1-G2	IV
P34 CN/CP	F	61	Colon sigmoideum	Colorectal cancer	pT4 pN1 M1-G2	IV
P38 CN/CP	M	67	Rectum	Colorectal cancer	pT2 pN0 M0-G3	I
Duo P39	M	72	Pancreas	Pancreatic carcinoma		
Duo P44	F	66	Pancreas	Pancreatic cyst		
Duo P47	F	57	Pancreas	Pancreatic carcinoma		
Jej P41	M	53	Pancreas	Pancreatic carcinoma		
Jej P44	F	66	Pancreas	Pancreatic cyst		
Jej P45	M	61	Pancreas	Bile duct cancer		
Ileum P40	F	78	Colon ascendens	Colorectal cancer		
Ileum P43	M	78	Colon ascendens	Colorectal cancer		
Ileum P46	M	64	Colon ascendens	Colorectal cancer		

primer (Promega, Mannheim, Germany) were preheated for 10 min at 70℃ and then chilled on ice for 2 min. A total of 5 × RT-avian myeloblastosis virus (AMV) buffer (Promega), dNTP (10 mmol/L), RNAsin, AMV-RT (avian myeloblastosis virus-reverse transcriptase) (all Promega) and DEPC-H_2O were added to a final volume of 20 μL. RT was carried out for 60 min at 42℃ and was stopped by heating to 95℃ for 5 min. The resulting cDNA was frozen at -20℃ until additional experimentation.

Semiquantitative RT polymerase chain reaction

Primer design was done with the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Cross-reaction of primers with the genes was excluded by comparison of the sequence of interest with a database (Blast 2.2, US National Centre for Biotechnology Information) and all primers used in our study were intron spanning. Polymerase chain reaction (PCR) reactions were undertaken with a 20 μL reaction mixture containing HotStarTaq Master Mix (Qiagen, Hilden, Germany), DEPC, 1 μL cDNA and 1.0 μmol/L concentration of the 3'- and 5'-specific oligomers (synthesized by Invitrogen, Hilten, Germany). PCR reactions were carried out on a thermal cycler (T3, Biometra). Detailed oligonucleotide sequence information and the PCR amplification protocol were published previously[5]. DNA contamination was determined by direct amplification of RNA extracts before conversion to cDNA. Contamination of RNA extracts with genomic DNA was determined by gel electrophoresis and by DNA digest prior to cDNA synthesis. PCR reactions were done within the linear range of amplification, and amplification products were separated using 1.5% agarose gel and stained with ethidium bromide. Gels were photographed on a transilluminator (Kodak Image Station 440), and amplicons were quantified using the Kodak 1D 3.5 network software.

Western blotting experiments

Western immunoblotting was done as follows: total protein (100 μg) or nuclear protein (30 μg) extracts of Caco-2 cell cultures were denaturated at 95℃ for 5 min, followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis on 12% polyacrylamide gels, and blotted onto a polyvinylidene difluoride membrane (NEN, Dreieich, Germany) at 350 mA for 2 h in a buffer containing 400 mmol/L glycine and 50 mmol/L Tris (pH 8.3). Non-specific binding sites were blocked with Rotoblock (Roth, Germany) in 1 × TBS buffer. After electroblotting of proteins, membranes were incubated with polyclonal antibodies for HNF1α (Santa Cruz sc6548), FOXA2 (Santa Cruz sc6554), FOXA3 (Santa Cruz sc5360), HNF4α (Santa Cruz sc 6556), and HNF6 (kind gift of Dr. Costa RH, Chicago, Illinois, USA) for 1 h and washed 3 times with 1 × TBS buffer containing 0.1% Tween-20 (Roth, Germany). Subsequently, the membranes were incubated with a 1:5000 diluted anti-α rabbit antibody (Chemicon, Hofheim, Germany) for 1 h at room temperature, followed by 3 successive washes with 1 × TBS buffer containing 0.1% Tween-20 (Roth, Germany). Immunoreactive proteins were visualized with a chemiluminescence reagent kit (NEN, Dreieich, Germany) according to the manufacturer's instructions, and bands were scanned with the Kodak Image Station CF 440 and analyzed using the Kodak 1D 3.5 imaging software (Eastman Kodak Company, USA).

Table 2 Expression pattern of liver-enriched transcription factors in the human intestine

	Duodenum	Jejunum	Ileum	Colon
HNF1	++	+++	++	+
HNF1α	++	+++	+++	+
HNF1β	+	-	+	++
FOXA2	+	++	-	++
HNF4	++	+++	++	+
HNF4α	+	+++	++	+
HNF4γ	++	++	++	+
HNF6	++	+	-	-
C/EBPα	+	+++	++	+
C/EBPγ	+	+++	++	+
MitATPase	++	++	++	++
PPARα	++	+	++	++
IGFβ	+	-	+	++
AHR	+	+	+	++
NGN3	+	+++	++	+
ALDH3A1	+	+++	-	+
ADH1A1	++	+	++	+
COL5A1	++	+	+	+
CYP51	++	++	++	+
UGT1A1	+++	+++	+	++
HSP105B	++	++	+	++
CDP	+	+	+	++
GATA4	++	+++	-	-

+++: Very strong expression; ++: Strong expression; +: Detectable; -: Not detectable. HNF: Hepatocyte nuclear factor; EBP: Enhancer binding protein.

Electrophoretic mobility shift assay

The procedure for electrophoretic mobility shift assays was adapted from a previously described method[11]. Briefly, 5 μg of Caco-2 nuclear extract were incubated with the binding buffer consisting of 25 mmol/L HEPES (pH 7.6), 5 mmol/L $MgCl_2$, 34 mmol/L KCl, 2 mmol/L DTT, 2 mmol/L Pefablock (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μL aprotinin (2.2 mg/mL, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 50 ng poly (dl-dC) and 80 ng bovine serum albumin (PAA Laboratories GmbH, Cölbe, Germany). The binding reaction was carried out for 20 min on ice, and free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel. For supershift studies, a specific HNF6 and/or HNF4α antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was added to the reaction mix 10 min before addition of the labeled probe. In the case of NGN3, no commercial antibody was available. Thus, a competition assay at 100- and 500-fold access of unlabeled oligonucleotide probe specific to NGN3 was used. Gels were blotted to Whatman 3 MM paper, dried under vacuum, exposed to imaging screens (Imaging Screen-K, Bio-Rad Laboratories GmbH, Munich, Germany) for autoradiography overnight at room temperature and analyzed using a phosphor imaging system (Molecular Imager FX pro plus; Bio-Rad Laboratories GmbH, Munich, Germany) and the Quantity One Version 4.2.2 software (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistical analysis

We applied the Wilcoxon signed rank test and the Student *t*-test to determine significance, with $P < 0.05$ being statistically significant.

RESULTS

Gene expression profiling of liver-enriched transcription factors in different segments of the human intestine

The gene expression of transcription factor was determined relative to mitochondrial ATPase, i.e. a housekeeping gene that was found to be stably expressed. As described in the Material and Methods section, tissue preparations were obtained from surgically removed but healthy gut segments, as part of the Whipple surgery for the removal of pancreatic cancers or alternatively from patients undergoing surgery for colonic cancer. The tissue material was derived from the mucosa and submucosa and processed further as described in the Material and Methods section.

Table 2 gives an overview of the segmental expression pattern of various liver-enriched transcription factors while Figure 1 depicts the expression of HNF6 and of genes targeted by this transcription factor. Note, the data is presented relative to the expression of the mitochondrial ATPase, which serves as a housekeeping gene (Figure 1A). The patterning of individual liver-enriched transcription factors in the duodenum, jejunum, ileum and colon is shown in Figure 1B. Additionally, in Figure 1C expression of some genes of interest in epithelial cell biology are given. In Figure 2 and as described in the Material and Methods section, representative RT-PCR gels are shown. Note, all PCR reactions were done within the linear range of amplification. Essentially, the abundance of transcript expression of HNF1, HNF4 and for some of its splice variants and for C/EBP was significantly more than that of mitochondrial ATPase. Specifically, HNF6 gene expression was most abundant in the duodenum ($P < 0.05$) when compared with other segments of the intestine, whereas expression of the zinc finger protein GATA4 and of the HNF6 target gene ALDH3A1 was most abundant in the jejunum (Figure 1B, $P < 0.05$). Likewise, expression of FOXA2 and the splice variants 2 and 4 of HNF4α were most abundantly expressed in the jejunum ($P < 0.05$). For most of the transcription factors and of genes targeted by these factors, expression of transcripts varied amongst patients rendering statistical significance impossible. With the exception of the colon we were unable to amplify transcripts for FOXA1 and FOXA2 in any of the human intestine tissues examined. Essentially, in the case of HNF6, gene expression declined from the duodenum towards colon, whereas FOXA2 expression was undetectable in the ileum. Likewise, HNF4 expression was most abundant in the jejunum as was expression of its splice variant HNF4α3 and HNF4α2+4 and of HNF4γ. Unlike HNF6 we observed expression of HNF4 and of C/EBPα and γ in colon tissue as well. The expression of HNF6 and of genes targeted by this factor, i.e. NGN3 was most abundant in jejunum followed by ileum and colon but for most of the other genes targeted by HNF6 such as ALDH1A1, ALDH3A1, COL5A1,

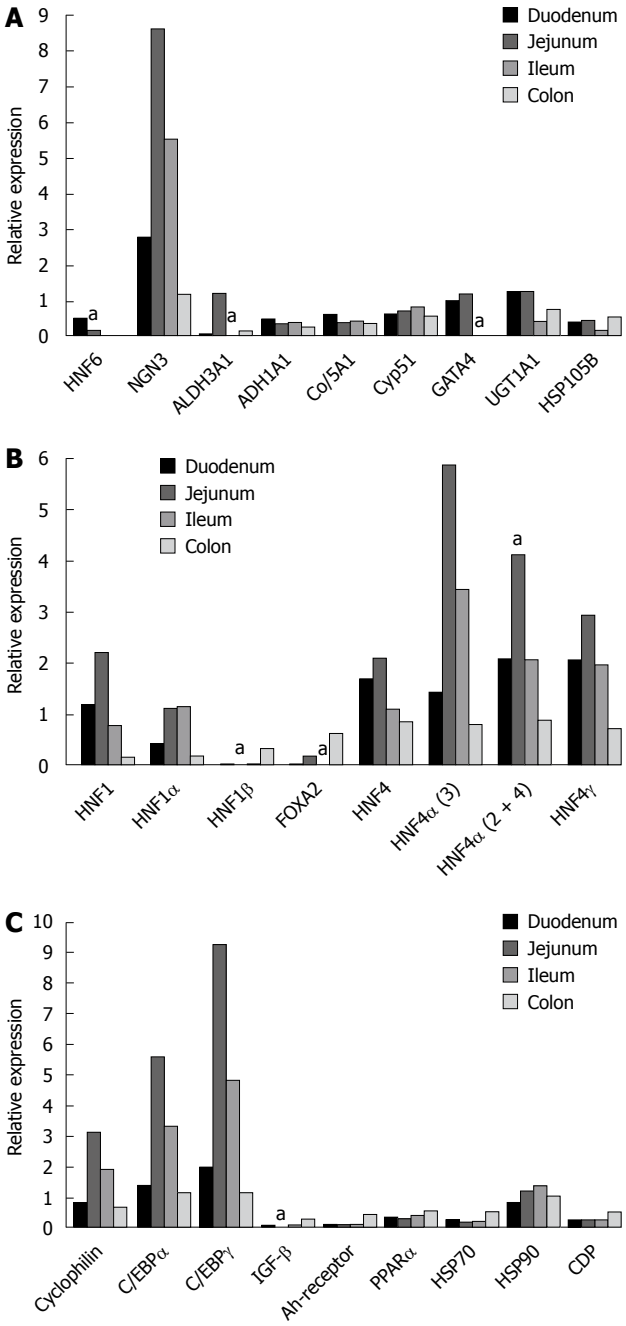

Figure 1 Gene expression of hepatocyte nuclear factor 6 and of other liver enriched transcription factors in the human intestine. A: Hepatocyte nuclear factor 6 (HNF6) and some of its target genes. Bars hallmarked by "a" refer to statistically significant changed expression ($P < 0.05$) when compared to other segments of the gut; B: Hepatic nuclear transcription factors. Bars hallmarked by "a" refer to statistically significant changed expression ($P < 0.05$) when compared to other segments of the gut; C: CAAT enhancer binding proteins and the transcription factors Ah-receptor, PPAR alpha as well as the heat shock proteins HSP70 and HSP90 and IGF-β. Bars hallmarked by "a" refer to statistically significant changed expression ($P < 0.05$) when compared to other segments of the gut.

CYP51, UGT1A1 and HSP105B, expression did not differ amongst the different human intestinal segments. With the exception of heat shock protein 90, the expression of the Ah-receptor and of the nuclear receptor PPARα, HSP70 and the CCAAT enhancer displacement protein CDP, did not differ amongst the different human intestinal segments studied. In contrast, the expression of the

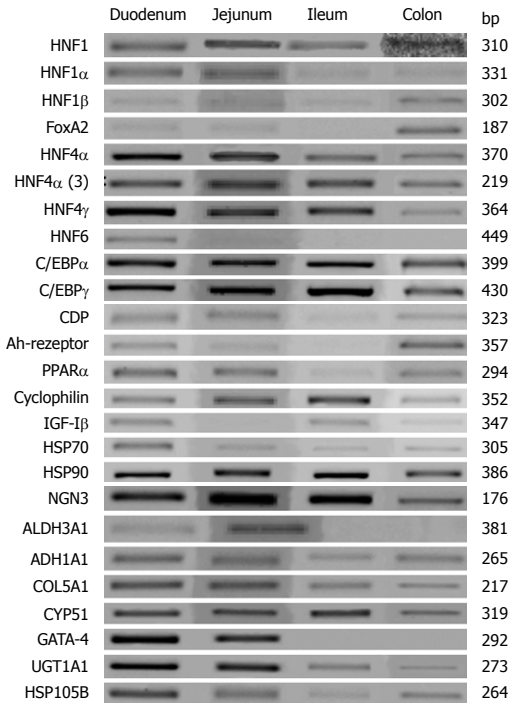

Figure 2 Representative reverse transcription polymerase chain reaction gels of gene expression of hepatocyte nuclear factor 6 and of other liver-enriched transcription factors in the human intestine. Note, all polymerase chain reaction reactions were done within the linear range of amplification.

insulin growth factor binding protein was more abundant in the colon ($P < 0.05$).

Studies using the human colon epithelial cancer cell line Caco-2

The expression of the aforementioned transcription factors was investigated in cultures of Caco-2 cells. Essentially, with time and cell culture confluency HNF6 gene expression increased up to day 11 but declined thereafter (see Figure 3 for microscopic images and Figure 4 for gene expression data). A similar rise in expression of transcription factor gene expression was seen for HNF1, some splice variants of HNF4 (2, 3 and 4) and of C/EBPγ (data not shown). None of these changes were, however, of statistical significance. Importantly, expression of HNF6 transcripts was unexpected, as neither healthy colon nor primary colonic cancers expressed HNF6. Likely, its expression in Caco-2 cells is the result of the cell culture environment with the supply of optimized culture media. We additionally determined the protein expression of liver-enriched transcription factor and observed expression of HNF1α, FOXA2, FOXA3 and HNF4α (Figure 5). In the case of FOXA3 and HNF4α, 2 immunoreactive bands were observed. Obviously the antibodies recognized these liver-enriched transcription factors posttranslational modifications. No HNF6 protein expression could be determined.

DNA binding studies with HNF6

We further investigated the DNA binding of HNF6 and of its downstream target NGN3 as well as HNF4α. As shown in Figure 6A, we were unable to detect HNF6

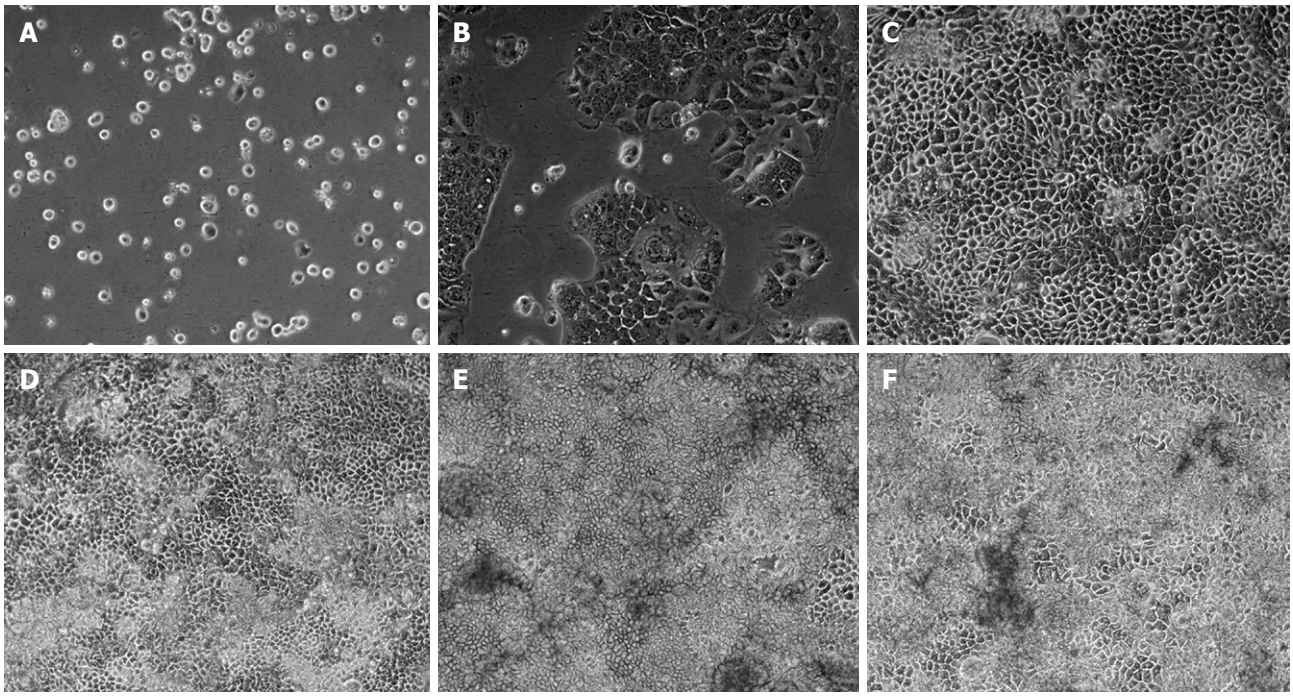

Figure 3 Microscopic images of time depended sequence of Caco-2 cells. Note, *HNF6* gene expression increased with time and cell culture confluency up to day 11 but declined thereafter (see Figure 2 for gene expression data). A: 0 d; B: 3 d; C: 5 d; D: 7 d; E: 11 d; F: 17 d.

DNA binding with nuclear extracts of Caco-2 cells. We previously reported abundant HNF6 protein expression in the liver[5], and therefore used nuclear extracts of the liver as a positive control for HNF6 DNA binding activity. In the past we demonstrated that expression of liver-enriched transcription factors can be modulated by treatment of Caco-2 cell cultures with Acrolor 1254[12]. This treatment did not induce DNA binding of HNF6 but increased expression of an unknown nuclear protein to an optimized HNF6 probe (Figure 6A). To further study HNF6 activity we investigated the DNA binding of the transcription factor NGN3. Essentially, NGN3 was reported to be a downstream target of HNF6 and is therefore regulated by this factor[13]. As shown in Figure 6C, NGN3 DNA binding is observed with nuclear extracts of Caco-2 cell cultures. Furthermore, treatment of Caco-2 cell cultures with Aroclor 1254, an inducer of transcription factors[12], resulted in increased DNA binding of NGN3. We also investigated DNA binding of HNF4α and observed abundant DNA binding activity as illustrated in Figure 6B. In the case of HNF4α a suitable antibody was available. The shifted band in the EMSA assay further documents specificity. As observed with NGN3 treatment of Caco-2 cell cultures with Aroclor 1254 resulted in increased HNF4α DNA binding. In the past we reported induction of HNF4α gene expression in cultures of Caco-2 cells[12]. We now extend our initial observation to DNA binding activity of this protein.

DISCUSSION

This study aimed for an improved understanding of the gene expression pattern of HNF6, FOXA2 and other nuclear transcription factors in the descending human intestine. Essentially, expression of liver-enriched transcription factors differed when the duodenum, jejunum, ileum and colon were compared. Mapping of liver-enriched transcription factors to different segments of the human intestine provided valuable insight into gene regulation that may have significant implications for physiology and disease. Indeed, numerous studies have established an important role of liver-enriched transcription factors in organ development and cellular function and there is conclusive evidence of nuclear transcription factors to act in concert in the orchestration of gene expression[1].

Here we report FOXA2 expression to be significantly upregulated in the colon but the coded protein of this transcription factor has been shown to inhibit HNF6 activity. As originally proposed by Rausa *et al*[14] an interplay of CBP coactivator protein with HNF6 and FOXA2 may regulate steady levels of these transcription factors. In human colorectal liver metastases FOXA2 expression was significantly induced while electromobility shift assays demonstrated HNF6 DNA binding activity to be prevented as previously reported[5].

Notably, no HNF6 DNA binding was observed with nuclear extracts of Caco-2 cells (Figure 6A) whereas DNA binding of HNF4α and of NGN3 was evident. Additionally, we observed increased DNA binding activity for HNF4α and NGN3 upon treatment of Caco-2 cell cultures with Aroclor 1254 (Figure 6B and C). Expression of HNF6 protein was below the limit of detection in untreated cultures of the Caco-2 cell line.

Of all transcription factors investigated, expression of HNF6 was most abundant in duodenum and jejunum whereas expression of GATA4 and of HNF4 including some of its splice variants in addition to C/EBPα and γ

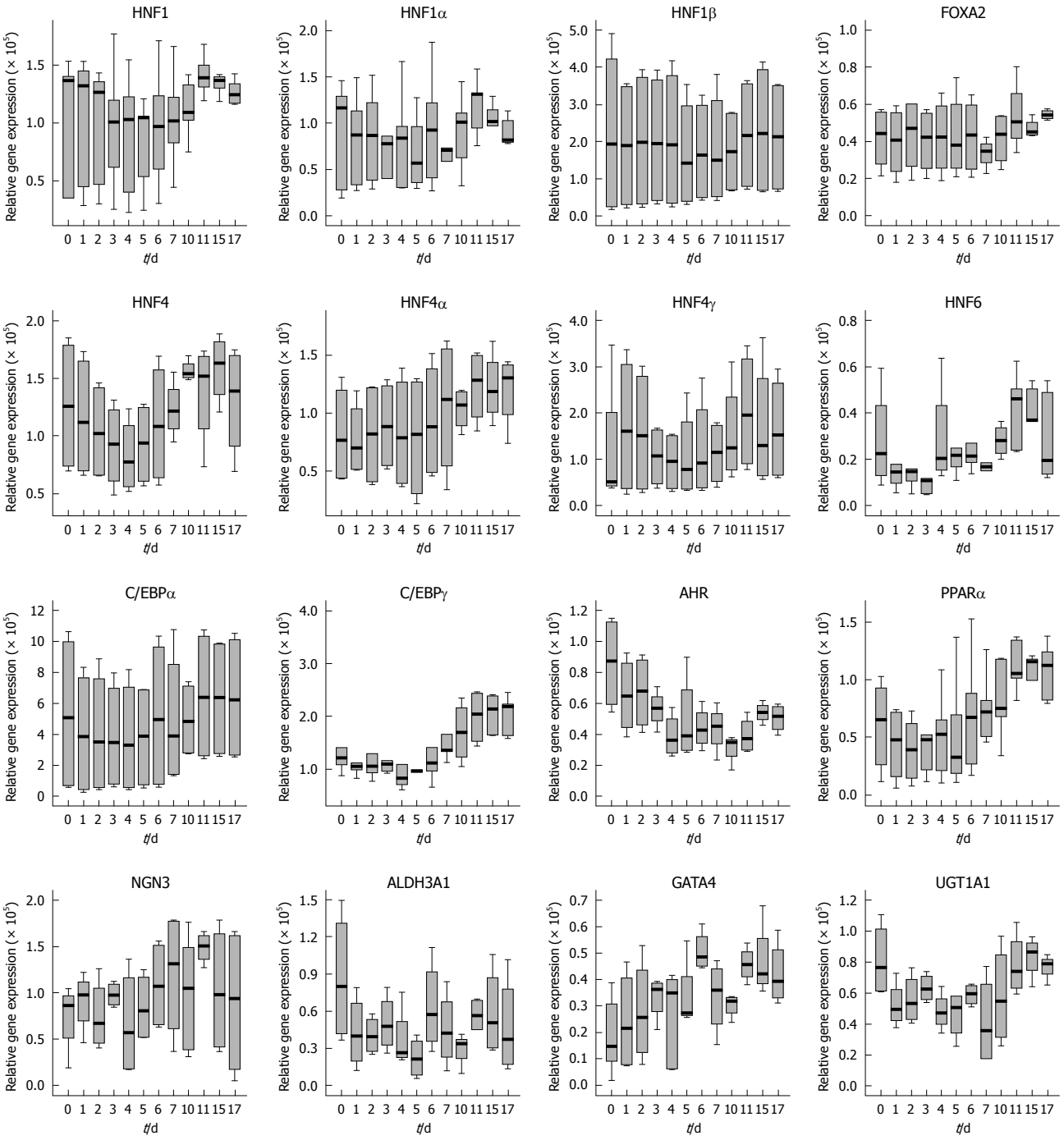

Figure 4 Time-dependent gene expression of liver-enriched transcription factors and some of its target genes in cultures of Caco-2 cells. HNF: Hepatocyte nuclear factor.

was most abundant in the jejunum.

The difference in expression pattern of individual transcription factors amongst the various segments of the human intestine is notable with HNF4 and C/EBP being abundantly expressed in the jejunum and the duodenum. Indeed, HNF4α has been shown to protect the gut against inflammatory bowel disease and there is clear evidence for a role of HNF4α in promoting differentiation of intestinal epithelial cells[15,16]. Furthermore, in the study of Stegmann *et al*[17] the metabolome, transcriptome and bioformatic analysis identified HNF4 as a central regulator of gene expression during enterocyte differentiation and crypt function, but recent evidence identified forkhead box transcription factors FOXA1 and FOXA2 to be important regulators of mucin expression in intestinal epithelial cells as well[18]. Additionally, the human C/EBPα gene was found to expressed at the highest level in the placenta followed by the liver, lungs, skeletal muscle, pancreas, small intestine, colon and in peripheral blood leucocytes[2]. As was reviewed elsewhere C/EBPα plays an important role in cell cycle control, cellular differentiation, many metabolic processes and the detoxification. The difference in C/EBPα expression may in part be the result of control of epithelial replacement through control of the p21

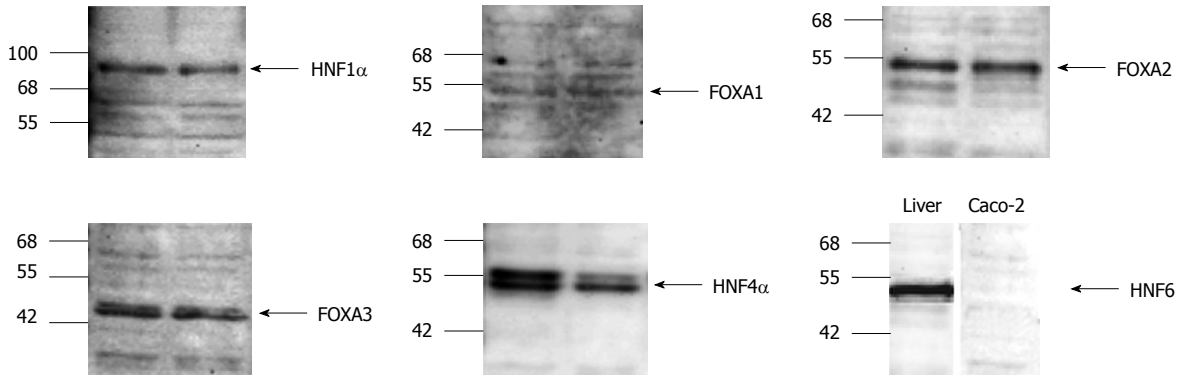

Figure 5 Western blotting of hepatocyte nuclear factor 1α, FOXA1, FOXA2, FOXA3, hepatocyte nuclear factor 4α and hepatocyte nuclear factor 6 in different human colon carcinoma Caco-2 cell line cultures. Note, in case of hepatocyte nuclear factor (HNF) 6 healthy human liver serves as control.

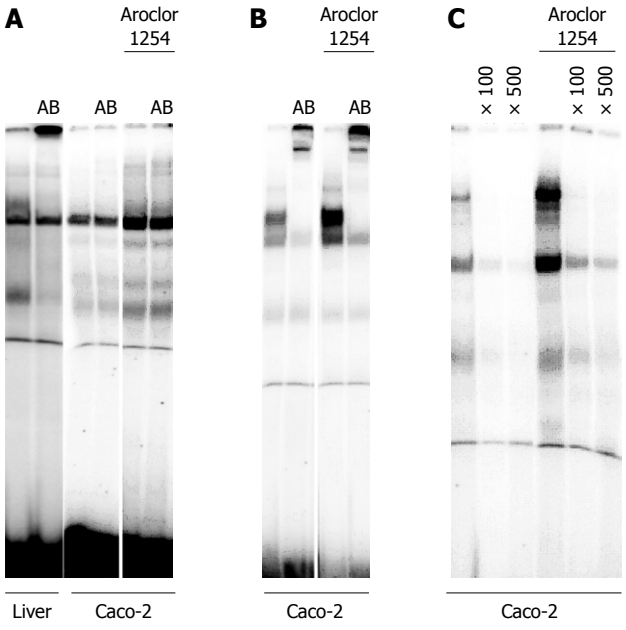

Figure 6 Electromobility shift assay with nuclear extracts of the human colon carcinoma Caco-2 cell line. A: Demonstrates the DNA binding of HNF6 to an optimized oligonucleotide probe using either nuclear protein extract of human liver tissue or Caco-2 cells. Note, the lane labeled as AB refers to the addition of a hepatocyte nuclear factor 6 antibody to demonstrate specificity of the DNA binding assay; B: Demonstrates the DNA binding of HNF4α to an optimized oligonucleotide probe using either nuclear protein extract of human liver tissue or Caco-2 cells. Note, the lane labeled as AB refers to the addition of a HNF4α antibody to demonstrate specificity of the DNA binding assay; C: Demonstrates the DNA binding of NGN3 (a HNF6 target gene) to an optimized oligonucleotide probe using either nuclear protein extract of human liver tissue or Caco-2 cells. Note, the lane labeled as × 100 or × 500 refers to competition assays with excess of unlabeled probe.

protein level thereby determining epithelial replacement[2].

In agreement with our previous study on colorectal liver metastases we were unable to detect HNF6 transcript expression in healthy and cancerous colonic tissue while the expression of splice variants of HNF4α in healthy colonic tissue was a significant finding and is likely to impact HNF4α activity, as we have shown recently in the case of human hepatocellular carcinoma[19]. Indeed, mice lacking HNF4α exhibited decreased levels of polysaccharides and acetic mucopolysaccharides with some altered expression of mucins and aquaporins[15].

The use of the human colon carcinoma cell line Caco-2 allowed us to compare expression of transcription factors in healthy colon and colonic epithelium of adenocarcinoma. Initially, we investigated expression of individual liver-enriched transcription factors as a function of time and confluency of the Caco-2 cell culture. With the exception of HNF6 there was no statistically significant difference in the expression of transcription factors as a function of cell culture time (up to 17 d) or confluency while cellular differentiation of Caco-2 cells was dependent upon the activity of the transcription factor NF-Y and E2F[20,21]. We also compared expression of transcription factors with those in the healthy colon but did not identify a significant difference.

Overall, our study identified local and segmental differences in the expression patterning of liver-enriched transcription factors in the human intestine. Tissue specific transcription factor expression provides a regulatory circuitry for the control of gene expression and cellular differentiation. The present study identifies significant differences in the expression of liver-enriched transcription factors amongst different segments of the human intestine that impacts on the epithelial cell biology of the gut.

COMMENTS

Background

Liver-enriched transcription factors are versatile proteins pertinent for cellular growth and differentiation of the liver. Growing evidence suggests these factors play a wider role in epithelial biology and cancerous diseases of the digestive tract. Indeed, recent studies provided evidence for hepatocyte nuclear factor 6 (HNF6) and FOXA2 as key regulators in colorectal liver metastases. As HNF6 was only expressed in colorectal liver metastases but not in primary colonic cancer or healthy colonic tissue, the authors investigated regulation of HNF6 and of other liver-enriched transcription factors in different segments of the human intestine thereby providing information on their expression pattern. Regional differences in the expression and regulation of transcription factors may be related to growth of intestinal tumors and may influence metastatic spread.

Research frontiers

The authors examined the gene expression pattern of liver-enriched transcription factors in different segments of the human intestine and compared the findings with results obtained from a human colon carcinoma cell line for a better understanding of their regulation in gut biology and disease.

Innovations and breakthroughs

This is the first study to investigate the expression of liver-enriched transcription factors in the human intestine and compare the data with the human colon

carcinoma cell line Caco-2. Knowledge of the expression pattern of liver-enriched transcription factors in different segments of the human intestine help to better understand the importance of HNF6 and FOXA2 in colorectal liver metastases.

Applications

Tissue specific transcription factor expression provides a regulatory circuitry for the control of gene expression and cellular differentiation of the gut epithelium. Restoring attenuated transcription factor DNA binding activity represents a novel strategy for the treatment of secondary malignancies of the liver.

Terminology

Liver-enriched transcription factors play a pivotal role in disease. Essentially, transcription factors are master regulatory proteins and interact with many different molecules including coactivators, repressors, enzymes, DNA and RNA to control gene expression. Such interactions will inevitably repress or activate gene expression and therefore determine cellular phenotype.

Peer review

This paper examines the distribution of liver-enriched transcription factors and related genes in human intestinal tissues. It represents a useful and novel contribution to the field.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3928

World J Gastroenterol 2010 August 21; 16(31): 3928-3935
ISSN 1007-9327 (print)

BRIEF ARTICLE

Liver sinusoidal endothelial and biliary cell repopulation following irradiation and partial hepatectomy

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Received: March 7, 2010 Revised: April 26, 2010
Accepted: May 3, 2010
Published online: August 21, 2010

Abstract

AIM: To investigate whether irradiation (IR) and partial hepatectomy (PH) may prepare the host liver for non-parenchymal cell (NPC) transplantation.

METHODS: Livers of dipeptidyl peptidase Ⅳ (DPP Ⅳ)-deficient rats were pre-conditioned with external beam IR (25 Gy) delivered to two-thirds of the right liver lobules followed by a one-third PH of the untreated lobule. DPPⅣ-positive liver cells (NPC preparations enriched for liver sinusoidal endothelial cells (LSECs) and hepatocytes) were transplanted via the spleen into the recipient livers. The extent and quality of donor cell engraftment and growth was studied over a long-term interval of 16 wk after transplantation.

RESULTS: Host liver staining demonstrated 3 different repopulation types. Well defined clusters of donor-derived hepatocytes with canalicular expression of DPPⅣ were detectable either adjacent to or in between large areas of donor cells (covering up to 90% of the section plane) co-expressing the endothelial marker platelet endothelial cell adhesion molecule. The third type consisted of formations of DPPⅣ-positive duct-like structures which co-localized with biliary epithelial CD49f.

CONCLUSION: Liver IR and PH as a preconditioning stimulus enables multiple cell liver repopulation by donor hepatocytes, LSECs, and bile duct cells.

Key words: Cell transplants; Dipeptidyl peptidase Ⅳ protein; Endothelial cells; Liver cell transplantation; Liver irradiation; Liver repopulation

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Krause P, Rave-Fränk M, Andreas Wolff H, Becker H, Christiansen H, Koenig S. Liver sinusoidal endothelial and biliary cell repopulation following irradiation and partial hepatectomy. *World J Gastroenterol* 2010; 16(31): 3928-3935 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3928.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3928

INTRODUCTION

Hepatocyte transplantation is considered to be a promising option for the treatment of both acute and chronic liver failure as well as for the correction of end stage metabolic liver disease[1]. However, host liver repopulation by transplanted hepatocytes requires a special preparative regimen combining the induced failure of endogenous cell proliferation with some strong mitogenic stimulus. There are a number of experimental protocols based on the application of DNA-damaging toxins such

as the pyrrolizidine alkaloids retrorsine[2] or monocrotaline[3], which efficiently inhibits the proliferative capacity of endogenous hepatocytes; however these systemically harmful and potentially carcinogenic substances are not suitable for preparation of the human liver.

Recently, we developed a preclinical rat model using external beam liver irradiation (IR) with 25 Gy administered to the right liver lobules (2/3 of liver mass) in combination with 1/3 partial hepatectomy (PH) of the untreated left liver lobule. In that study, the aim was to prime the residual host liver mass for selective donor cell growth, leading to significant liver repopulation by donor hepatocytes[4]. In a further study, we investigated the underlying molecular effects, demonstrating that IR suppressed the regeneration of endogenous hepatocytes in response to PH through a persistent block of the cell cycle[5]. Additionally, we were able to reveal considerable damage to the liver sinusoidal endothelial cells (LSECs), contributing to the notion that these cells are particularly vulnerable to IR[6,7]. However, there is a paucity of data regarding the possible IR damage to bile duct cells and its consequences for cell therapy purposes.

Therefore, the aim of this study was to investigate whether liver injury following IR and PH could not only enable donor hepatocyte proliferation, but more importantly the growth of transplanted non-parenchymal cells (NPCs) in the host parenchyma. With this in mind, we hypothesized that host liver IR permits the replacement and reconstitution of endogenous endothelial as well as biliary cells by donor cells.

MATERIALS AND METHODS

Reagents

Medium and buffers were supplied by Gibco Brl, Germany. All further chemicals were reagent grade and, unless specified otherwise, were supplied by Sigma-Aldrich (Munich, Germany). Primary antibodies were used in this study as summarized in Table 1. Secondary peroxidase-conjugated antibodies (EnVision Kit) were purchased from DAKO Diagnostica, Germany. Secondary species-specific fluorescence conjugated antibodies (Alexa Fluor 488, Alexa Fluor 555) were obtained from Molecular Probes (Goettingen, Germany).

Isolation of primary hepatocytes and NPC preparation

Hepatocytes and NPCs were isolated in a 2-step *in situ* collagenase digestion of the liver[8]. The hepatocytes were segregated using gradual centrifugation steps at 35 *g* for 10 and 5 min and processed separately. Freshly isolated hepatocytes (purification grade approx 98%) displaying a vitality of greater than 90% (tested with trypan blue exclusion) and cell attachment greater than 70% proved to be sufficient for further transplantation experiments. NPCs in the supernatant were centrifuged for 7 min at 400 *g* and resuspended in 20 mL phosphate-buffered saline (PBS). The suspension was further processed by a 2-step Percoll gradient centrifugation (25% and 50%) for 10 min at 1000 *g* aimed at enriching the endothelial cell content. The cells of the interface were washed for 10 min at 200 *g* and resuspended in 800 μL PBS for transplantation. Cytospins were performed to characterize the transplanted cells with immunohistochemistry.

Animals, liver preconditioning, and transplantation experiments

As recipients, a strain of dipeptidyl peptidase Ⅳ (DPP Ⅳ)-deficient Fisher 344 rats was established in the animal care facility of the University Medical Centre Goettingen, Germany. Syngeneic donor Fisher 344 rats were purchased from Charles River, Germany. All animal breeding, care, and experimentation procedures were in accordance with German national legislation on animal protection. All procedures were performed under constant sevoflurane/oxygen inhalation. Buprenorphine (0.1 mg/kg body weight) was applied intraperitoneally during anesthesia, and was repeated subcutaneously 8-12 h later.

Recipient livers of rats were preconditioned with external beam, computed tomography-based partial liver IR (25 Gy) of the right liver lobules (2/3 of hepatic mass) 4 d prior to 1/3 partial hepatectomy (PH) as described previously[4]. For transplantation experiments, the spleen was mobilized and the cell suspension was slowly injected over 3 min into the parenchyma, from where they are known to migrate via the portal vein into the recipient liver (all residual liver lobules). In experimental group 1, rats were transplanted with 20×10^6 NPCs and additionally received 12×10^6 hepatocytes. Experimental group 2 was only transplanted with NPC preparations. Control animals were transplanted with hepatocytes only.

Rats were sacrificed for tissue analysis after 1 wk and after the long-term interval of 16 wk following transplantation. Tissue samples from each liver lobe were excised and snap frozen in 2-methylbutane at -80℃. Cryosections of 5 μm thickness were fixed in ice-cold acetone for 10 min.

Immunofluorescence analysis

Cytospins of cell preparations (3×10^4 cells were centrifuged onto a glass slide at 28 *g* for 5 min) or cryosected tissues were immunostained for the first antigen (incubation with the first primary antibody [anti-CD49f, anti-CD45, anti-desmin, anti-CX32, anti-platelet endothelial cell adhesion molecule (PECAM) or anti-hepatic sinusoidal endothelial (HSE) marker], using Alexa 488-conjugated goat anti-mouse IgG or anti-rabbit for fluorescence detection [1:400, 1 h at room temperature (RT)] and then further processed with the second immunostaining protocol. After rehydration in Tris buffer, specimens were blocked and subsequently incubated with the second primary antibody (anti-DPPⅣ), rinsed with Tris buffer and exposed to the second Alexa Fluor 568 goat anti-mouse IgG2a (1:400, 1 h at RT). Slides were finally covered with Vectashield® mounting medium with DAPI (1 μL/mL) (Vector Laboratories, UK) to visualize the cell nuclei. Negative controls were used for each antibody by omitting the primary an-

Table 1 Antibodies used for immunofluorescence analysis

Antibody/detected antigen	Species	Manufacturer	Cat. No.	Dilution
DPPIV (dipeptidyl peptidase IV = CD26)	Mouse monoclonal IgG2a	BD pharmingen	559639	1:100
CD45 (leukocyte common antigen)	Mouse monoclonal IgG1	BD pharmingen	554875	1:20
CD49f (integrin α6)	Mouse monoclonal IgG1	Serotec	MCA 2034	1:500
Desmin (hepatic stellate cells)	Rabbit	Lab vision	RB-9014	1:500
Connexin 32 = CX32 (gap junction protein)	Rabbit	Sigma-aldrich	C3595	1:5000
HSE (hepatic sinusoidal endothelial cells)	Mouse monoclonal IgG2a	IBL	10078	1:500
CD31 (PECAM-1 = platelet endothelial cell adhesion molecule-1)	Mouse monoclonal IgG1	BD pharmingen	55025	1:1000

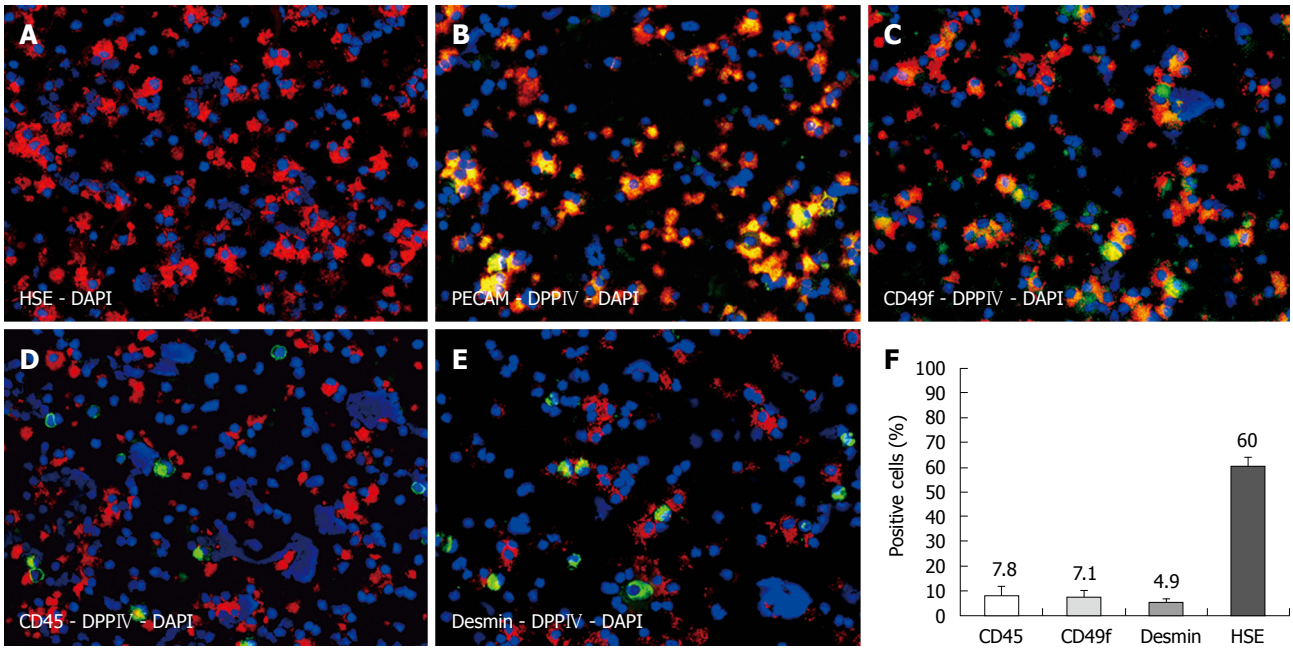

Figure 1 Characterization of non-parenchymal cell preparations by immunofluorescence on cytospins. Hepatic sinusoidal endothelial (HSE) (red) identifying approximately 60% of all cells as hepatic sinusoidal cells (A), donor specific dipeptidyl peptidase IV (DPPIV) (red) co-localized with platelet endothelial cell adhesion molecule (green) (B), and with CD49f as bile duct marker (green) (C). Donor cells were negative for CD45 (D) and desmin (E). Labeling indices (F), nuclear counterstaining with DAPI (blue), original magnification × 200.

tibody from the protocol. Multiple immunofluorescence-labeled specimens were serially excited and observed with the TEXAS Red-, FITC- and UV-filter sets on an inverted confocal microscope (LEICA DM IRE2, Bensheim, Germany). Pictures of each filter set were digitally merged using image layering software (Leica FW 4000, Version 1.1). The labeling index was expressed as a percentage of positive cells counted.

RESULTS

Characterization of NPC preparations

Immunofluorescence co-localization studies were performed on cytospins of freshly isolated NPC preparations enriched with LSECs (Figure 1A), of which 80% were immunoreactive for DPPIV. The majority of all cells displayed the hepatic sinusoidal endothelial marker HSE (60%). For technical reasons, the anti-HSE could not be co-stained with DPPIV, as both antibodies are mouse monoclonal of the same subtype (IgG2a). We therefore used anti-PECAM to co-localize the endothelial marker with the donor-specific antigen in sections of transplanted livers. Indeed, when examining cytospins of NPC preparations, all PECAM-positive cells co-expressed donor specific DPPIV (Figure 1B). Biliary epithelial cells expressing CD49f represented the second largest fraction (7.1%), and these cells were colocalized with DPPIV too (Figure 1C). Furthermore, we assessed co-staining with CD45 and desmin to identify other NPCs. We detected CD45-positive cells (7.8%), representing hematopoietic cells, and were mostly negative for the donor cell antigen (apart from very few activated T lymphocytes) (Figure 1D). Desmin-positive cells (4.9%) did not display DPPIV either (Figure 1E). Figure 1F depicts the labeling indices as stated. We could not detect any cytokeratin 18-positive cells in the NPC preparations, indicating there was no contamination with hepatocytes (data not shown).

Qualitative assessment of liver repopulation

NPC preparations and hepatocytes from donor wild-type rats (DPPIV-positive) (experimental group 1) were transplanted into DPPIV-deficient recipients following the repopulation stimulus of partial IR and PH. Immunofluorescence co-localization studies assessed the extent and

quality of liver repopulation after 1 and 16 wk. One week following transplantation, single DPPIV-positive cells and small clusters were detectable in the host liver parenchyma (data not shown). However, after 16 wk, extensive repopulation by donor cells and descendents was documented in the transplanted livers of all groups. On gross examination, the repopulated areas appeared histologically identical to normal, unharmed liver tissue. Morphological evaluation revealed 3 different types of donor-derived cell [Figure 2A (overview) and more detailed in Figure 2B]. Firstly, well-defined clusters of donor cells displaying DPPIV in a canalicular (garland-like) pattern were found in close proximity to the portal areas. These compact clusters appeared to comprise mature hepatocytes with the characteristic enzyme expression of DPPIV in the basolateral membranes. Their size varied and ranged from 40-50 to several hundred cells in diameter. Cells in these clusters co-expressed the hepatocyte differentiation markers cytokeratin 18, connexin 32 (CX32) (gap junction protein enabling intercellular communication) and cytochrome p450 subtype 2B1, revealing intact metabolic function (data not shown). Secondly, large areas of DPPIV-positive donor cells were arranged in a string-like pattern and emerged from the portal veins. They covered a maximum 90% of the section plane and expressed the donor specific antigen as it is known from LSECs (longitudinal cells expressing DPP IV in the cytoplasm as well in the membranes). Thirdly, formation of duct-like structures expressing DPPIV could be detected. These donor-derived cells were mostly found in association with the bile duct system of the recipient liver and co-stained with an antibody detecting the biliary epithelial CD49f (Figure 2C). The red fluorescent DPP IV on the apical side partially overlapped with the green cytoplasmatic staining of CD49f resulting in a yellow ring. These duct-like structures were also found as individual formations outside the donor hepatocyte clusters, mostly but not always encircled by endothelial donor cells.

In experimental group 2 (transplantation of NPC preparations only), the extent of repopulation was similar, apart from the fact that no clusters of hepatocytes could be detected in these host livers. Subtotal repopulation by DPPIV-positive endothelial cells could be visualized in these animals and donor-derived bile duct structures were as frequent as in group 1, being scattered throughout the parenchyma but generally in close proximity to the portal triads.

Phenotypic analysis of NPC repopulation

Subsequent analysis revealed the phenotypic characteristics of the transplanted cells and their descendents. The pan-endothelial marker PECAM (CD31) is most commonly used to detect LSECs *in situ*. In the present study, this marker was also employed to co-localize LSECs with donor specific DPPIV. Co-staining of DPPIV and PECAM resulted in a yellow-orange overlay, suggesting the endothelial phenotype of the string-like repopulation areas (Figure 3A-C). The merged figure clearly illustrates that both antigens were present in the squamous layer of cells that lined the interior surface of the sinusoids. It has to be pointed out that there was no donor-specific DPPIV co-expression in the endothelial cells of the portal vessels.

When the repopulating LSECs were double-labeled for DPPIV and CX32, the punctuated canalicular pattern seen in hepatocytes was not apparent (Figure 3D). Additionally, the LSECs were negative for desmin, a marker of hepatic stellate cells (Ito cells) (Figure 3E). Indeed, desmin-positive host cells could be clearly visualized in between the repopulating donor endothelial cells but also in the surrounding tissue. In both cases, the distribution pattern was not different from healthy liver. Furthermore, there was no evidence of CD45 co-expressing donor blood cells in transplanted livers (data not shown). Figure 3F shows DPPIV-positive ductular cells co-expressing CD49f. In this picture, the donor biliary epithelial cells form duct-like structures of different sizes, which is representative of the overall repopulation of this kind. Some ductules only have a narrow diameter, whereas others form large ducts consisting of some dozen surrounding cells. On examination of serial sections, it appears that the duct formations possess a 3-dimensional structure of communicating tubes.

DISCUSSION

Hepatocyte transplantation has been used in many animal models. Preconditioning of the host liver prior to cell transfer is regularly used to enhance proliferation of the transplanted cells, up to a near total repopulation of the host liver by donor hepatocytes and their descendents. However, little is known as to whether the replacement of endogenous NPCs may be facilitated in terms of a preparative regimen for cell therapy.

Hepatic IR is an established stimulus for priming the host liver for hepatocyte repopulation purposes in preclinical models[4,9]. Moreover, IR is already being considered for human application in the upper abdomen for the high dose treatment of gastrointestinal and hepatic primary malignancies or metastases[10-13]. IR can be therapeutically targeted to a whole organ or to small portions, but photons always affect the variety of cells present. The liver comprises different cell types, of which the majority represents hepatocytes, making up approximately 80% of hepatic cells[14]. The NPC fraction (20%) comprises endothelial cells, Kupffer cells, stellate cells, epithelial cells of bile ducts, and some neural cells. However, when considering therapeutic strategies, the endothelium and bile duct cells are known to be highly susceptible to injuries following microenvironmental changes (e.g. caused by ischemia/reperfusion, endotoxemia, tumor growth, angiogenesis or the response to cytotoxic treatment)[15-17]. Radiation also induces oxidative stress through the mitochondria-dependent generation of reactive oxygen species and is therefore another potent candidate that may harm NPCs of the liver[18,19]. Considering liver IR as a preparative regimen with clinical prospects, we wanted to demonstrate whether the suggested "multi-cell" damage may be compensated for by subsequent transplantation of liver cell suspensions explicitly containing NPCs solely or in addition to hepato-

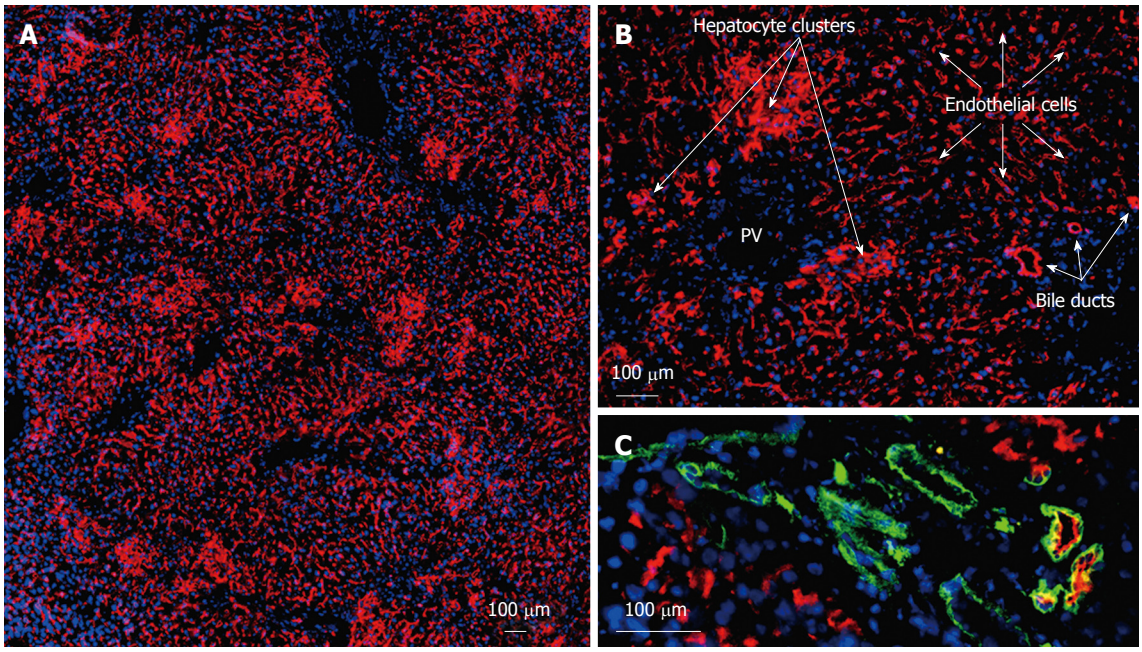

Figure 2 Characteristics of dipeptidyl peptidase Ⅳ-positive cells in the liver following transplantation of non-parenchymal cell preparations and hepatocytes. Frozen sections were obtained from recipient livers present at 16 wk post-transplantation. Acetone-fixed frozen sections were single or double-labeled by indirect immunofluorescence analysis with an antibody detecting the donor cell specific dipeptidyl peptidase Ⅳ (red) (A-C) and bile-duct specific anti-CD49f (green) (C). Merged images are combined with blue nuclear DAPI-staining. Circumscribed and compact clusters of hepatocyte repopulation could be distinguished from large areas of endothelial repopulation which covered a maximum of 90% of the section plane. Formation of bile duct-like structures could be detected by co-staining with the duct marker CD49f. PV: Portal vein.

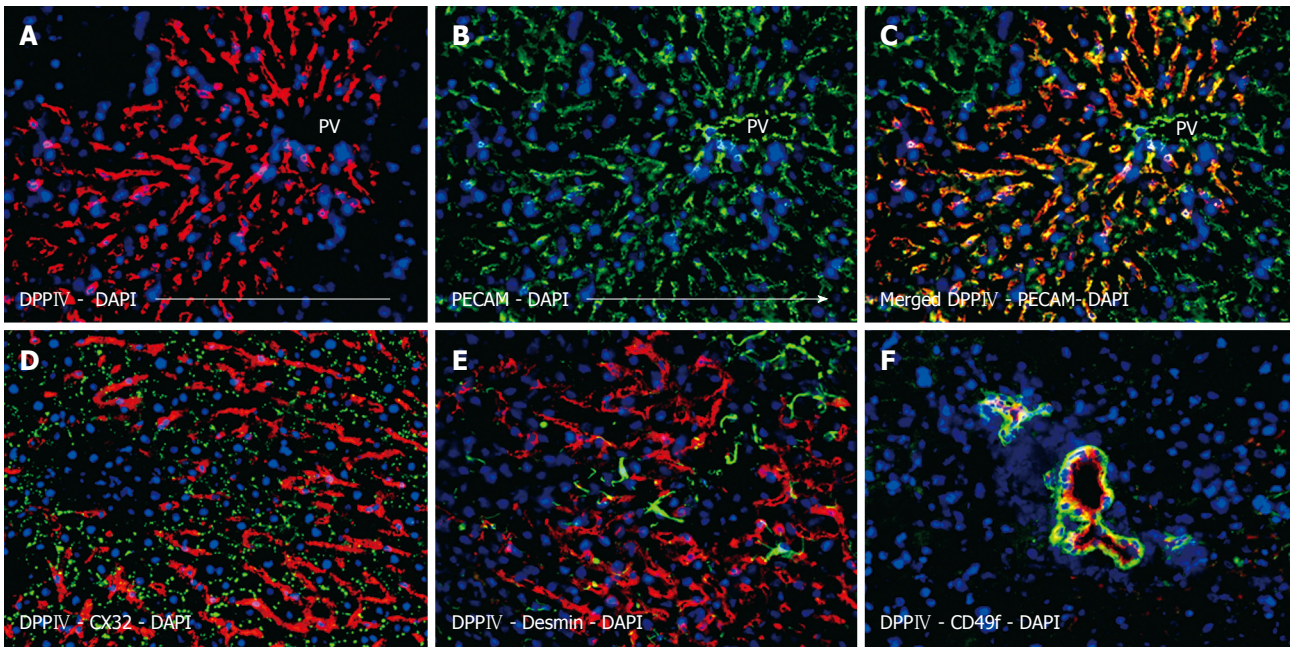

Figure 3 Phenotypic assessment of the non-hepatocytes liver repopulation showing extensive and string-like repopulation by liver sinusoidal endothelial cells emerging from the portal veins. Donor derived cells were identified by dipeptidyl peptidase Ⅳ (DPPⅣ) immunofluorescence staining (red) (A), which co-localized with the endothelial marker PECAM (green) (B), overlay (C). Donor endothelial cells could be clearly distinguished from endogenous hepatocytes which were outlined by the hepatic differentiation marker CX32 (green) (D), desmin-positive cells (green) were detectable with a regular staining pattern for normal (unharmed) liver (E). Additionally, DPPⅣ-positive donor cells formed bile duct structures which expressed the specific marker CD49f (green) (C), nuclear counterstaining with DAPI (blue), original magnification × 200.

cytes. We used a reliable transplantation model (hepatocytes isolated from wild type Fisher 344 rats were transplanted into DPPⅣ-negative hosts) to assess the extent and quality of liver repopulation by donor cells[20].

As proof of concept, our study demonstrated that the preparative regimen of IR and PH led to recipient liver repopulation by sinusoidal endothelial and biliary cells. Replacement of the endothelium was extensive, covering

up to 90% of the section plane. Bile duct-forming structures could often be detected in the neighborhood of endogenous bile ducts, but biliary cells derived from donors were also seen as individual ductular arrangements in the recipient parenchyma. It has to be pointed out that both types of cell engraftment (endothelial and biliary) were not necessarily bound to additional hepatocyte transplantation. In the experimental group of NPC transplantation only, engraftment and repopulation occurred to the same level and extent as following transplantation in addition to hepatocytes. In the latter group, repopulation by all 3 cell types was rarely seen separated from each other, but more frequently at the same site.

Our results are in line with recent literature reporting that hepatic IR is a highly desirable preparative stimulus[9,21,22]. However, IR in those studies was always performed as an invasive procedure (by laparotomy) with doses of 15-50 Gy administered to the whole liver. We focused on a more clinically acceptable approach of non-invasive external beam IR administered to selected lobules of the liver. The workgroup of Guha also demonstrated the outstanding role of the hepatic sinusoidal endothelial barrier as a key player supporting subsequent donor hepatocyte engraftment[9]. They found that IR caused a transient disruption of the endothelial cell lining with exfoliation facilitating the subsequent passage of donor hepatocytes. Indeed, this report confirms our previously published results revealing the significant damage to LECs following IR [prolonged detection of double strand breaks (phosphorylated histone H2AX)][5]. These observations encouraged us to perform the present transplantation study elucidating the feasibility of NPC replacement in irradiated liver.

There are a few reports in the literature confirming the engraftment of NPCs in preconditioned liver. Brilliant and co-workers used injections of mitomycin C to demonstrate that bile ducts and endothelial cells could be generated within 4 wk following transplantation[23]. This substance is commonly known as a cytotoxin widely used as an effective anti-cancer agent, e.g. to treat bladder carcinoma or gastrointestinal tumors[24,25]. Mitomycin C and PH offered a rapid protocol to assess the engraftment efficiency of fetal liver as well as adult liver isolates. However, the preconditioning protocol resulted in a high morbidity (up to 100%) which could be reduced, though not totally eradicated by the additional application of antibiotics (gentamycin). In our study, both morbidity and mortality in all groups prior to and following hepatocyte transplantation remained low overall (1%-2%). The cases of mortality examined were determined to be caused by individual narcotic or surgical complications than to the preconditioning by IR.

The workgroup of Gupta reported using a murine knockout transplantation model in which the liver endothelium was repopulated sufficiently following the administration of the genotoxic pyrrolizidine alkaloid monocrotaline[26]. They demonstrated that transplanted LECs proliferated and reconstituted 9% of the host liver NPCs after 3 mo, thereby correcting the bleeding phenotype of NOD/SCID hemophilia A mice by elevating the Factor Ⅷ activities to over 10% (13 of 15 animals). This study clearly demonstrated the functional correction of a genetic defect as an excellent example for the feasibility of targeted cell therapy. Once again, the preparative agent monocrotaline could not be specifically targeted at the liver. When applied systemically, monocrotaline is known to cause clinically relevant injuries to the endothelium of the lung, resulting in pulmonary hypertension which may obviate its human application[27].

It is well known that high dose IR of the liver is hindered by the induction of radiation induced liver disease (RILD) and more severely by fatal veno-occlusive disease, a non-thrombotic obliteration of the lumina of small intrahepatic veins initially triggered by endothelial injury leading to the deposition of fibrin-related aggregates in the subendothelial zone[28]. These aggregates, and the intramural entrapment of fluid and cellular debris, progressively occlude the hepatic venous flow and generate intrahepatic hypertension. Owing to the risk of RILD, whole liver IR doses exceeding 30 Gy have to be generally avoided in humans[13]. However, we may address the question as to whether endothelial reconstitution following autologous transplantation may allow for the higher IR doses necessary in the local treatment of advanced intrahepatic tumors and multi-lobular metastases.

Endothelial cell reconstitution may also be used to reduce the host immune reaction in liver transplantation[29]. Taking into account that endothelial cells play a pivotal role in both acute and chronic rejection, the transplant immunogenicity could be significantly reduced by endothelial chimerism. A possible strategy would be to generate a chimeric liver, in which damaged endothelial cells are replaced by host cells. Two different mechanisms may be considered: firstly, endothelial damage can result from ischemia/reperfusion injury *sui generis* in whole organ transplantation from cadavers, or secondly may be generated by limited dose IR of the donor liver organ. The workgroup of Murase already demonstrated that circulating endogenous bone marrow-derived cells routinely contributed to LSEC repopulation between 1% and 5% in a rat model of naïve orthotopic liver transplantation[30]. Further experimentation might elucidate whether the rate of engraftment is enhanced by exogenously delivered endothelial cells or progenitors[31,32] or by additional IR.

In the present report, we show for the first time that the preconditioning stimulus of liver IR and PH triggers the repopulation of hepatocytes, LSECs and bile duct cells. We may conclude from our results that this "multicell" repopulation compensates for the liver tissue damage following IR. The extensive engraftment of endothelial cells in particular offers a variety of new therapeutic concepts concerning high dose IR of the liver, immunological cell chimerism in liver allografts, and the treatment of genetic disorders based upon endothelial cells (e.g. lack of coagulation factors such as Factor Ⅷ and von Willebrand Factor).

ACKNOWLEDGMENTS

The authors would like to thank Sabine Wolfgramm and

Sabrina Goldmann for their excellent work in preparing the immunolabeling figures for this article. We would furthermore like to express our appreciation to Andrew Entwistle for his critical review of the manuscript.

COMMENTS

Background

Hepatocyte transplantation is regarded as a promising option to correct acute liver failure and hereditary metabolic liver disease. However, the liver not only constitutes mature hepatocytes, but also a multitude of non-parenchymal cells. So far, little is known concerning the supportive role of non-parenchymal cells in cell therapy studies and whether their engraftment and subsequent proliferation in the host parenchyma may be triggered.

Research frontiers

Liver repopulation is based on the preferential proliferation of engrafted donor cells in response to some mitogenic stimulus. The aim of this study was to investigate whether irradiation, known to suppress endogenous cell proliferation, and partial hepatectomy as the powerful mitogenic stimulus could prepare the host liver not only for hepatocyte but also for non-parenchymal cell transplantation. Both pretreatment techniques may be considered as suitable in the clinical setting and may be targeted to the implantation site, thereby limiting side effects.

Innovations and breakthroughs

The precondition stimulus of liver irradiation and partial hepatectomy prompted the engraftment of hepatocytes, liver sinusoidal cells and bile duct cells. This "multi-cell" repopulation may not only compensate for irradiation-induced liver damage, thus enabling new therapeutic concepts such as high dose radiotherapy, but also generate immunologically relevant cell chimerism and finally facilitate the correction of genetic disorders based upon endothelial cells. This would broaden the therapeutic potential of liver cell therapy.

Applications

As a proof of concept, the study demonstrated that transplanted non-parenchymal cells repopulate the host liver by forming sinusoidal endothelium as well as bile duct-like structures.

Peer review

This is a well-conducted experimental study that deserves early publication in the journal.

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S- Editor Wang JL **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3936

World J Gastroenterol 2010 August 21; 16(31): 3936-3943
ISSN 1007-9327 (print)

BRIEF ARTICLE

Helicobacter pylori CagA protein polymorphisms and their lack of association with pathogenesis

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Supported by Sciences Faculty, Los Andes University, Bogotá, Colombia and National Cancer Institute, Bogotá, Colombia, Grant No. 41030310-28 (to Bravo MM)
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Received: February 21, 2010 Revised: April 26, 2010
Accepted: May 3, 2010
Published online: August 21, 2010

Abstract

AIM: To investigate *Helicobacter pylori* (*H. pylori*) CagA diversity and to evaluate the association between protein polymorphisms and the occurrence of gastric pathologies.

METHODS: One hundred and twenty-two clinical isolates of *H. pylori* cultured from gastric biopsies obtained from Colombian patients with dyspepsia were included as study material. DNA extracted from isolates was used to determine *cagA* status, amplifying the C-terminal *cagA* gene region by polymerase chain reaction. One hundred and six strains with a single amplicon were sequenced and results were used to characterize the 3′ variable region of the *cagA* gene. To establish the number and type of tyrosine phosphorylation motifs Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine (EPIYA) bioinformatic analysis using Amino Acid Sequence Analyzer-Amino Acid Sequence Analyzer software was conducted. Analysis of the association between the number of EPIYA motifs and the gastric pathology was performed using χ^2 test and analysis of the presence of EPIYA-C motifs in relation to the pathology was made by logistic regression odds ratios. Comparisons among EPIYA types found and those reported in GenBank were performed using a proportion test in Statistix Analytical Software version 8.0.

RESULTS: After amplification of the 3′ of the *cagA* gene, 106 from 122 isolates presented a single amplicon and 16 showed multiple amplicons. As expected, diversity in the size of the *cagA* unique fragments among isolates was observed. The 106 strains that presented a single amplicon after 3′ *cagA* amplification came from patients with gastritis (19 patients), atrophic gastritis (21), intestinal metaplasia (26), duodenal ulcer (22) and gastric cancer. DNA sequence analysis showed that the differences in size of 3′ *cagA* unique fragments was attributable to the number of EPIYA motifs: 1.9% had two EPIYA motifs, 62.3% had three, 33.0% had four and 2.8% had five motifs. The majority of tested clinical strains (62.3%) were found to harbor the ABC combination of EPIYA motifs and a significant statistical difference was observed between the frequencies of ABCC tyrosine phosphorylation motifs and Western strains sequences deposited in GenBank.

CONCLUSION: The present report describes a lack of association between *H. pylori* CagA-protein polymorphisms and pathogenesis. ABCC high frequency variations compared with Western-strains sequences deposited in GenBank require more investigation.

Key words: *Helicobacter pylori* CagA-protein polymorphisms; Molecular characterization; Bioinformatic analysis; Pathogenesis; Cancer

Acosta N, Quiroga A, Delgado P, Bravo MM, Jaramillo C. *Helicobacter pylori* CagA protein polymorphisms and their lack

of association with pathogenesis. *World J Gastroenterol* 2010; 16(31): 3936-3943 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3936.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3936

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral gram-negative microaerophilic bacillus[1]. This bacteria is one of the most common worldwide human pathogens[2], it is present in at least 50% of the world's population, with the highest incidence recorded in industrially underdeveloped areas, including Asia, Africa and South America[3]. *H. pylori* colonizes the human stomach and persists for several decades, causing chronic gastritis and peptic ulcer diseases[4]. Studies have suggested that chronic infection by *H. pylori* is an important risk factor for the development of gastric carcinoma[5,6]. For this reason, *H. pylori* was defined as a type I carcinogen by The International Agency for Research on Cancer - IARC[7].

The cytotoxin-associated antigen A, CagA, was identified in 1989[8]. It is encoded in the cag pathogenicity island (cag PAI), a segment of 40 kb that codifies components required to ensemble a type IV secretion system (TFSS)[9]. More than 90% of isolated strains from East Asia including Korea, Japan, and China are known to harbor cagA, while 50%-60% of isolated strains from Western countries are positive for it[10]. This gene shows variation which is explained by adaptive evolution, where a genetically diverse *H. pylori* population provides the host with a repertoire of varied phenotypes from which a subpopulation with optimal fitness may be selected[11]. This evolution would operate through recombination between *H. pylori* direct DNA repeats that result in deletion (or duplication) of phosphorylation sites in *cag*A gene[12,13] or the entire genomic cag PAI[14].

The *cagA* gene product, CagA, is directly translocated from *H. pylori* into the gastric epithelia cells the bacteria are attached to *via* TFSS[15-18] and upon localizing in the inner surface of the plasma membrane, CagA undergoes tyrosine phosphorylation by Ab1 and Src family kinases on specific tyrosine residues within a Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine (EPIYA) motif[19-25]. Once CagA is phosphorylated, it interacts with Src homology phosphatase 2 (SHP-2) which stimulates downstream signaling cascades involved in the reorganization of the cytoskeleton, resulting in cellular morphological changes such as the "hummingbird" phenotype[16]. Among the various CagA activities that disturb cellular functions, deregulation of SHP-2 by CagA is of potential importance in gastric carcinogenesis because mutations in PTPN11, the gene encoding human SHP-2, have been identified in human malignancies[26,27].

CagA protein varies in size according to the strain[28,29]. The structure of the gene reveals a 5' highly conserved region and CagA size variation is due to the presence of different types and/or numbers of repeat sequences containing the EPIYA motifs within the C-terminal variable region[28]. Four types of EPIYA segments have been described: A, B, C, and D, each of which contains a single EPIYA motif[30]. Moreover, Panayotopoulou *et al*[22] and Kanada *et al*[31] described the pattern around the EPIYA motif to determine the type to which it corresponds as follows: EPIYA-A, EPIYAKVNKKK(A/T/V/S)GQ; EPIYA-B, E(S/P)IY(A/T)(Q/K)VAKKVNAKI; EPIYA-C, EPIYATIDDLG and EPIYA-D, EPIYATIDFDEANQAG. Earlier studies have shown that CagA protein nearly always contains EPIYA-A and EPIYA-B sites, followed by one to three EPIYA-C repeats in Western-type *H. pylori* isolates[32] or by one EPIYA-D motif in East Asian-type isolates[33]. Src kinase in gastric epithelial cells phosphorylates CagA on EPIYA-C tyrosine residue[34,35]. Consequently, among Western CagA strains, the number of EPIYA-C sites is directly associated with the level of tyrosine phosphorylation. Thus, Western CagA proteins with a greater number of EPIYA-C sites are pathophysiologically more virulent and probably more carcinogenic[36]. The tyrosine phosphorylation status of CagA is important for the pathogenicity of *H. pylori*[22] and this variable number of EPIYA could be of clinical relevance in gastroduodenal diseases. For the above stated reasons it is important to analyze the genetic variability of *cag*A gene from clinical strains in relation to the associated pathologies. In this study, we used a polymerase chain reaction (PCR)-sequencing-bioinformatics strategy to characterize the CagA variable region of *H. pylori* from Colombian isolates. Additionally, the association between CagA diversity and the severity of gastroduodenal disease was analyzed.

MATERIALS AND METHODS

Clinical strains and culture conditions

A total of 122 *H. pylori* strains obtained from the stock collection at the Instituto Nacional de Cancerología, in Bogotá, Colombia, were grown on blood agar plates, supplemented with 7% horse serum (Invitrogen, Grand Island, NY), 1% Vitox (Oxoid, Basingstoke, UK), and Campylobacter selective supplement (Oxoid, Basingstoke, UK), at 37℃ for 3 d in microaerophilic conditions. Three *cagA* positive control strains NCTC 11637, NCTC 11638 and ATCC 43579, were also included. Isolates belonged to patients with different types of gastric pathologies including benign, mild and severe conditions associated with *H. pylori* infection. Histopathology diagnosis was recorded for all voluntary participants.

DNA extraction and PCR assay

Genomic DNA was extracted using AquaPure Genomic DNA isolation kit, BIO-RAD, according to manufacturer's instructions and obtained DNA was stored at -20℃ until PCR amplification. In order to amplify the variable *cagA* region, a PCR assay was carried out in a volume of 50 μL containing 50 mmol/L KCl, 20 mmol/L Tris-HCl, pH (8.4), $MgCl_2$ 1.75 mmol/L, 0.2 mmol/L of each dNTP, 1 pmol/μL of each primer (CAGTF 5-3: ACCCTAGTCGGTAATGGG and CAGTR 5-3: GCTTTAGCTTCTGAYACYGC), previously reported by Yamaoka *et al*[37], 1.25 units of Taq DNA Polymerase [Invitrogen, Carlsbad

(California), USA] and 4 μL of DNA (positive controls with GenBank accession numbers: *H. pylori* NCTC 11637 (AF202973.1), NCTC 11638 (AF282853) and ATCC 43579 (AB015414.1). The PCR conditions included an initial denaturation step: 92℃ for 5 min, followed by 35 cycles of 92℃ for 1 min, 61℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. PCR products were run on 1% agarose gels in 0.5 × TAE buffer at 100 V in a BIO-RAD® electrophoresis system and purified using the Wizard SV Gel and PCR Clean-Up System Kit [Promega, Madison (Wisconsin), USA] according to the manufacturer's instructions, prior to sequencing.

Sequencing of 3' variable region of the cagA region

Sequencing was performed using an ABI PRISM® 310 Genetic Analyzer [Applied Biosystems, Foster City (California), USA], BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The reactions were done in a volume of 10 μL containing 0.5 × of Premix, 0.5 × of buffer, 0.16 μmol/L of each primer and 2 μL of purified DNA. The conditions were: one cycle at 96℃ for 1 min, followed by 25 cycles of 10 s at 96℃, 5 s at 50℃ and 4 min at 60℃. All of the 106 sequences obtained were deposited into GenBank/EMBL/DDBJ database with the following accession numbers: FJ755476 and FJ915841 to FJ915945.

Bioinformatics analysis

The sequences (forward and reverse) were edited and assembled using CLC DNA Workbench [CLC Bio A/S, (Aarhus C), Denmark]. For the characterization and quantification of the EPIYA motifs located in the C-terminal of CagA protein, a software called Amino Acid Sequence Analyzer (AASA) was designed for the study, in order to look for the type and number of EPIYA motifs, using the 6 open reading frames of each sequence. The characterization of tyrosine phosphorylation motifs, which contains EPIYA sequences, was done as previously described by Higashi *et al*[30]. Clustal W program[38] was used to generate a multiple alignment from the amino acid sequences of each strain.

In order to test the capability of the software to establish phosphorylation motifs in an accurate manner and the facility of its use, all controls were processed as a first phase. Sequence data results was as expected according to GenBank databases, so based on these results evaluation of clinical strain was done.

Statistical analysis

Analysis of association between the number of EPIYA motifs and the gastric pathology described was performed using χ^2 test. Analysis of the presence of one or more than one EPIYA-C motifs in relation to the pathology was made by logistic regression odds ratios (OR); a 95% confidence interval (CI) was calculated using SPSS statistical software package version 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons among EPIYA types found and those reported in GenBank were performed using a Proportion test in Statistical Analysis Software, version 8.0 (Software, 1985-2003).

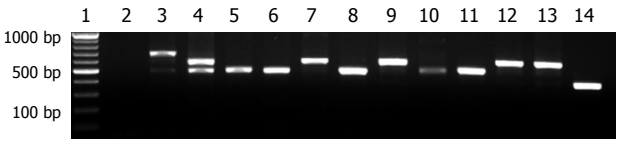

Figure 1 Electrophoretic analysis of the *cagA* 3' variable region by polymerase chain reaction. The polymerase chain reaction amplicons were analyzed on a 1% agarose gels. From left to right, lane 1 shows a 100 base pair DNA ladder, lane 2 negative control, lane 3 reference strain *Helicobacter pylori* NCTC 11637, lanes 4-14 show products of isolates from different patients.

RESULTS

PCR assay

After amplification of the 3' of the *cagA* gene, 106 clinical isolates from 122 isolates presented a single amplicon (a unique band), and 16 showed multiple amplicons (two or more fragments) (Figure 1, lane 4). As expected, diversity in the size of the *cag*A fragment among isolates was observed, and PCR products ranged from 343 to 811 bp. The 106 strains that presented a single amplicon after 3'-*cagA* amplification came from patients with superficial gastritis (19 patients), atrophic gastritis (21), intestinal metaplasia (26), duodenal ulcer (22) and gastric cancer (18).

Bioinformatics analysis of peptide sequences

After sequencing of *cagA* 3' region gene, corresponding peptide sequences from all of the strains with single amplicons were deduced. The combination of the different EPIYA motifs was determined using the ClustalW and AASA software, based on the classification defined by Higashi *et al*[30] and Panayotopoulou and collaborators[22]. The pattern EPIYA(K/Q)VNKKK(A/T)GQ that corresponds to EPIYA-A; the pattern E(P/S)IY(A/T)(Q/K)VAKKV(N/T)(A/Q)KI, to EPIYA-B; and the pattern EPIYATIDDL(G/R) to EPIYA-C were found (Figure 2). All the EPIYA motifs were Western type (Table 1), we did not found strains harboring EPIYA-D, a characteristic pattern of Eastern type.

DNA sequence analysis revealed that 2 of 106 strains (1.9%) had two EPIYA motifs, 66 strains (62.3%) three EPIYA motifs, 35 strains (33.0%) four EPIYA motifs and 3 strains (2.8%) five EPIYA motifs. In 66 out of the 106 (62.3%) strains, the pattern of the EPIYA motifs was ABC type (Table 1). Moreover, 49 of 106 strains had a modified EPIYA-B motif (EPIYT) instead of EPIYA (Figure 2) and there was no association between this type of tyrosine phosphorylation motif (EPIYT) containing a threonine residue instead of an alanine residue and the studied pathologies ($P = 0.51$) (data not shown).

Studies have shown that CagA proteins with more EPIYA-C motifs are expected to be more active biologically than those with a small number of EPIYA-C motifs because they interact more effectively with SHP-2 phosphatases and therefore it could perturb SHP-2-dependent signaling pathways, inducing greater morphological changes or probably contributing to generation of gastric cancer[30,36]. For this reason, in order to determine if there was any relation between the severity of the gastroduodenal

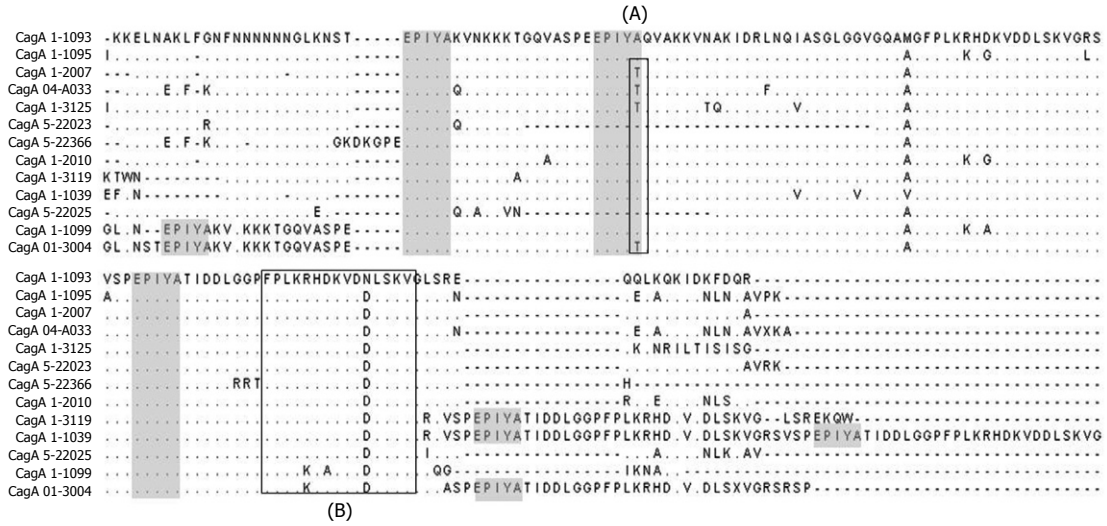

Figure 2 Multiple alignment of the amino acid sequence of the 3' region from *cagA*. First Square (A) shows the Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine (EPIYA) motifs which had a modified EPIYA-B motif (EPIYT) instead of EPIYA. Second Square (B) shows the CagA multimerization (CM) motif which contains the amino acid sequence FPLXRXXXVXDLSKVG. The shaded squares represent the localization of EPIYA motif in the alignment. Amino acids are designated by standard one-letter codes. (·) same residue; (-) gap. GenBank accession numbers and EPIYA motif of each isolate in this figure: CagA 1-1093 (FJ915841) (ABC), CagA 1-1095 (FJ915842) (ABC), CagA 1-2007 (FJ915843) (ABC), CagA 04-A033 (FJ915904) (ABC), CagA 1-3125 (FJ915893) (ABC), CagA 5-22023 (FJ915937) (AC), CagA 5-22366 (FJ915913) (ABC), CagA 1-2010 (FJ915844) (ABC), CagA 1-3119 (FJ915891) (ABCC), CagA 1-1039 (FJ915944) (ABCCC), CagA 5-22025 (FJ915878) (BC), CagA 1-1099 (FJ915912) (AABC) and CagA 01-3004 (FJ915917) (AABCC).

Table 1 Prevalence of different types of CagA tyrosine phosphorylation motifs compared with data obtained from Argent *et al*[39] and Colombian strains reports in GenBank *n* (%)

	Type(s) of EPIYA motif									
	BC	AABC	ABC	ABCC	ABCCC	AABCC	ABABC	AC	D	Total
Gastritis	1	0	10	6	0	1	0	1	0	19
Atrophic gastritis	0	0	15	5	0	0	1	0	0	21
Intestinal metaplasia	0	1	16	9	0	0	0	0	0	26
Duodenal ulcer	0	0	14	8	0	0	0	0	0	22
Gastric cancer	0	1	11	5	1	0	0	0	0	18
This study total	1 (0.9)	2 (1.9)	66 (62.3)	33 (31.1)	1 (0.9)	1 (0.9)	1 (0.9)	1 (0.9)	0 (0.0)	106
Colombian[1]	2 (2.2)	2 (2.2)	49 (53.3)	29 (31.5)	1 (1.1)	2 (2.2)	1 (1.1)	1 (1.1)	0 (0.0)	87
Argent *et al*[39][2]	3 (0.7)	3 (0.7)	262 (63.3)	81 (19.6)	19 (4.6)	1 (0.2)	1 (0.2)	4 (1.0)	[3]	374

[1]CagA sequences of Colombian strains recovered on GenBank; [2]Data for Western CagA EPIYA types (-A, -B, -C); [3]The EPIYA –D was not included in the analysis of this table because it was absent in the Colombian strains. EPIYA: Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine.

disease and the number and type of the EPIYA motifs, statistical analyses were applied.

Statistical tests

A χ^2 test performed showed that in this group of Colombian isolates, the variation of all EPIYA motifs in CagA was not directly associated to the outcome of the disease caused by *H. pylori* ($P = 0.52$). Then, atrophic gastritis, intestinal metaplasia, duodenal ulcer and gastric cancer risks were estimated with respect to a reference group made up of patients with gastritis, infected by *cagA* positive strains with one EPIYA-C motif and those with more than one EPIYA-C motif. No association between the number of EPIYA-C motifs and the pathology was found using logistical regression (Table 2). Proportion test frequencies of the EPIYA genotypes obtained in this study were compared with those of CagA sequences including the C-terminal variable region reported by Argent *et al*[39] and sequences collected from Colombia deposited in GenBank. Statistical differences between the frequencies of ABCC pattern in Western strains previously reported by Argent *et al*[39], and our results were established, $P < 0.002$ (Table 1 and Figure 3).

DISCUSSION

The present work considered amplification and sequencing of the 3' variable *cagA*-gene region and a final bioinformatics analysis of the corresponding C-terminal of CagA protein using AASA software as a single and rapid method for *H. pylori CagA* characterization. Determination of number and type of *H. pylori* CagA phosphorylation motifs has been suggested by some researchers as a way to predict clinical outcome of *H. pylori* associated pathologies and as a prognosis tool by others. So, in order to examine the viability of mentioned approach, 122 Co-

Table 2 Association between *cagA* Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine-C motif number and gastric pathology *n* (%)

Pathology	No more than 1 EPIYA-C motif	At least 2 or more EPIYA-C motif	OR	95% CI
Gastritis	12 (16.9)	7 (20)	1.000	-
Atrophic gastritis	16 (22.5)	5 (14.2)	1.867	0.47-7.34
Intestinal metaplasia	17 (23.9)	9 (25.7)	1.102	0.32-3.78
Duodenal ulcer	14 (19.7)	8 (22.8)	1.021	0.30-4.51
Gastric cancer	12 (16.9)	6 (17.1)	1.167	0.28-3.65
Total	71	35		

EPIYA: Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine.

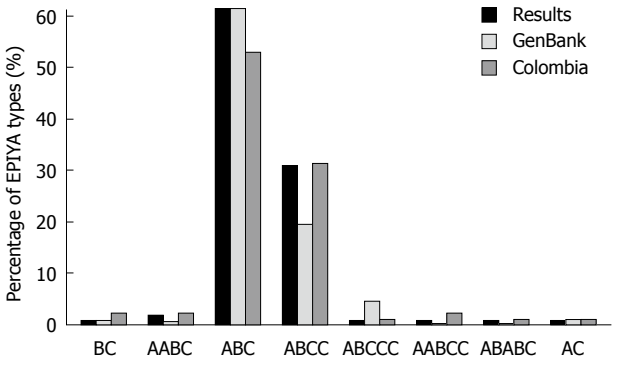

Figure 3 Comparison between the prevalence of different types of CagA tyrosine phosphorylation motifs from this study compared with data obtained from Argent *et al*[39] and Colombian strains reports in gene bank. There is a statistical difference between the frequency of ABCC pattern in Western strains reported in GenBank (19.6%) and our results (31.1%). EPIYA: Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine.

lombian clinical isolates, all of them derived from adults, were analyzed. From 122 initial strains included, 106 isolates that presented a single PCR product were considered for latter analysis. As expected, diversity in the size of the *cag*A fragment among isolates was observed, and PCR products ranged from 343 to 811 bp. This is explained by the fact that *cagA* varies in size depending on the number of EPIYA motifs it has, and because each type of EPIYA adds a certain number of amino acids, since each type is surrounded by a specific amino acid sequence. EPIYA-A represents an additional 32 amino acids in the protein, EPIYA-B 40 more amino acids, and EPIYA-C 34 more amino acids for Western CagA type[40,41]. The remaining 16 strains that showed two or more PCR products were excluded. However, they were considered for further analysis due to: (1) they could represent subclones attributable to a clone microevolution process demonstrating the high recombination rate of *H. pylori* plastic genome; or (2) they could represent a coinfection process with multiple *H. pylori* strains coexisting in the same host. A microevolution process at *cag*A gene has been described previously in the same individual and also in different family members[12,42,43]. Otherwise strains with a polymorphism in the *cagA* variable region have been also observed by Panayotopoulou *et al*[22], Aras *et al*[12] and Reyes-Leon *et al*[42]. The EPIYA patterns found in this study match with those reported by Panayotopoulou *et al*[22], but there were differences for EPIYA-A in which the amino acid Lysine (K) in the 6th position (being the amino acid E of the EPIYA motif the 1st position) changed to K/Q as previously reported[19], EPIYA-B in which the pattern in positions 12 and13 changed from being only Asparagine and Alanine (N, A) to (N/T) and (A/Q) respectively and finally EPIYA-C in which the amino acid in the 11th position changed from Glycine (G) to (G/R) (data not shown, accession number: FJ915913), which had been previously reported by Occhialini[44]. There was no association between the phosphorylation motif, EPIYT, which has a threonine residue instead of an alanine residue and the studied pathologies ($P = 0.51$). However, it has been reported that isolates that harbor the ABCC genotype and have a modification in the 5th residue (EPIYT) in the B type may induce lower levels of cellular elongation and interleukin-8 secretion than isolates with the normal ABCC pattern[42].

EPIYA results from this study were compared with those reported by Argent *et al*[39]. The analyses showed that in our samples the ABC pattern of EPIYA motifs is the most common (62.3%) (Table 1 and Figure 3), which is in agreement with Western sequences reported before (63.3%)[39]. Interestingly, we found a statistical difference between the frequency of ABCC patterns in Western strains reported in GenBank (19.6%) and our results (31.1%) (Table 1) ($P = 0.002$). A search in the GenBank data base was performed in order to look for sequences reported in Colombia[45,46] that included the C-terminal variation region of CagA. A comparison between the frequencies of the different EPIYA patterns with our results showed that there is not a statistically significant difference (Table 1) ($P = 0.98$). Current research showed that the variation of the EPIYA motifs in CagA protein is not directly associated with the outcome of the disease caused by *H. pylori* and that there is no association between the number of EPIYA-C motifs and the pathology (Table 2). Similar results had been reported before in Iranian and Iraqi populations[47], where it seems there is no explicit positive correlation between the number of EPIYA motifs and various gastroduodenal diseases associated to *H. pylori* infection. In contrast, Yamaoka *et al*[37] showed that 7 out of 8 *H. pylori* Colombian strains (87.5%) with more than 3 EPIYA-C motifs were from gastric cancer patients. More recently Sicinschi *et al*[45] evaluated the 3' cagA region in 66 isolates from Colombian patients with gastric precancerous lesions, from areas with low (31 strains) and high risk of gastric cancer (35 strains) in the south of the country. The proportion of strains bearing one EPIYA-C (62.2%), two EPIYA-C (34.3%) and three EPIYA-C (1.5%) motifs were similar to the ones observed in our population of strains. In contrast with our results, they observed a significant association between the presence of two or three EPIYA-C motifs and more severe lesions. Particularly they found a very high prevalence of strains with more than two EPIYA-C motifs in individuals with intestinal metaplasia (16/27, 59%). Sicinschi *et al*[45] included in the study only isolates

from men aged between 39 and 69 years in order to have high prevalence of *H. pylori* infection and preneoplastic lesions; 40% of the analyzed strains were from intestinal metaplasia. Our study included isolates from the Andes Mountains in the central part of the country, a high risk gastric cancer area; we included strains from men and women aged between 19 and 80 years, furthermore we included strains from gastric cancer patients. These differences in the design of the studies and also variations in geographic areas could explain the different results obtained. A limitation of our study is the small size of the analyzed population of strains. Although we could not find differences between EPIYA-C motifs and clinical outcomes, high prevalence of strains with more than one EPIYA-C motif might explain in part the high incidence of gastric cancer in Colombia.

It also must be considered that the absence of association between the CagA polymorphisms and the pathogenesis could be due to other factors such as genetic features of the host and cellular and extracellular environmental variables, which could influence the development of gastric cancer in combination with the type and number of EPIYA motifs in CagA protein. Moreover, another possibility as stated by Püls *et al*[48], using site-specific mutagenesis, suggests that tyrosine phosphorylation at EPIYA-C is sufficient, but not exclusive, to activate translocated CagA, suggesting that other motifs besides EPIYA-C are used for phosphorylation of CagA proteins as well. For this reason it is not only important to study the tyrosine phosphorylation motif, EPIYA, but also other new repetitions that have been discovered recently, such as the 7-AA sequence KIDQLNQ, which occurs in and near the CagA variable region[49]. Another important amino acid sequence is FPLXRXXXVXDLSKVG (Figure 2, second square), which surrounds the EPIYA-C motif and EPIYA-D in Western and East Asian CagA species, this was called CagA multimerization (CM) motif[50]. This CM motif was characterized in all of our strains, and interestingly in the strain 5-22019 (GenBank access number: FJ915938) the mentioned motif does not only surround the EPIYA-C motif but also the EPIYA-A. For this reason, it is important to study the molecular interaction between CagA proteins through the CM motif, because CagA multimerization is critically involved in the formation of the CagA-SHP2 signaling complex, during *cagA*-positive *H. pylori* infection[50]. Thus, it will be important to study the biological activity of Colombian CagA variants that harbor a variation in the C-terminal of the CagA protein, in order to comprehend how the interaction between the EPIYA-C motif and the SRC-2 protein in the epithelial cells works.

At the structural level it is important to determine how the EPIYA motifs are located in the CagA protein and if it influences CagA-SRC interaction, and consequently contribute to elucidate the biological activity of the CagA protein in the development of carcinogenesis in epithelial cells through this interaction. It is also important for future studies to check the evolution of the *cagA* gene in patients with atrophic gastritis, because this pathology has been reported as an early step in the development of gastric cancer[51] and this combined with genetic variability on the *cagA* gene generated by multiple rearrangements such as insertions, deletions, and substitution events on a 102nt region encoding the EPIYA-C motif could increase the probability of developing a gastric cancer pathology[52]. This kind of study has already been developed through hybridization assays with whole-genome DNA microarrays studies from Colombian strains[53] and monitoring the acquisition of additional EPIYA-C motifs throughout adulthood to determine if they contribute to *H. pylori* biological activity at a later age[22].

Our findings suggest that CagA polymorphisms EPIYA motifs, in Colombian patients are not clearly associated with the outcome of the disease. For this reason, it could be important to evaluate if the EPIYA motifs variation can cause an effect in the CagA protein structure and if so, if it can be correlated with its interaction inside the gastric epithelial cells. CagA has a striking functional similarity with Gab, Grb2 associated binder adaptor, in terms of being a protein that recruits SHP-2 to the plasma membrane and activates SHP-2 phosphatase by forming a physical complex[54]. Accordingly, it could be important to understand the role of CagA variants inside the gastric epithelial cells at protein-protein interaction level. On the other hand, microevolution in *cagA* gene has important implications for *H. pylori* pathogenesis because changing CagA type may provide a partial explanation of why studies examining the relationship between bacterial virulence and disease do not show tighter associations[43]. Finally, the CagA variable region characterization was successfully achieved and it gave an important register of the CagA protein polymorphisms in Colombian patients.

ACKNOWLEDGMENTS

We would like to thank colleagues who developed the bioinformatics software (AASA): Eric Gerard Rothstein Morris, Miguel Andrés Yáñez Barreto and Sonia Vivas from the Engineering Faculty, Los Andes University, Bogotá, Colombia.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is one of the most common worldwide human pathogens and it is present in at least 50% of the world's population. Studies have suggested that chronic infection by *H. pylori* is an important risk factor for the development of gastric carcinoma, due to the presence of an important virulence factor, CagA protein.

Research frontiers

CagA protein is a potential factor in the development of gastric carcinogenesis in human gastric epithelial cells. However, the relation between the polymorphisms on CagA and the pathogenesis of *H. pylori* in gastric cells has not been addressed in terms of number and type of Glutamine acid-Proline-Isoleucine-Tyrosine-Alanine (EPIYA) motif located in the C-terminal of CagA protein.

Innovations and breakthroughs

Some studies argue that there is an association between the EPIYA motifs in CagA and the pathology presented by the patients. In this study, the authors show that this association is not present in Colombian patients. Furthermore, their results suggest that this association is not equal in different regions of the world because there are others factors such as structural conformation and

protein-protein interactions that could affect the final outcome of the translocation of CagA in the gastric epithelial cells.

Applications

Because there is no association in Colombian samples between the motifs in the C-terminal of CagA and the pathology, it may be important to understand how these motifs interact with their target on gastric cells (Src kinase and Src homology phosphatase 2 proteins) in order to elucidate the function of CagA in the development of cancer gastric.

Terminology

CagA is one of the major virulence factors of *H. pylori*. This protein is involved in the development of gastric cancer because when this protein is translocated into gastric cells it causes changes in the signal transduction pathway which are involved in the proliferation of the cells.

Peer review

The paper by Acosta *et al* assesses EPIYA in Colombian patients with dyspepsia and finds no association with pathology.

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S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3944

World J Gastroenterol 2010 August 21; 16(31): 3944-3949
ISSN 1007-9327 (print)

BRIEF ARTICLE

Steatosis and steatohepatitis in postmortem material from Northwestern Greece

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Received: December 21, 2009 Revised: January 26, 2010
Accepted: February 2, 2010
Published online: August 21, 2010

Abstract

AIM: To determine the prevalence of steatosis and steatohepatitis in a series of autopsies in Northwestern Greece.

METHODS: Liver biopsy material from a total of 600 autopsies performed over a period of 2 years (2006-2008) to define the cause of death was subjected to histological examination. Patient demographic data were also collected. Tissue sections were stained with different dyes for the evaluation of liver architecture, degree of fibrosis and other pathological conditions when necessary.

RESULTS: Satisfactory tissue samples for histological evaluation were available in 498 cases (341 male, 157 female) with a mean age of 64.51 ± 17.78 years. In total, 144 (28.9%) had normal liver histology, 156 (31.3%) had evidence of steatosis, and 198 (39.8%) had typical histological findings of steatohepatitis. The most common causes of death were ischemic heart disease with or without myocardial infarction (43.4%), and traffic accidents (13.4%).

CONCLUSION: A high prevalence of steatosis and steatohepatitis was detected in postmortem biopsies from Northwestern Greece. Since both diseases can have serious clinical consequences, they should be considered as an important threat to the health of the general population in Greece.

Key words: Fatty liver; Non-alcoholic liver disease; Steatosis; Steatohepatitis; Autopsy

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Zois CD, Baltayiannis GH, Bekiari A, Goussia A, Karayiannis P, Doukas M, Demopoulos D, Mitsellou A, Vougiouklakis T, Mitsi V, Tsianos EV. Steatosis and steatohepatitis in postmortem material from Northwestern Greece. *World J Gastroenterol* 2010; 16(31): 3944-3949 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3944.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3944

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the accumulation of fat within liver cells in individuals who deny a significant history of alcohol ingestion, is emerging as the most frequent liver disease in developed countries[1,2]. The diagnosis of NAFLD and non-alcoholic steatohepatitis (NASH) is based on histological findings of either fat alone, fat plus inflammation, fat plus hepatocyte injury (ballooning degeneration), or fat with fibrosis and cirrhosis[3]. The significance of these histological categories rests on the fact that both the prevalence and clinical outcome vary by histological category[4].

NAFLD is commonly associated with severe obesity, defined as body mass index (BMI) > 35 kg/m^2, with 74%-90% of liver biopsies showing fatty change[5,6]. NASH, first named by Ludwig *et al*[7], is probably a less aggressive condition than alcoholic hepatitis, but nevertheless might progress through necro-inflammatory change and early fibrosis to cirrhosis[8,9]. In contrast, NAFLD without necro-inflammatory change is generally a benign condition[10,11].

There are few published data on the frequency of NASH and NAFLD in Greece, but without biopsies[12]. The present study aimed to determine the prevalence of these diseases in a series of autopsy material from Northwestern Greece.

MATERIALS AND METHODS

Study protocol

The aim of the present study was to estimate the prevalence of steatosis and steatohepatitis in postmortem material from Northwestern Greece. For this purpose, a number of liver specimens from people who died suddenly or in a traffic accident and had undergone autopsy to define the cause of death, were investigated.

The survey took place at the regional University Hospital of Ioannina, from 15 March 2006 until 20 December 2008. The collaborating centers were the Department of Internal Medicine, the Laboratory of Pathology and the Department of Forensic Medicine and Toxicology. The study protocol was approved by the Hospital Ethical Committee.

Autopsy material from 600 people (391 men and 209 women) aged 3-94 years was investigated. All autopsies were forensic cases, being performed in those with unnatural death, such as sudden death and accidents (especially traffic accidents). No academic cases were included.

Demographic information, history of alcohol use and previous hepatic and non-hepatic chronic diseases, such as diabetes mellitus, and use of medication, were obtained from the first-degree relatives of the cases. Gross appearance of the liver was also recorded. Sampling was performed by the same physician.

Cases with a history of liver disease of any type and those with autolysed liver samples were excluded. Wedge biopsies (2 cm × 2 cm × 2 cm) from the right and left lobes and one biopsy of the same size from deeper areas of the liver parenchyma were obtained in each case. Any visually grossly abnormal areas were also sampled.

Isolation of liver specimens and histological assessment

Liver tissue sections from the necrotomic material were fixed in 40 g/L neutral formaldehyde for 24 h. After that, the tissues were dehydrated, embedded in paraffin, cut at 4 μm, and stained with hematoxylin-eosin for light microscopy.

For histochemical staining, additional 4-μm thick tissue sections were cut from paraffin blocks and were stained with silver reticulin and Masson's trichrome methods for the evaluation of liver architecture and the degree of fibrosis, when necessary. In addition, Perl's Prussian blue staining was performed to evaluate iron load based on a grading system of 0-4, as previously described[13].

Immunohistochemical study for the hepatitis B virus surface and core antigens was performed on formalin-fixed, paraffin-embedded liver tissue sections by the labeled streptavidin-avidin-biotin method, using appropriate antibodies (anti-HbsAg monoclonal mouse antibody, clone 3E7; DAKO, Germany; anti-HbcAg polyclonal rabbit antibody; DAKO). Immunohistochemical cytoplasmic and nuclear staining of hepatocytes was shown as HbsAg and HbcAg positivity, respectively.

Each biopsy was scored on a scale for steatosis, injury/inflammation and fibrosis. The grading and staging of steatohepatitis was based on criteria developed by Brunt *et al*[14]. Most specimens contained 12-24 portal tracts.

The two histopathologists involved in this study agreed on terms, definitions and the histological criteria of the pathologic lesions. The liver sections were examined under an Olympus optical microscope and the histopathologists were blind to the patients' personal history. All positive findings were reevaluated for grading and staging by both histopathologists. In cases of diagnostic discrepancy, the result was reported according to the consensus of a joint slide review session. Histological findings were recorded in a standard form.

Statistical analysis

Data are presented as mean ± SD, unless otherwise stated. One-way ANOVA or ANOVA based on ranks, followed by multiple pairwise comparisons, was used for multigroup comparisons. Correlations between variables were determined with the use of the *f* factor. All statistical tests were performed with the use of the Statistica software, version 6.0. $P < 0.05$ was considered significant unless otherwise noted.

An overall statistical analysis was performed at first. After that, the cases were divided into subgroups based on sex, and comparison was performed in correlation with the degree of steatosis and steatohepatitis. The cases were also divided into four age subgroups: < 45 years (65 cases, 13%, age group a); 45-60 years (97 cases, 19.4%, age group b); 60-75 years (187 cases, 37.6%, age group c); and > 75 years (149 cases, 30%, age group d). Statistical analysis based on the histological findings was performed

Table 1 Detailed causes of death in the population study

Cause of death	*n* (%)
Ischemic heart disease and/or myocardial infarction	237 (47.59)
Traffic accidents	67 (13.45)
Pneumonic embolism	41 (8.23)
Cerebrovascular incidents	21 (4.2)
Trauma	16 (3.2)
Rupture of aortic aneurysm	16 (3.2)
Neoplasm	14 (2.8)
Cerebrovascular trauma	12 (2.4)
Drowning	12 (2.4)
Heroin overdose and other toxic agents	11 (2.2)
Hanging	7 (1.4)
Cirrhosis	6 (1.2)
Severe burns	5 (1.0)
Thrombosis of the mesentery with peritonitis	4 (0.8)
Sepsis	3 (0.6)
Lower gastrointestinal bleeding	3 (0.6)
Upper gastrointestinal bleeding	2 (0.4)
Inhalation of CO	2 (0.4)
Subdural hematoma	2 (0.4)
Endocarditis	2 (0.4)
Lobular pneumonia	1 (0.2)
Other (infections, electrocution, *etc*.)	14 (2.8)

in each age subgroup. Finally, the cases were divided in to two subgroups according to the cause of death: in the first subgroup the cause of death was ischemic heart disease with or without myocardial infarction, and in the second, all other vascular diseases. Further correlations with liver histology, age and sex were also performed.

RESULTS

During the study period, 600 cases were assessed. One hundred and two cases were excluded because of moderate to severe autolysis in liver tissue, positive immunohistochemical findings for hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) or a history of known chronic liver disease. Thus, 498 cases were evaluable and included 341 men and 157 women with a mean age of 64.51 ± 17.78 years. Most of the cases (255) were residents of Ioannina, the largest town in Northwestern Greece.

The most common causes of death were: ischemic heart disease with or without myocardial infarction (47.6%), traffic accidents (13.4%), pneumonic embolism (8.2%), cerebrovascular incidents (4.2%), rupture of aortic aneurysm and trauma (3.2%), neoplasm (2.8%), and others (21%). Table 1 presents a detailed description of the causes of death in our study population.

From the 498 cases, 144 (28.9%) had normal liver histology, whilst 156 (31.3%) had evidence of steatosis, and 198 (39.8%) had histological findings typical of steatohepatitis. In the subgroup with steatosis, 112 (71.8%) had steatosis < 33%, 28 (18%) had steatosis between 33 and 66%, and 16 (10%) had steatosis of > 66%. In the steatohepatitis subgroup, 113 (57%) cases were classified as grade 1, 61 (30.8%) as grade 2, and 24 (12.1%) as grade 3. Staging was categorized as stages 1, 2, 3 and 4, respectively. Thus, 69 (35%) were stage 1, 69 (35%) stage 2, 36 (18%) stage 3, and 24 (12) stage 4. Correlations of the grades and the stages of the 198 cases with steatohepatitis are depicted in Table 2. As in other types of chronic hepatitis, the grade and stage of the disease might be disparate, as they are meant to reflect different aspects of liver injury. The degrees of steatosis are presented.

Table 2 Correlation of grade and stage in patients with steatohepatitis

Grade	Stage					Totals
	0	1	2	3	4	
Mild (Ⅰ)	0	55	44	13	1	113
Moderate (Ⅱ)	0	13	21	14	13	61
Severe (Ⅲ)	0	1	4	9	10	24
Totals	0	69	69	36	24	198

The analysis of steatosis, steatohepatitis and normal liver tissues in the aging groups was statistically significant ($P = 0.0005$, $f = 6$, Figure 1). The same analysis in correlation with sex was not significant ($P = 0.148$). Overall analysis of the age groups and sex in correlation with steatosis, steatohepatitis and normal liver tissues showed borderline significance ($P = 0.043$, $f = 3$, Figure 1). Table 3 presents the number of subjects by sex and age group.

History of diabetes mellitus was found in 18 cases with steatosis and in 30 cases with steatohepatitis. Mild iron loading (1+ to 2+) was detected in 41 cases (23 men, 18 women), which accounted for 8.2% of the study population. From these, 39 cases had also steatohepatitis and therefore only two cases of steatosis were detected. Furthermore, analyses based on the age subgroups revealed mild iron loading (1+ to 2+) in eight cases between 45 and 60 years, in 18 cases between 60 and 75, and in 15 cases > 75 years.

Finally, from the 236 ischemic heart disease deaths with or without myocardial infarction, 47 (20%) had normal liver histology, 90 (38%) had steatosis, and 99 (42%) had histological findings typical of steatohepatitis. In the same subgroup, only 11 cases had mild iron loading.

DISCUSSION

The present study demonstrated that steatosis and steatohepatitis are emerging as common silent liver diseases in Northwestern Greece. The true incidence and prevalence of these diseases in different populations is largely unknown[15], even if it is believed that they are the most common liver diseases in the general population[2,16,17]. This might be explained partly because liver histology is required as the gold standard for precise diagnosis of these conditions, and the relatively invasive procedure of liver biopsy is still not considered essential for the management of NAFLD by many physicians. In addition, most of the reports on the prevalence rates of NAFLD are based on ultrasonographic studies and/or the presence of elevated serum levels of aminotransferases, in the

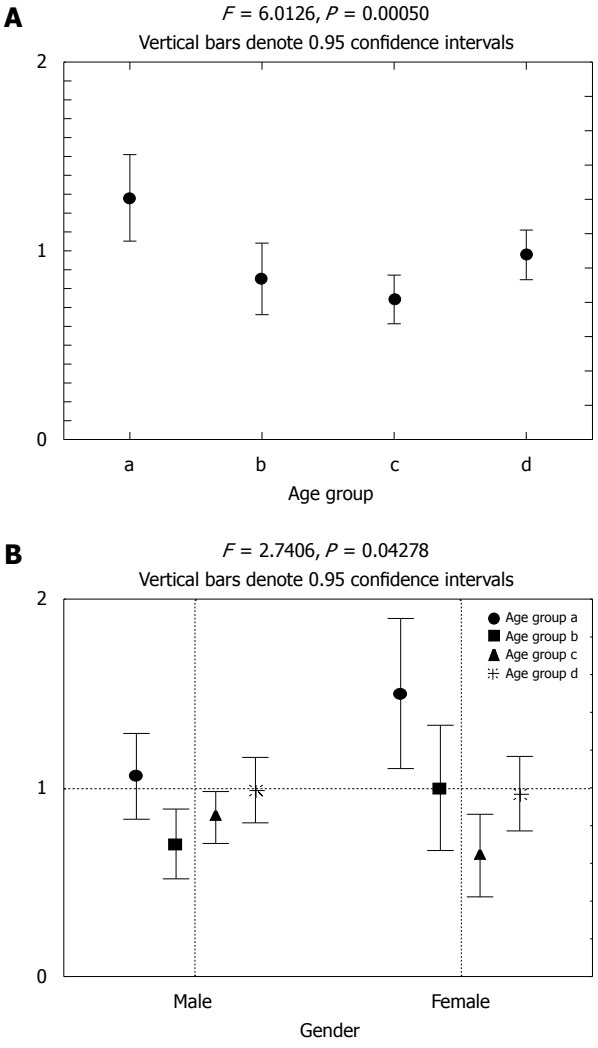

Figure 1 Correlations of age subgroups in different subjects. A: Correlation of age subgroups (a, b, c and d) in subjects with steatohepatitis (0) and steatosis (1), and normal subjects (2). Mean values for each group are presented. The four age subgroups were defined as follows: < 45 years (group a), 45-60 years (group b), 60-75 years (group c), and > 75 years (group d); B: Correlation of sex and age in subjects with steatohepatitis (2) and steatosis (1), and in normal subjects (0). Data are shown for male and female subjects separately, according to age group.

absence of any other known liver disease or significant alcohol consumption[2,18].

In clinical practice, diagnostic liver biopsy is performed only for highly selected patients. For this reason, the reported rates that are based on liver biopsies cannot reflect the true prevalence of NAFLD and NASH in the general population[19,20]. In addition, histological lesions of NASH are unevenly distributed throughout the liver parenchyma; therefore, sampling errors of liver biopsies can result in misdiagnosis and staging inaccuracies[21]. Furthermore, NASH and alcoholic steatohepatitis can share common histological findings[22,23].

Autopsies, performed for those who have passed away for reasons other than liver diseases, are certainly better sources for the determination of a more reliable prevalence for NAFLD and NASH[24]. The particular characteristics of forensic autopsies are the relatively young age of

Table 3 Number of subjects in every age group according to sex

Age groups	a	b	c	d	Totals
Male	49	74	134	84	341
Female	16	23	53	65	157
Total	65	97	187	149	498

a: < 45 years; b: 45-60 years; c: 60-75 years; d: > 75 years.

the subjects and their usually better general health before death. Among our cases, male sex was predominant; most of the subjects had no history of chronic diseases, and in > 80%, the causes of death were acute events such as cardiovascular diseases, traffic accidents and trauma. To the best of our knowledge, this is the first report concerning liver diseases based on an autopsy series in Greece.

Obesity is a major clinical problem in Greece[25]. Unfortunately, we were not able to measure BMI in the study population. As a result, no correlations of steatosis and steatohepatitis with BMI could be performed. In addition, although 10 patients had positive immunohistochemical findings for the HBsAg and HBcAg, serological viral markers for hepatitis B and hepatitis C virus infection were not performed in the present study. However, the prevalence of both infections is low in Greece[26-28].

In none of our cases was the use of medication that induced or predisposed to the progression of NASH[29]. The higher prevalence of NAFLD and NASH in patients with diabetes mellitus has been well documented before[30-32] and is in accordance with our findings. Silent cirrhosis was found in only six of our patients, which indicates a low prevalence of this condition in Northwestern Greece, in comparison with other autopsy series[24,33]. The correlation of cirrhosis with NAFLD and NASH is one of the most controversial issues. Although there is substantial evidence that NASH might lead to cirrhosis in about 20% of cases, and is the most common etiology for cryptogenic cirrhosis[34], well-designed, prospective long-term studies for determination of outcome in patients with NAFLD are lacking[2,3]. However, it seems that older age, obesity and the presence of diabetes mellitus are factors that predispose towards progression of NASH to cirrhosis[35-37]. In particular, the aggressive form of NAFLD is best based on the pathological diagnosis of steatonecrosis with Mallory hyaline and fibrosis[38,39]. The clinical implications of this alarming prevalence of NAFLD and NASH are derived from the fact that these liver conditions may progress to end-stage liver disease and cancer[40,41].

Mild iron loading was detected in 41 cases in our study population. It is clear that, in our cases with steatohepatitis, the role of increased iron stores in the development of fibrosis seemed to be of less importance. The role of lipogenesis and fatty acid oxidation in the development of NAFLD and NASH has been well established[42]. In particular, the complex progression of NAFLD is related to metabolic abnormalities associated with massive steatosis, insulin resistance and reductions

in fatty acid oxidation[43-45]. NASH is attributed to tumor necrosis factor-α, free fatty acid toxicity, toxicity caused by dicarboxylic acids, a decrease in mitochondrial and peroxisomal β-oxidation, generation of reactive oxygen species, lipid peroxidation and many other events that lead to apoptosis and inflammation[46,47].

The reported prevalence of steatosis in our study is in accordance with other studies[24]. However, the reported prevalence of steatohepatitis in this Greek population is much higher in comparison with other population studies[48-51]. Although results from this autopsy study cannot be extrapolated to the general healthy population, the reason for such a high rate could possibly be attributed to the decreased physical activity and the alterations in dietary habits in the Greek population over recent decades.

In conclusion, it seems that steatosis and steatohepatitis are very common liver diseases among postmortem material in Northwestern Greece. Since both diseases can have serious clinical consequences, they should be considered as an important threat to the health of the general population in Greece. Further studies to confirm this prevalence and assess the etiology and natural history of these lesions in the general healthy Greek population, in combination with possible therapeutic strategies, are therefore mandatory.

COMMENTS

Background

The incidence of steatosis and steatohepatitis is currently rising faster than any other liver disease in the western world, although the cause of this increase is largely unknown. These diseases progress from necro-inflammatory change and early fibrosis to cirrhosis and liver cancer. Both diseases can result in end-stage liver disease. Liver biopsy remains the gold standard for diagnosis and treatment. The present study aimed to determine the prevalence of steatosis and steatohepatitis in a series of autopsies in Northwestern Greece.

Research frontiers

Many predisposing factors for the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have been proposed. Age seemed to be an important one in our population study, whereas the role of sex remains to be investigated. In addition, further studies are important to define the role of genetic factors in both diseases.

Innovations and breakthroughs

Many reports have highlighted the evolution of steatosis and steatohepatitis in liver fibrosis and carcinogenesis. This is believed to be the first study in Greece to report the prevalence of steatosis and steatohepatitis, based on liver histology in the general population. Among our cases, male sex was predominant, most of the subjects had no history of chronic diseases (reported healthy subjects), and in > 80% of the cases, the causes of death were acute events such as cardiovascular diseases, traffic accidents and trauma.

Applications

By understanding the rising prevalence of NAFLD and NASH, this study could represent a future strategy for early therapeutic intervention in the treatment of patients with steatosis and steatohepatitis. Screening of the general population and detecting the possible risk factors is also advisable.

Terminology

Tissue sections were stained with different dyes for the evaluation of liver architecture, degree of fibrosis and other pathological conditions when necessary. Simple steatosis of the liver was staged as follows: < 33%, 33%-66%, > 66%. NASH was diagnosed after excluding other causes of liver inflammation.

Peer review

This paper is interesting and well written. Finding a steatohepatitis prevalence of about 40% is important news in Greece and could have implications throughout Europe.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3950

World J Gastroenterol 2010 August 21; 16(31): 3950-3956
ISSN 1007-9327 (print)

BRIEF ARTICLE

Mechanism underlying carbon tetrachloride-inhibited protein synthesis in liver

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Received: May 5, 2010 Revised: June 17, 2010
Accepted: June 24, 2010
Published online: August 21, 2010

Abstract

AIM: To study the mechanism underlying carbon tetrachloride (CCl_4)-induced alterations of protein synthesis in liver.

METHODS: Male Sprague-Dawley rats were given CCl_4 (1 mL/100 g body weight) and ^{3}H-leucine incorporation. Malondialdehyde (MDA) level in the liver, *in vitro* response of hepatocyte nuclei nucleotide triphosphatase (NTPase) to free radicals, and nuclear export of total mRNA with 3′-poly A^+ were measured respectively. Survival response of HepG2 cells to CCl_4 treatment was assessed by methyl thiazolyl tetrazolium. Km and Vmax values of nuclear envelope NTPase activity in liver of rats treated with CCl_4 were assayed by a double-reciprocal plot.

RESULTS: The protein synthesis was inhibited while the MDA level was significantly increased in liver of rats treated with CCl_4. In addition, CCl_4 decreased the NTPase binding capacity of nuclear envelope (Km value) in cultured HepG2 cells. Moreover, *in vitro* ferrous radicals from Fenton's system suppressed the NTPase activity of liver nuclear envelope in a dose-dependent manner. Down-regulation of the nuclear envelope NTPase activity indicated a lower energy provision for nucleocytoplasmic transport of mRNA molecules, an evidence in CCl_4-treated HepG2 cells correspondingly supported by the nuclear sequestration of poly $(A)^+$ mRNA molecules in morphological hybridization research.

CONCLUSION: Inhibition of mRNA transport, suggestive of decreased NTPase activity of the nuclear envelope, may be involved in carbon tetrachloride-inhibited protein synthesis in liver.

Key words: Carbon tetrachloride; Nuclear envelope nucleotide triphosphatase; Nucleocytoplasmic transport inhibition; Hydroxyl radical

Peer reviewers: Jordi Camps, PhD, Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, 43201 Reus, Catalunya, Spain; Islam Khan, PhD, Professor, Department of Biochemistry, Faculty of Medicine, Kuwait University, PO box 24923, 13110 Safat, Kuwait

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INTRODUCTION

Carbon tetrachloride (CCl_4) is an organic solvent widely

used in chemical industry. Its hepatotoxicity includes fatty liver and hepatic necrosis[1,2]. Moreover, CCl_4 has also an ability to inhibit protein synthesis in liver with early hepatotoxic damage[3]. Early studies showed that free radicals, such as trichloromethyl ($\cdot CCl_3$) and oxygen-centered lipid radicals (LO$\cdot$ or LOO, or both), are generated during CCl_4 metabolism by hepatic cellular cytochrome P450. These radicals can cause hepatic injury resulting from CCl_4 exposure. Protein synthesis is a cytoplasmic event in eukaryotic cells, in which mRNA templates in the form of a genetic code are employed to guide protein synthesis. A nuclear envelope (NE) of eukaryotic cells causes spatial separation between mRNA and protein synthesis. Thus, mRNA templates in nuclei have to be transported into cytoplasm, a limiting process of protein synthesis. Nuclear membrane-associated nucleotide triphosphatase (NTPase), a specific energy driver of mRNA nucleocytoplasmic export, enables mRNA cytoplasmic accumulation[4,5]. Our previous work demonstrated that reduced NTPase activity in the NE correspondingly decreases mRNA transport[6]. In this report, we studied the role and mechanism of NTPase activity in CCl_4-induced inhibition of protein synthesis in liver.

MATERIALS AND METHODS

Materials

Trypsin, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), adenosine triphosphate (ATP), guanine triphosphate (GTP), cytosine triphosphate (CTP), thymine triphosphate (TTP), and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Company (St. Louis, MO, USA). DMEM medium was purchased from Invitrogen (Cat.12100-046, Carlsbad, CA, USA), and fetal bovine serum (FBS) was purchased from Beijing New Probe Biotechnology Co., Ltd. (Beijing, China). Trichloride acetic acid (TCA, analytic purity grade), a precipitating agent for protein, and CCl_4 (analytic purity grade) were obtained from Beijing Chemical Reagent Factory (Beijing, China). L-[3, 4-^{3}H]-leucine was purchased from Beijing Atom High Tech., Ltd. (Beijing, China).

Cell culture

HepG2 cells (Cat. HB-8065, human hepatic carcinoma cell line) were obtained from American Type Culture Collection (Manassas, VA, USA). HepG2 cells were grown in a DMEM supplemented with 10% newborn calf serum and maintained at 37℃ in a humidified atmosphere containing 5% CO_2. The 80% confluent-cultured HepG2 cells were treated for 2 h with fresh DMEM with or without CCl_4, the former containing a final concentration at 4 mmol/L CCl_4 (CCl_4 group), and the latter with the same volume of DMSO (control group).

Animals

Sprague-Dawley rats were supplied by the Experimental Animal Center, Peking University Health Science Center. The following procedure was approved by the Medical Animal Ethics Committee of the Health Science Center, Peking University. Male rats weighing 180-220 g were fasted for 14 h prior to the experiment, and then had free access to standard animal food and tap water. The animals were housed in plastic cages and maintained at 23 ± 1℃ under a natural light-dark cycle in a well-ventilated room.

^{3}H-leucine incorporation

Rats (n = 6) in the CCl_4 group were given CCl_4 (dissolved in an equal part of vegetable oil) at 1 mL/100 g of body weight under anesthesia. Rats in the control group (n = 6) received the same volume of vegetable oil. Two weeks following CCl_4 treatment, 50 μCi ^{3}H-leucine (100 g of body weight) was injected into each animal *via* the tail vein. Rats were dispatched by cervical dislocation 1 h later with their livers quickly isolated and perfused in ice-chilled phosphate buffer saline (PBS). Liver tissue samples, free of blood, were divided into two parts and stored at -80℃. To measure the total liver radioactivity, one aliquot of liver tissue was baked at 60℃ until its weight remained constant. The liver tissue samples were then ground into powder and digested at 70℃ in 5 mL 70:30 mixture of perchloric acid/H_2O_2 for 30 min. To measure the radioactivity retained at the TCA-insoluble portion of the liver protein, another aliquot of liver tissue was homogenized in ice-chilled PBS using a Teflon glass homogenizer. Homogenates were precipitated with 10% TCA, and TCA-insoluble sediments were digested at 70℃ in the same volume of perchloride acid/H_2O_2 mixture for 30 min. Protein level in TCA-insoluble sediments was measured using the Bradford method. Radioactivity remaining either in the entire liver or in TCA-insoluble portion was determined with a Beckman liquid scintillation counter. ^{3}H-leucine incorporations in the entire liver or in the TCA-insoluble protein were expressed as count per minute (CPM) over mg of dry liver tissue or TCA-insoluble protein portion[7].

MDA level in rat liver

Twenty male rats were divided into control group, CCl_4-treatment group, taurine group, and CCl_4/taurine group, 6 rats in each group. The rats were treated with or without CCl_4 (dissolved in vegetable oil) *via* subcutaneous injection, and oral administration of taurine if necessary. Two weeks after treatment, the rats were sacrificed under anesthesia with urethane (1 g/kg, ip). Their livers were perfused *in situ* at 37℃ with PBS *via* the left ventricle. Liver tissue samples were homogenized in approximately 10 times the volume of saline to tissue weight. MDA level was measured by assaying rat liver homogenates with a MDA kit (Bio-lab Materials Institute, Beijing) following its manufacturer's instructions. Results were calibrated to their protein content and expressed as nmol MDA/mg protein.

MTT metabolic viability

Cytotoxicity of HepG2 cells to CCl_4 was detected by MTT metabolic viability assay as previously described[8].

Briefly, HepG2 cells were seeded into a 96-well plate (1×10^5 cells/well), incubated for 12 h, and then switched to a DMEM containing CCl_4 at the indicated doses (0, 2, 10, and 50 mmol/L). After incubated for another 4 h at 37℃ and further incubated with 1 mg/mL MTT for 2 h, the activity of cellular mitochondrial succinic dehydrogenase was measured. Absorbance at 570 nm wavelength with a reference wavelength of 655 nm reflected the number of surviving cells.

Isolation and characterization of nuclei

Twenty-four hours after incubation, HepG2 cells were harvested to isolate their nuclei as previously described[5]. Suspended cells were homogenized in Teflon (10 strokes) and centrifuged at 800 *g* for 10 min. The nuclei were suspended in DS/PMSF buffer, layered over cushions in the same buffer, and spun down at 70 000 *g* for 60 min. Isolated nuclei were resuspended in STM/PMSF buffer, relayered over cushions of DS/PMSF buffer, and centrifuged at 70 000 *g* for 30 min. The final pellet was resolved with STM/PMSF to obtain a final protein concentration of 1 mg/mL and stored at -70℃. The marker enzyme activities specific to nuclei or cytoplasm were determined for monitoring the purity of isolated NE preparations.

Kinetics assay of NE NTPase activity

NE NTPase activity was assayed as described by Ramjiawan *et al*[9] with some modifications. In the assay, 40 μL nuclear extracts (containing 40 μg protein) of HepG2 cells with 2 mmol/L CCl_4 or DMSO treatment was preincubated in 200 μL buffer for 10 min at 30℃. After different levels of ATP or GTP substrates were added into the nuclear extracts to initiate NTPase reaction and incubated at 30℃ for 10 min, reactions were terminated by adding 10% SDS and test tubes were immediately placed in ice. Inorganic phosphates produced in these reactions were detected according to the method of Tiffany[10]. The two characteristic constants, i.e. maximum velocity (Vmax) and Michaelis constant (Km) for NTPase, were obtained using the Line weaver-Burke equation, and expressed as nmol Pi/mg, Pr/min and nmol/L, respectively, for ATP or GTP. Preliminary experiments showed a linear relationship between NTPase activity and incubation time.

Nuclear export of total mRNA with 3'-poly A^+ tailing

Double staining of HepG2 cells with a fluorescein isothiocyanate (FITC) probe conjugated with oligo-$(dT)_{15}$ specific for mature mRNA with 3'-poly A^+ tailing and 4, 6-diamidino-2-phenylindole (DAPI) for localization of nuclei was carried out as previously described[11]. Briefly, after formaldehyde fixation, HepG2 cells cultured on cover slips were permeabilized with PBS/0.5% Triton X-100 for 10 min at room temperature. Cover slips were equilibrated in 2 × SSC for 10 min, and incubated with a FITC-labeled oligo-$(dT)_{15}$ probe (showing green fluorescence) for 30 min. The cells on cover slips were washed three times with PBS, and incubated for another 30 min at room temperature with a DAPI solution (showing blue fluorescence). The cells were then washed once with PBS/0.2% Triton X-100 and twice with PBS. To ensure that the FITC-hybridization of mRNA was not resulted from DNA binding, RNase A was introduced into the sample to digest all remaining RNA. Total mature mRNA with 3'-poly A^+ tailing was clearly observed either in nuclei or in cytoplasm with FITC-oligo $(dT)_{15}$ labeling under a fluorescence microscope.

NTPase response of hepatocyte nuclei to in vitro hydroxyl radicals

Hepatocyte nuclei from rats were prepared and characterized using the method described in section "Isolation and characterization of nuclei". Different levels of hydroxyl radicals from *in vitro* Fenton reaction[12] system were freshly prepared by mixing $FeSO_4$ and H_2O_2 in the ratio of 0/0, 0.1/0.5, 0.5/2.5, 1/5, and 5/25 [(μmol/L)/(μmol/L)], respectively. The mixture was immediately added to the hepatocyte nuclei. After hepatocyte nuclei were incubated with different levels of hydroxyl radicals for 30 min at 30℃, NE NTPase activity for the ATP or GTP was determined according to the method described in section "Kinetics assay of NE NTPase activity".

Statistical analysis

All data were expressed as mean ± SE (n = 4-6). Statistical analysis was performed by one-way analysis of variance followed by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

$CC1_4$ inhibited protein synthesis and increased MDA production in liver

Electron micrographs showed an early and widespread dislocation of ribonucleoprotein particles from membranes of the rough endoplasmic reticulum in hepatocytes of rats 3 h after CCl_4 administration. Furthermore, amino acid incorporations into two liver-produced proteins, albumin and fibrinogen, were decreased in the same CCl_4-treated rats. These findings suggest that protein synthesis in liver is influenced by CCl_4 treatment[3].

To confirm that the incorporation of amino acid into liver protein is impaired, 50 μCi ^{3}H-leucine (100 g of body weight) was administered to the rats through the tail vein 2 h after CCl_4 treatment. Trichloride acetic acid (TCA), a general protein-precipitating agent, was used in tissue homogenates to separate total protein from rat liver tissues. The rates of ^{3}H-leucine incorporation into the liver homogenate and TCA-precipitated liver protein are shown in Figure 1A. Treatment with CCl_4 significantly decreased ^{3}H-leucine incorporation into TCA-precipitated liver protein (103.7 ± 5.4 cpm/mg in control group *vs* 71.8 ± 3.8 cpm/mg in CCl_4 treatment group).

No significant difference was observed in total radioactivity in the entire liver between CCl_4 treatment and control groups (66.7 ± 2.4 cpm/g *vs* 64.2 ± 1.8 cpm/g), indicating that the decreased total protein synthesis in CCl_4 treatment group is not due to the difference in leu-

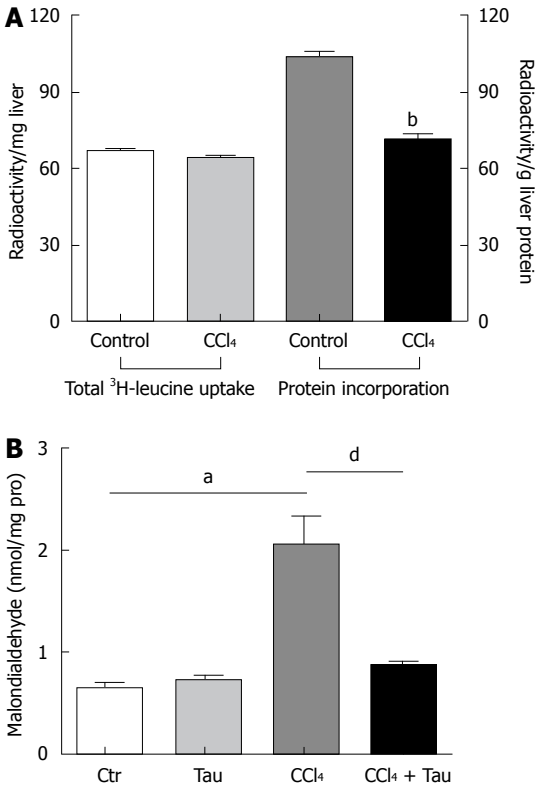

Figure 1 CCl_4 inhibits protein synthesis (A) and increases malondialdehyde production (B) in liver of Sprague-Dawley rats. Data are expressed as mean ± SE. $^aP < 0.05$ *vs* control group; $^bP < 0.01$ *vs* their counterpart control group; $^dP < 0.01$ *vs* CCl_4 treatment group.

cine uptake between the two groups.

· CCl_3 radicals produced in reactions by liver microsomes of animals exposed to CCl_4 were assumed to attack the membrane lipid in hepatocyte endoplasmic reticulum. When these free radicals attacked the membrane lipid in hepatocyte endoplasmic reticulum, malondialdehyde was brought out quickly, suggesting that MDA formation is recognized as an indicator of lipid peroxidation initiated by reactive radicals. Taurine has been well-known to be an effective scavenger of reactive radicals *in vivo* and *in vitro* through an unknown mechanism[5], which ameliorates MDA production in oxidative damaged liver. In the current work, the MDA level was 220% higher in liver of rats treated with CCl_4 than in liver of rats not treated with CCl_4. Meanwhile, the MDA level was 42% lower in liver of rats treated with combined CCl_4 and taurine than in liver of rats treated with CCl_4 alone (Figure 1B), indicating that reactive radicals have formed in liver of rats during CCl_4 metabolism.

$CC1_4$ inhibited nuclear NTPase affinity of HepG2 cells after in vitro incubation

To further investigate the mechanism underlying CCl_4-inhibied protein synthesis in liver, the CCl_4 dose acting on cultured HepG2 cells was maximized by measuring the hepatotoxicity of CCl_4 in a MTT cell survival assay. CCl_4 at the dose of 10 mmol/L and 50 mmol/L decreased the survival rate of HepG2 cells to 32% (0.290 ± 0.08 *vs* 0.196 ± 0.04) and 97% (0.290 ± 0.08 *vs* 0.009 ± 0.01), respectively ($P < 0.01$, Figure 2A). Therefore, CCl_4 at a dose of lower than 10 mmol/L was used to explore the mechanism underlying CCl_4-inhibited protein synthesis in liver of rats in subsequent experiments.

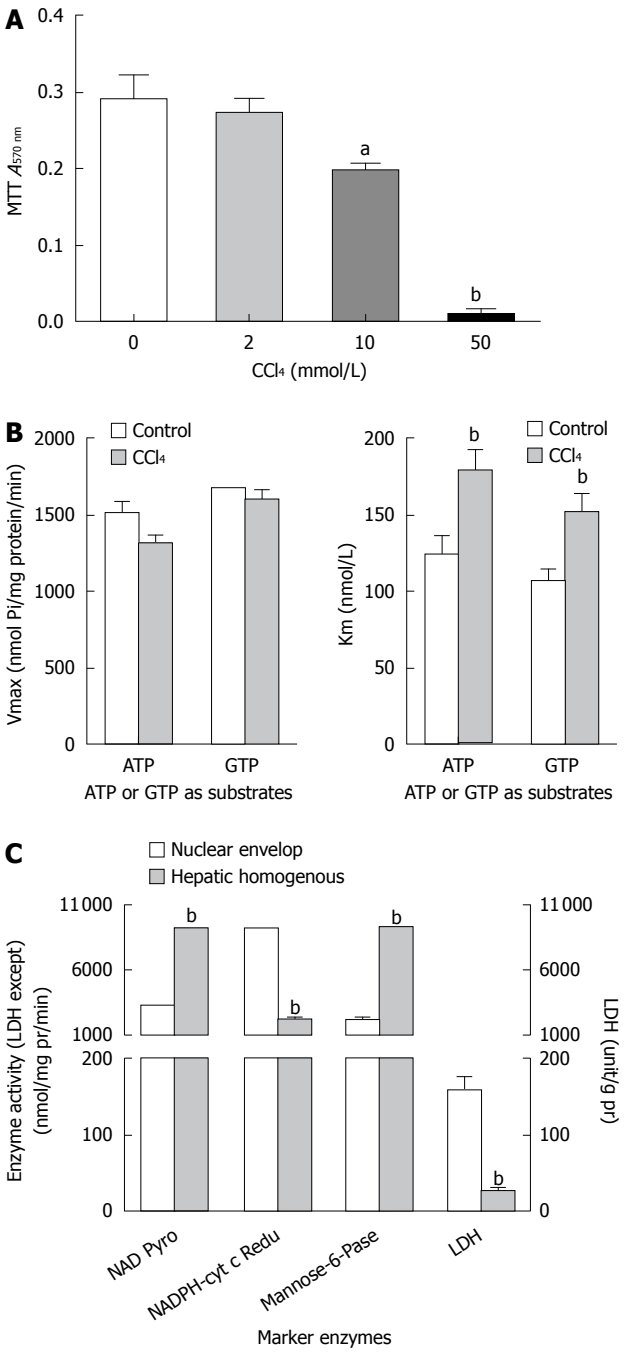

Figure 2 $CC1_4$ inhibits nucleotide triphosphatase affinity in nuclei of HepG2 cells. A: Methyl thiazolyl tetrazolium (MTT) metabolic viability assay at a dose of 10 mmol/L showing the inhibitory effect of CCl_4 on growth of HepG2 cells; B: Substrate-affinity (Km, B2) is decreased without any alterations in the substrate binding sites (Vmax,B1) for adenosine triphosphate (ATP) or guanine triphosphate (GTP) in nuclear nucleotide triphosphatase of HepG2 cells treated with CCl_4 (2 mmol/L); C: Enzyme' activities of nuclei extracts and homogenates from HepG2 cells in control and CCl_4 treatment groups. $^aP < 0.05$ *vs* control group, or nuclear envelop group, respectively; $^bP < 0.01$ *vs* their counterpart control group, or nuclear envelop group, respectively. LDH: Lactose dehydrogenase.

Protein synthesis in liver, regulated by the richness of

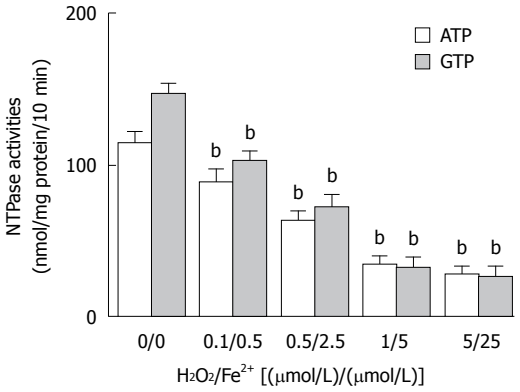

Figure 3 Involvement of reactive radicals in decreased nucleotide triphosphatase activity of nuclei. Data are represented as mean ± SE. $^bP < 0.01$ *vs* H_2O_2/Fe^{2+} group [(0 μmol/L)/(0 μmol/L)]. NTPase: Nucleotide triphosphatase.

cytoplasmic template of mRNA, is a key event in cell survival and metabolism. Down-regulation of nuclear export of mature mRNA into cytoplasm is related to its depressed NE NTPase activity in aged rats (data not shown). NTPase, embedded in eukaryotic nuclear envelope, provides nucleocytoplasmic transport energy and promotes shuttling of macromolecules between nuclei and cytoplasm *via* its hydrolysis action on NTP substrates, such as ATP, GTP, CTP, and TTP. In this study, nuclear envelopes of HepG2 cells were isolated to study the effect of CCl_4 on mRNA export into cytoplasm through NE NTPase action in HepG2 cells.

To characterize the purity of nuclei, several marker enzymes for different organelles were used to assess the quality of isolated nuclear envelopes with reference to their hepatic homogenous counterparts (Figure 2C). The activity of NAD pyrophosphorylase was 6.9-fold higher in the prepared nuclear envelope, previously assumed to be exclusively located in nuclei, than in homogenates of HepG2 cells. The activity of NADPH cytochrome-C reductase (as a marker enzyme in microsome) was only 22% higher in the prepared nuclear envelope than in homogenates of HepG2 cells. The activities of other marker enzymes, such as mannose-6-phosphatase (an indicator of both nuclei and cytoplasm) and lactose dehydrogenase (an indicator of cytoplasm) had a similar changing tendency as NAD pyrophosphorylase and NADPH cytochrome-C reductase, showing that these nuclear envelopes, prepared from HepG2 cells in this experiment, have less cytoplasmic contamination and are comparable with the prepared nuclear envelopes, and can be used in analysis of NTPase activity.

In the present study, CCl_4 significantly depressed the NTPase activity of hepatic nuclei, regardless of whether the ATP or GTP acted as a substrate, in terms of Km but not Vmax value. An estimation using the double-reciprocal plot method (Figure 2B) showed that CCl_4 interfered with the HepG2 NE NTPase Km but not the Vmax, in ATP or GTP acting as a substrate, suggesting that the Km value of liver nuclear membrane NTPase contributes to a lower hydrolysis efficiency on NTP molecules in the CCl_4 treatment group than in the control group, and decreases energy provision for nucleocytoplasmic transport.

Involvement of reactive radicals in decreased NTPase activity of nuclei

The elevated MDA level in liver tissue of Sprague-Dawley rats treated with CCl_4 was significantly correlated with membrane lipid peroxidation initiated by reactive radicals. To observe the involvement of reactive radicals produced by CCl_4 metabolism in hepatic nuclear NTPase activity, Fenton reaction system, an *in vitro* mimic system, was used. In brief, ferrous sulfate and hydroperoxide were simultaneously mixed in different ratios [0.1/0.5, 0.5/2.5, 1/5, and 5/25, (μmol/L)/(μmol/L)] and placed into cultured media to produce unstable reactive radicals. HepG2 nuclear envelope fractions were incubated with an equal volume of *in vitro* radicals at different concentrations. The results showed that the *in vitro* radicals decreased the nuclear NTPase activity in a dose-dependent manner, regardless of whether ATP or GTP was used as a substrate (Figure 3). After nuclei of HepG2 cells were incubated with reactive radicals at a mixing ratio of (5 μmol/L)/(25 μmol/L) of Fe^{2+}/H_2O_2, the NTPase activity on the NE was decreased by 75.4% (for ATP) and 82.2% (for GTP), respectively ($P < 0.01$), indicating that the decreased nuclear NTPase activity in HepG2 cells treated with CCl_4 may be attributed to hepatic microsome CCl_4 metabolites through the action of its reactive radicals.

Total mRNA nuclear export from HepG2 cells blocked by CCl_4 treatment

As described above, the nuclear NTPase activity in HepG2 cells was sharply suppressed by CCl_4 treatment, the exporting process of mRNA templates into cytoplasm was thus assumed to be influenced in CCl_4-treated HepG2 cells. To investigate whether the concurrent export inhibition of mRNA occurs in CCl_4-treated HepG2 cells, the poly $(A)^+$ RNA molecular (i.e. mRNA) shuttling between nuclei and cytoplasm in cultured HepG2 cells treated or not treated with CCl_4 was morphologically observed by fluorescence *in situ* hybridization with a FITC-labeled oligo-$(dT)_{15}$ probe, a specific targeting probe for endogenous 3'-poly$(A)^+$ tailing maturing mRNA (Figure 4). The FITC-labeled oligo-$(dT)_{15}$ hybridization in HepG2 cells not treated with CCl_4 was almost evenly distributed throughout the whole cells. Moreover, a weaker hybridization was observed in cytoplasm of HepG2 cells treated with CCl_4. Pretreatment with RNase A nearly abolished the observed results mainly from the specific hybridization to poly$(A)^+$ mRNA with FITC-labeled oligo-$(dT)_{15}$ staining, demonstrating that CCl_4 treatment inhibits the export of poly $(A)^+$ mRNA molecules from the nuclear site by inhibiting nuclear NTPase activity.

DISCUSSION

In 1987, Enzan[13] conducted a study to observe the pro-

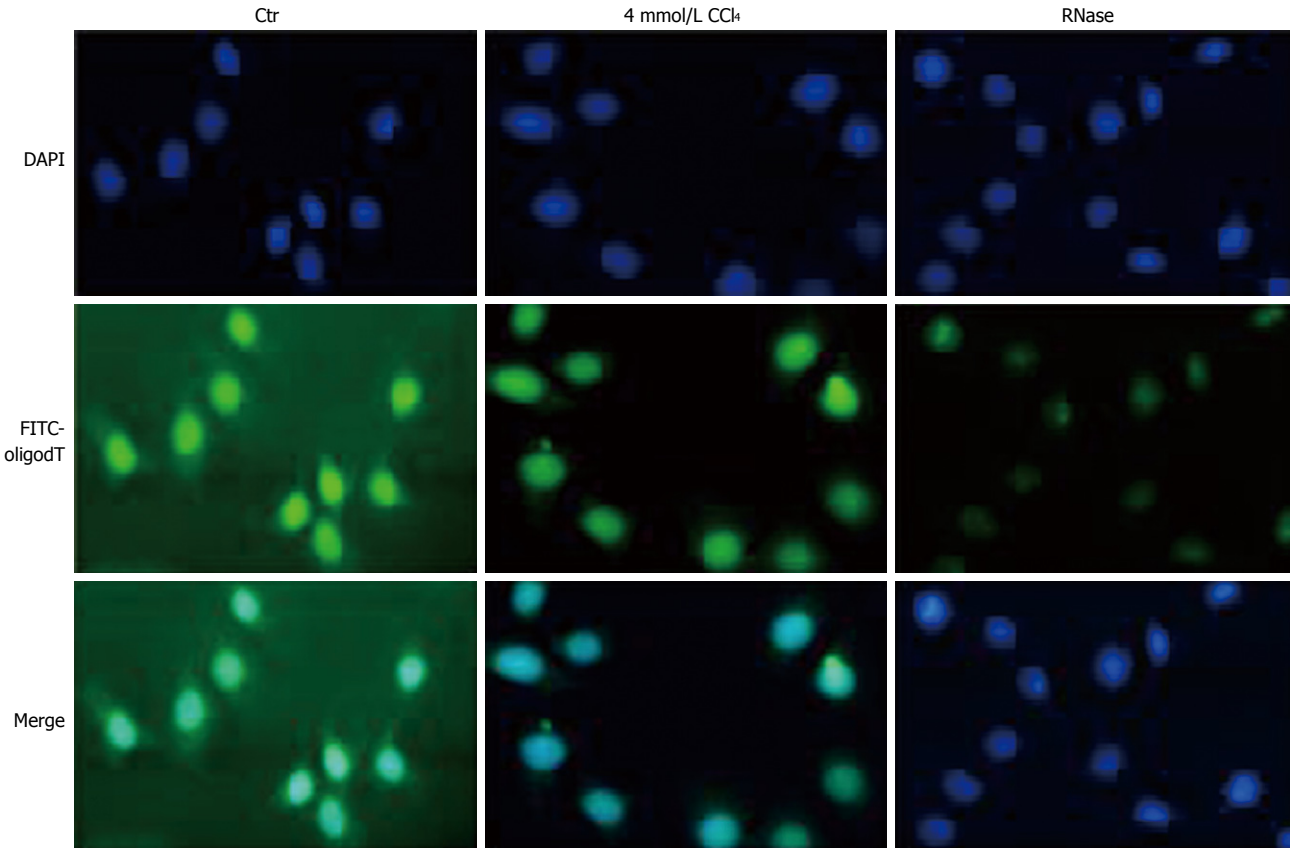

Figure 4 CCl_4 decreases total mRNA nuclear export of HepG2 cells. DAPI: 4,6-diamidino-2-phenylindole dihydrochloride; FITC: Fluorescein isothiocyanate.

tein synthesis of Ito cells in acute liver injury induced by CCl_4. He used small liver tissue blocks from normal and CCl_4-treated mice that was incubated in cultured medium supplemented with ^{3}H-leucine for one hour, and found that a few Ito cells in normal mouse liver tissues were labeled with ^{3}H-leucine, while no labeling over the Ito cells was observed in CCl_4-treated mouse liver without any cellular degeneration, revealing that protein synthesis is blocked in Ito cells after CCl_4 treatment[13]. In this study, ^{3}H-leucine incorporation into liver protein was decreased 30% in CCl_4-treated rats, which is consistent with the findings of Enzan[13].

Both protein synthesis and degradation occur actively in liver in order to maintain the normal metabolism through appropriate steady-state levels of liver proteins. Among the liver protein homeostasis, mature 3'-poly $(A)^+$ mRNA acting as a template appears to be an important determinant regulator for protein synthesis. Thus, all eukaryotic cytoplasmic mature 3'-poly $(A)^+$ mRNA molecules have to be transported from their original nuclei by traversing through the nuclear pore, an active process in which the nuclear membrane-associated NTPase can provide mechanic energy by catalyzing the hydrolysis of nucleoside tri-phosphates[4,10]. Moreover, the effect of changes in nuclear membrane phosphatidylcholine composition by phospholipase A_2 (PLA_2) on mRNA transport is related to the changes in NTPase activity responded to PLA_2[6]. Based on this assumption, we further explored whether protein synthesis in liver inhibited by CCl_4 is related to alterations in nuclear NTPase activity. In brief, the Km value of NE NTPase in CCl_4-treated HepG2 cells increased to 44% (ATP as a substrate) and 42% (GTP as a substrate), respectively (Figure 2B), indicating that NE NTPase in CCl_4-treated HepG2 cells has a lower substrate binding affinity and a lower energy provision for the export process.

Although the nuclear NTPase activity was inhibited by CCl_4 treatment, whether inhibition of protein synthesis in liver inhibited by CCl_4 is also brought out by the decreased the nucleocytoplasmic transport of total mRNA remains unknown. A fluorescent FITC-probe conjugated with 5'-oligomerization with 15 consequent thymine bases was designed to test this hypothesis (Figure 4). The probe can specifically bind to mature mRNA with 3'-poly A^+. Human HepG2 cells treated with CCl_4 decreased the fluorescence strength in cytoplasm, suggesting that CCl_4 inhibits mature mRNA nuclear export and that decreased NE NTPase activity is involved in CCl_4-induced inhibition of total mRNA nucleocytoplasmic transport and protein synthesis in liver.

In this study, the MDA level was 220% higher in CCl_4 treatment group than in control group. However, taurine, a potent scavenger of reactive oxidative radicals, clearly reduced the MDA level in liver of rats treated with CCl_4 (Figure 1B), displaying that CCl_4-induced liver oxidative damage is caused by reactive radicals *via* liver cytochrome P450 metabolism of CCl_4. To further study the effect of free radical levels on alteration in nuclear NTPase activity,

the nuclear extracts were co-incubated with Fenton's radicals at different concentrations for 30 min. The nuclear NTPase activity was inhibited by Fenton's radicals in a dose-dependent manner (Figure 3), suggesting that metabolism of CCl_4 radicals is an intermediate step in inhibiting nuclear NTPase activity, mRNA nucleocytoplasmic transport, and protein synthesis.

The cellular concentration of a protein is determined by a delicate balance of at least seven processes, each one under several potential points of regulation. It has been shown that gene expression is down-regulated by CCl_4 and other chemicals. However, the mechanical mechanism underlying inhibition of protein synthesis in liver induced by CCl_4 is correlated to the decreasing nucleocytoplasmic transport of mature mRNA. Advances in modern chemical industry demand to evaluate the safety of various chemical substances. The comprehensive evaluation of these chemical substances has to be further studied in hepatotoxicity.

COMMENTS

Background

Many proteins produced in liver, such as albumin and prothrombin, are involved in coordination in the body's metabolism. The importance of protein function in pathogenesis of liver disease has been increasingly recognized. Carbon tetrachloride (CCl_4) is an organic solvent widely used in chemical industry. Its hepatotoxicity includes fatty liver and hepatic necrosis, and evidence also shows that CCl_4 inhibits protein synthesis in liver with early hepatotoxic damage. The mechanism underlying alterations in hepatic protein synthesis induced by CCl_4 treatment, however, is largely unknown.

Research frontiers

Among the steady-state levels of liver protein, mature mRNA acting as a template is an important determinant regulator for protein synthesis. The mRNA nucleocytoplasmic transport has to be promoted by mechanic energy from the nuclear membrane-associated nucleotide triphosphatase (NTPase), which can catalyze the hydrolysis of nucleoside triphosphate. Inhibition of protein synthesis in liver is correlated with down-regulation of nucleocytoplasmic transport in total mRNA during hepatotoxicity induced by CCl_4.

Innovations and breakthroughs

This study focused on the effect of mRNA nucleocytoplasmic transport on protein synthesis in liver. The results showed that the decreased NTPase activity of hepatic nuclear envelope inhibits mRNA nucleocytoplasmic transport during protein synthesis in liver inhibited by CCl_4.

Applications

This study highlighted the importance of nucleocytoplasmic mRNA transport in regulation of protein synthesis in liver. Nucleocytoplasmic mRNA transport and gene transcription are deserved in assessment of hepatotoxicity of prospective chemical substances.

Terminology

A nuclear envelope NTPase is a key enzyme involved in nucleocytoplasmic transport by catalyzing the hydrolysis of nucleoside triphosphate. When the activity of injured NTPase in nuclear envelope is decreased, the number of mRNA templates in cytoplasm is influenced.

Peer review

The manuscript is well written and the hypothesis contributes to our understanding of the mechanism underlying CCl_4-induced liver damage.

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S- Editor Wang YR **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3957

World J Gastroenterol 2010 August 21; 16(31): 3957-3963
ISSN 1007-9327 (print)

BRIEF ARTICLE

Usefulness of CT angiography in diagnosing acute gastrointestinal bleeding: A meta-analysis

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Supported by Shanghai Leading Academic Discipline Project, No. S30203 and Shanghai Jiaotong University School of Medicine Leading Academic Discipline Project
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Received: March 1, 2010 Revised: April 27, 2010
Accepted: May 4, 2010
Published online: August 21, 2010

Abstract

AIM: To analyze the accuracy of computed tomography (CT) angiography in the diagnosis of acute gastrointestinal (GI) bleeding.

METHODS: The MEDLINE, EMBASE, Cancerlit, Cochrane Library database, Sciencedirect, Springerlink and Scopus, from January 1995 to December 2009, were searched for studies evaluating the accuracy of CT angiography in diagnosing acute GI bleeding. Studies were included if they compared CT angiography to a reference standard of upper GI endoscopy, colonoscopy, angiography or surgery in the diagnosis of acute GI bleeding. Meta-analysis methods were used to pool sensitivity and specificity and to construct summary receiver-operating characteristic.

RESULTS: A total of 9 studies with 198 patients were included in this meta-analysis. Data were used to form 2 × 2 tables. CT angiography showed pooled sensitivity of 89% (95% CI: 82%-94%) and specificity of 85% (95% CI: 74%-92%), without showing significant heterogeneity (χ^2 = 12.5, P = 0.13) and (χ^2 = 22.95, P = 0.003), respectively. Summary receiver operating characteristic analysis showed an area under the curve of 0.9297.

CONCLUSION: CT angiography is an accurate, cost-effective tool in the diagnosis of acute GI bleeding and can show the precise location of bleeding, thereby directing further management.

Key words: Angiography; Gastrointestinal hemorrhage; Meta-analysis; Sensitivity and specificity; Tomography

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Wu LM, Xu JR, Yin Y, Qu XH. Usefulness of CT angiography in diagnosing acute gastrointestinal bleeding: A meta-analysis. *World J Gastroenterol* 2010; 16(31): 3957-3963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3957.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3957

INTRODUCTION

Acute gastrointestinal (GI) bleeding represents a common medical emergency with an annual incidence of 40-150 episodes per 100 000 persons for upper GI hemorrhage and 20-27 episodes per 100 000 persons for lower GI hemorrhage[1]. GI bleeding is usually classified as upper or

lower based on whether the bleeding source is proximal or distal to the ligament of Treitz[2].

Nearly 75% of bleeding will cease spontaneously, however, bleeding can recur in 25% of cases, causing significant morbidity and mortality[3,4]. Mortality rates are generally 3%-5%, but can reach up to 23% with massive bleeding or bleeding that recurs after hospitalization[5].

There are multiple modalities that are currently being used in the evaluation and treatment of acute GI hemorrhage, each with its own strengths and weaknesses[6,7].

Esophagogastroduodenoscopy (EGD) and colonoscopy are currently considered the first-line diagnostic procedures of choice for both upper and lower GI bleeding[8,9]. Endoscopic evaluation allows relatively safe, direct localization and characterization of bleeding lesions within the majority of the upper GI tract as well as in the colon and distal ileum. The distal duodenum and the majority of the small bowel cannot be adequately assessed with conventional endoscopy. The reported sensitivity and specificity of EGD for upper GI bleeding are 92%-98% and 30%-100%, respectively[4].

Noninvasive imaging with technetium-99m (Tc-99m)-labeled red blood cell (RBC) or Tc-99m sulfur colloid scintigraphy can be used to detect and localize GI bleeding. Tc-99m RBC scintigraphy is 93% sensitive and 95% specific for detecting a bleeding site with active arterial or venous bleeding rates as low as 0.2 mL/min[10] anywhere within the GI tract. An advantage of red cell scintigraphy is the ability to carry out delayed scans up to 24 h after radioisotope injection to detect rebleeding. Radionuclide scintigraphy has a false localization rate of approximately 22%, which limits its value as a diagnostic test[11].

Mesenteric angiography can detect bleeding rates greater than 0.5 mL/min and has the advantage of therapeutic intervention through transcatheter embolization. Angiography has a sensitivity of 40%-86%[12,13].

Initial experience indicates that multidetector computed tomography (CT) angiography is a promising first-line modality for the time-efficient, sensitive, and accurate diagnosis or exclusion of active GI hemorrhage and may have a profound impact on the evaluation and subsequent treatment of patients who present with acute GI bleeding.

In this article, we discuss and illustrate the emerging role of CT angiography in the evaluation and localization of acute, active GI hemorrhage.

MATERIALS AND METHODS

Literature search

A comprehensive computer literature search was performed to identify studies assessing the diagnostic value of CT angiography for acute GI bleeding.

The MEDLINE and EMBASE databases, from January 1995 to December 2009, were searched with the following key words: "gastrointestinal hemorrhage" OR "gastrointestinal bleeding" AND "CT angiography" OR "X-ray computed" AND (sensitivity OR specificity OR false-negative OR false-positive OR diagnosis OR detection OR accuracy).

Other databases, such as Sciencedirect, Springerlink, Scopus and the Cochrane library were also checked for relevant articles using the same keywords. The references reported in all retrieved articles were also supplemented with extensive checking.

Selection of studies

Two investigators (Wu LM and Xu JR), who were blinded to the journal, author, institution and date of publication, independently checked retrieved articles. According to a standardized data extraction form, we read all the abstracts to obtain potentially eligible articles, and then we managed to get the full text of these articles to determine whether they were exactly eligible. Disagreements were resolved by consensus.

The inclusion criteria were (1) articles were published in English; (2) CT was used as the index test in the diagnosis of GI bleeding; (3) The reference test had to be angiography, endoscopy, colonoscopy, surgery or a combination; (4) For per-patient statistics, sufficient data were presented to calculate the true-positive (TP), false-negative (FN), false-positive (FP) and true-negative (TN) values; (5) 5 or more patients were included; (6) With regard to the quality of the study design, only articles in which the number of "yes" answers for the 14 questions in the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) quality assessment tool[14] was more than 9 were included; and (7) When data or subsets of data were presented in more than one article, the article with most details or the most recent article was chosen. The authors of abstracts and studies not reporting sufficient data were contacted to request additional information.

The excluded criteria were as follows: (1) Articles which did not include raw data such as reviews, case reports, comments, editorials, letters and congress; (2) Articles which used CT as the tool for diagnosis without information on CT alone; (3) Diagnosis of GI bleeding for other existing diseases and could not be differentiated; and (4) Reports specializing in the etiology of bleeding.

Quality assessment

A quality assessment tool for diagnostic accuracy studies, named "QUADAS", was used to evaluate the quality and to extract relevant study design characteristics of included studies[15]. This tool is an evidence-based quality assessment tool developed for use in systematic reviews of studies of diagnostic accuracy and was fully described by Whiting *et al*[14].

Statistical analysis

2 × 2 tables were extracted on per-patient basis, including the numbers of TP, TN, FP and FN results in each study. Pooled sensitivity and specificity, with 95% CI, were obtained. A value of 0.5 was added to all cells of studies that contained a count of zero to avoid potential

Table 1 Study characteristics

Author	Yr	*n*	Age (average, yr)	Study design	Patient selection	Type of CT	Reference standard	Male/ female	QUADAS score
Ettorre *et al*[16]	1997	18	N/A	Prospective	Unselected	Single slice	Conventional angiography or surgery	N/A	9
Ernst *et al*[17]	2003	24	59 (18-85)	Prospective	Unselected	Single slice	Colonoscopy, enteroscopy or surgery	15/4	10
Tew *et al*[18]	2004	13	N/A	Retrospective	Unselected	4-slice	Conventional angiography, surgery or clinical follow-up	15/4	9
Miller *et al*[19]	2004	18	69 (43-83)	Prospective	Selected	Dual slice	Endoscopy, colonoscopy or conventional angiography	9/9	6
Sabharwal *et al*[20]	2006	7	69 (48-83)	Prospective	Selected	4-slice	Conventional angiography or colonoscopy	2/5	10
Yoon *et al*[21]	2006	26	66 (18-89)	Prospective	Unselected	4-slice	Digital subtraction angiography	17/9	12
Jaeckle *et al*[22]	2008	36	51 (4-85)	Retrospective	Selected	16, 40-slice	Endoscopy, or surgery	22/14	10
Zink *et al*[23]	2008	41	55 (21-92)	Prospective	Selected	16-slice	Labeled red blood cell scan or surgery	N/A	9
Lee *et al*[24]	2009	15	72 (42-90)	Retrospective	Unselected	16, 64-slice	Conventional angiogram, colonoscopy, capsule enteroscopy, labeled red blood cell scan or surgery	9/6	9

Quality Assessment of Diagnostic Accuracy Studies (QUADAS) score: study quality was evaluated using the 14 items specified in the QUADAS tool. One point was given for each criterion met, giving a total quality score out of 14. N/A: Data not available; CT: Computed tomography.

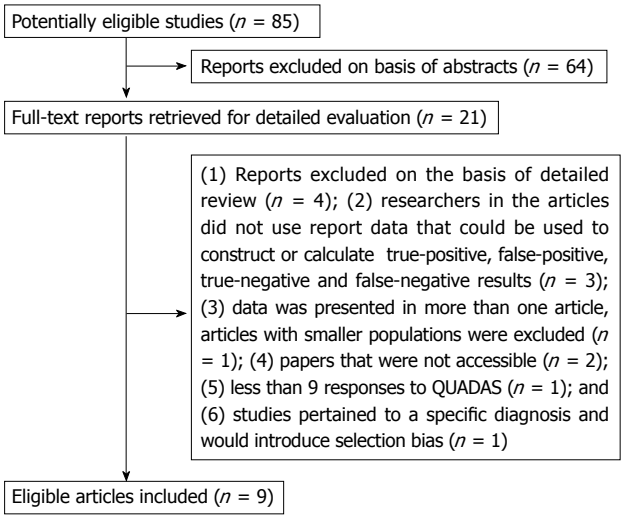

Figure 1 Reports evaluated for inclusion in the meta-analysis. QUADAS: Quality Assessment of Diagnostic Accuracy Studies.

problems in odds calculations for studies with sensitivities or specificities of 100%.

Heterogeneity was assessed with the χ^2-test using a random effects model (DerSimonian and Laird). A summary receiver operating characteristic (SROC) curve was fitted using the Moses and Littenberg model and a weighted area under the curve (AUC) obtained to measure the diagnostic performance of CT angiography. Statistical analysis was conducted using META-DISC software (version 1.4; Clinical Biostatistics Unit, Ramo´ny Cajal Hospital, Madrid, Spain).

RESULTS

Literature search and study design characteristics

The detailed procedure of study selection for the meta-analysis is shown in Figure 1. Eighty five initial studies were searched from all the databases. After reading the abstracts, we reviewed 21 studies in detail. Of these articles, 12 were excluded because: (1) Reports excluded on the basis of detailed review (n = 4); (2) researchers in the articles did not use report data that could be used to construct or calculate TP, FP, TN and FN results (n = 3); (3) data was presented in more than one article, articles with smaller populations was excluded (n = 1); (4) paper was not accessible (n = 2); (5) less than 9 responses to QUADAS (n = 1); and (6) studies pertained to a specific diagnosis and would introduce selection bias (n = 1).

Finally, 9 articles fulfilled all the inclusion criteria and were selected for data extraction and data analysis. We obtained the full text for all 9 eligible studies, some features of each are shown in Table 1.

Study description and study quality

The median number of participants per study was 18 (range 7-41). Our study reported the results by using an individual patient as the unit of analysis. Seven studies used multidetector CT (MDCT), which ranged from a dual-slice CT to a 64-slice CT. Protocols for CT scanning varied among studies, with one study using water as an oral contrast[19], and another study using intraarterial contrast[16]. The criterion for a positive CT was generally extravasation of contrast into the bowel lumen. The study by Ernst *et al*[17] used a broader definition by also including contrast enhancement of the bowel wall, presence of vascular abnormalities, polyp, tumor or spontaneous hyperdensity of peribowel fat. The reference standard among studies consisted of angiography (either digital subtraction or conventional angiography), upper GI endoscopy, colonoscopy and surgery, either alone or in combination.

Of the 9 studies, six[16,17,19,20-23] enrolled patients prospectively. Three studies[18,22,24] were retrospective database reviews.

The severity of GI bleeding varied and included patients who did not require blood transfusion to patients who required multiple transfusions. One study focused on massive GI bleeding - which was defined as hemodynamic

Table 2 Study results

Study	*n*	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Ettorre *et al*[16]	18	13	0	3	2	0.81 (0.54-0.96)	1.00 (0.16-1.00)
Ernst *et al*[17]	24	15	0	4	5	0.79 (0.54-0.94)	1.00 (0.48-1.00)
Tew *et al*[18]	13	7	0	0	6	1.00 (0.59-1.00)	1.00 (0.54-1.00)
Miller *et al*[19]	18	14	0	2	2	0.88 (0.62-0.98)	1.00 (0.16-1.00)
Sabharwal *et al*[20]	7	5	0	0	2	1.00 (0.48-1.00)	1.00 (0.16-1.00)
Yoon *et al*[21]	26	20	1	2	3	0.91 (0.71-0.99)	0.75 (0.19-0.99)
Jaeckle *et al*[22]	36	26	0	0	10	1.00 (0.87-1.00)	1.00 (0.69-1.00)
Zink *et al*[23]	41	5	5	1	30	0.83 (0.36-1.00)	0.86 (0.70-0.95)
Lee *et al*[24]	15	7	5	2	1	0.78 (0.40-0.97)	0.17 (0.00-0.64)

FN: False negative; FP: False positive; NA: Not applicable; TN: True negative; TP: True positive.

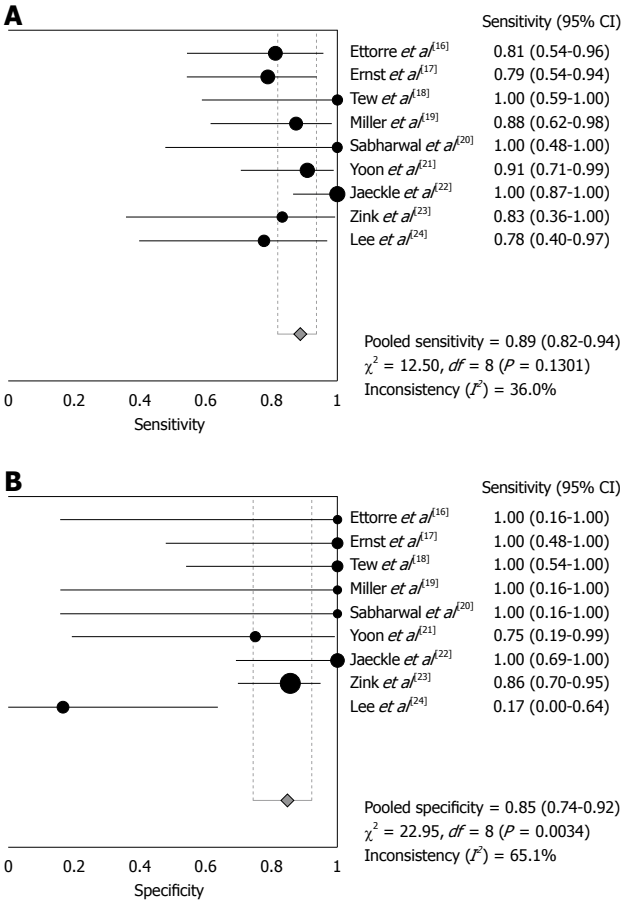

Figure 2 Forest plot of pooled sensitivity and specificity of computed tomography angiography in diagnosis of site of acute gastrointestinal bleeding. A: Sensitivity; B: Specificity.

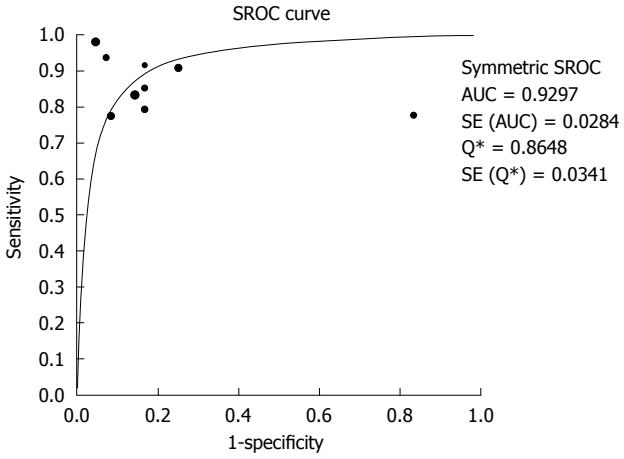

Figure 3 Summary receiver-operating characteristic curve for computed tomography angiography in the diagnosis of site of acute gastrointestinal bleeding. SROC: Summary receiver-operating characteristic; AUC: Area under the curve.

instability or a transfusion requirement of greater than 4 units of packed cells within a 24-h period[21]. Two primary studies pertained to lower GI bleeding exclusively[18,20].

The quality and completeness of the reporting of studies was variable (quality scores in Table 1). Three studies were retrospective in nature and interpretation of the tests was blinded in only two studies. The reference test was not standardized among studies, raising the possibility of differential verification bias affecting estimates of accuracy.

Pooled sensitivity, pooled specificity and AUC

CT angiography showed pooled sensitivity of 89% (95% CI: 82%-94%) without significant heterogeneity (χ^2 = 12.5, P = 0.13). Pooled specificity was 85% (95% CI: 74%-92%) without showing significant heterogeneity (χ^2 = 22.95, P = 0.003). Using the fitted SROC curve, overall AUC was 0.9297, indicating very good diagnostic accuracy. TP, FN, FP and TN results are shown in Table 2. Sensitivities and specificities are showed in Figure 2, and the SROC curve is shown in Figure 3.

In the study by Miller *et al*[19], six patients had a source of bleeding suggested on CT that was not detected with endoscopy or surgery. One of these patients had a 99mTc red cell scan, which showed a bleeding site in the cecum. The bleeding sites of the other five patients were attributed to bleeding that subsequently stopped or bleeding from a small bowel source, which was difficult to detect with endoscopy. In the absence of any alternative diagnoses, the five positive CT studies were classified as true positives.

DISCUSSION

Acute GI bleeding is an emergency situation with high mortality rates. Therefore, fast detection and localization of the bleeding site are required for effective hemostatic therapy. Currently, the first diagnostic procedure in pa-

tients with upper GI tract bleeding is endoscopy, whereas colonoscopy, conventional angiography or 99mTc-RBC scintigraphy are the standard procedures in patients with lower GI tract hemorrhage. Unfortunately, effective endoscopy may be hampered by blood clots or feces obscuring the bleeding site in unprepared patients, while potential disadvantages of conventional invasive angiography include an increased risk of complications[13]. Inherent drawbacks of scintigraphy are that it is a time-consuming method with only limited sensitivity for the identification of bleeding sites.

Fortunately, with high sensitivity, as shown in our review, CT angiography is a possible option for the investigation of GI bleeding. In an animal model of colonic hemorrhage, Kuhle *et al*[25] reported that single detector helical CT angiography can depict active hemorrhage with a rate of 0.3 mL/min, thus exceeding the sensitivity of mesenteric angiography of 0.5 mL/min and approaching values of RBC scintigraphy of 0.2 mL/min.

Due to higher spatial and temporal resolution provided by MDCT technology, faster scanning times and improved multiplanar reformatted images, the limited number of studies available precludes subgroup analysis to assess whether this has improved the diagnostic accuracy of CT.

Numerous studies have been performed to evidence the role of CT relative to conventional angiography for the diagnostic workup of patients suffering from acute GI bleeding[15,25-27]. These reports include case studies by Krestan *et al*[26], and Singer[27] as well as single-detector row helical CT studies performed in patients[16] and animals[25]. Several studies in our series have shown that CT can diagnose bleeding where angiography failed to locate the source[16,20,28]. In the study by Sabharwal *et al*[20], CT showed the site of bleeding in three patients for whom conventional angiography was negative. Subsequent emergency colonoscopy confirmed the presence of a blood-filled colon without showing the site of bleeding.

Jaeckle *et al*[22] evaluated the accuracy of MDCT for detection and localization of acute upper and lower GI hemorrhage or intraperitoneal bleeding. Thirty-six consecutive patients with clinical signs of acute bleeding underwent biphasic MDCT. MDCT findings were correlated with endoscopy, angiography or surgery. Among the 36 patients evaluated, 26 were examined for GI bleeding and 10 for intraperitoneal hemorrhage. Confirmed sites of GI bleeding were the stomach ($n = 5$), duodenum ($n = 5$), small bowel ($n = 6$), large bowel ($n = 8$) and rectum ($n = 2$). The correct site of bleeding was identifiable on MDCT in 24/26 patients with GI bleeding.

The sensitivity of CT may be higher than reported because GI bleeding is intermittent in nature and the rate of bleeding can vary from minute to minute[29]. A patient may have active GI bleeding shown on CT, which ceased by the time colonoscopy or angiography was carried out. The article by Miller *et al*[19] had five cases with a source of bleeding visible on CT that other methods of investigation failed to detect.

CT angiography is an excellent diagnostic tool for fast and accurate detection and localization of acute GI hemorrhage and intraperitoneal bleeding. Advantages of MDCT for the diagnostic workup of patients with suspected acute abdominal hemorrhage include widespread availability, speed, reproducibility and minimal invasiveness. The complications of invasive angiography, such as groin hematoma, dissection and distal embolization are reported to occur in 1.3%-2.2% of procedures[30,31]. Moreover, CT scanning of the abdomen can be performed immediately during hemorrhagic episodes, and bleeding can be depicted within the small bowel, an anatomic region not readily accessible to endoscopy. Furthermore, when a bleeding site is shown on CT and the initial mesenteric angiographic examination is negative, delayed selective angiography can be carried out to locate and embolize the bleeding vessel[32].

Limitations of CT angiography for detection of acute GI bleeding are the lack of therapeutic options in comparison to those that are available with endoscopy, colonoscopy and angiography, radiation dose, and risks affiliated with contrast material such as allergy, nephropathy, or hyperthyreosis. Another disadvantage of CT angiography is that metallic artifacts can interfere with visualization of contrast in the bowel lumen and lead to false positive results on CT angiography, as shown in the study by Yoon *et al*[33].

In this meta-analysis, bias was considered. To avoid selection bias, not only the MEDLINE and EMBASE databases but also the Sciencedirect, Springerlink, Scopus, and the Cochrane library were searched for relevant articles.

To minimize bias in the selection of studies and in data extraction, reviewers who were blinded to the journal, author, institution and date of publication, independently selected articles on the basis of inclusion criteria. In addition, scores were assigned to study design characteristics and examination results by using a standardized form that was based on the QUADAS tool. The QUADAS tool is an evidence-based quality assessment tool, which was developed for use in systematic reviews of studies of diagnostic accuracy[14].

To ensure all the selected articles were high quality articles, only articles in which the number of "yes" answers for the 14 questions in the QUADAS quality assessment tool[14] was more than 9, were selected. If the number of "no" or "unclear" answers was more than 4, the article was excluded. In this way, we excluded low quality articles to make sure the results of this research are credible.

However, some limitations still exist in this meta-analysis. A potential limitation of any meta-analysis is the possibility of publication bias because studies with optimistic results may be published easier than studies with unfavorable results, and studies with large sample size may be published easier than studies with small sample size. We attempted to examine publication bias by evaluating whether the size of the studies was associated with the results for diagnostic accuracy. No association was found between sample size and diagnostic accuracy.

The inclusion bias should be considered in our study.

Only the studies published in English were selected in this meta-analysis. This could cause unavoidable bias. However, this bias was small because most studies of high quality were published in English. Besides, all primary studies had relatively low numbers of subjects ($n = 7$ to $n = 41$). In practice, larger numbers of subjects are difficult to obtain because CT angiography is not commonly used as an investigation for GI bleeding at most institutions[33]. Thirdly, the overall quality of included studies, as defined in the QUADAS tool, was only moderate. Finally, clinical heterogeneity among studies is also an issue. The severity of GI bleeding varied, with one study focusing on "massive" GI bleeding. The primary different definitions for a positive CT may have resulted in misclassification.

We propose the routine use of CT angiography in the initial radiological investigation of patients who meet the criteria for acute GI hemorrhage. Because it is accurate in the diagnosis of acute GI bleeding and can show the precise location and etiology of bleeding, thereby directing further management. However, further large prospective studies are needed to define the role of CT in acute GI bleeding when other investigations are unable to provide a diagnosis.

COMMENTS

Background

Acute gastrointestinal (GI) bleeding is an emergency situation with high mortality rates. Fast detection and localization of the bleeding site are required for effective hemostatic therapy. Due to higher spatial and temporal resolution provided by multi-row detector computed tomography (MDCT) technology, acquisition of arterial- and portal-venous phase images as well as depiction of active extravasation of contrast material have become feasible.

Research frontiers

MDCT is an excellent diagnostic tool for fast and accurate detection and localization of acute GI hemorrhage and intraperitoneal bleeding. Advantages of MDCT for the diagnostic workup of patients with suspected acute abdominal hemorrhage include widespread availability, speed, reproducibility and minimal invasiveness. Moreover, computed tomography (CT) scanning of the abdomen can be performed immediately during hemorrhagic episodes, and bleeding can be depicted within the small bowel, an anatomic region not readily accessible to endoscopy.

Innovations and breakthroughs

Currently, CT angiography is not widely used in the diagnosis of acute GI bleeding. This meta-analysis confirmed that it has the advantage of being a noninvasive test, which diagnoses the site and cause of bleeding, thereby guiding definitive treatment.

Applications

Invasive selective interventional angiography or surgery can be performed immediately after MDCT has identified the bleeding site. This diagnostic algorithm may lead to a significant reduction in time and may substantially decrease the rate of negative angiographies in the future.

Peer review

This is a systematic review concerning the usefulness of CT angiography on acute GI bleeding. Authors have nicely performed the meta-analysis for the "usefulness of CT angiography in diagnosing acute GI bleeding". This article is well written and beneficial for many physicians.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3964

World J Gastroenterol 2010 August 21; 16(31): 3964-3969
ISSN 1007-9327 (print)

BRIEF ARTICLE

Multiple primary malignant tumors of upper gastrointestinal tract: A novel role of ^{18}F-FDG PET/CT

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Supported by The Fund from the Bureau of Public Health of Xiamen City for creative research by young scientists, No. WQK0704; and the fund from the Health Department of Fujian Province for young scholars, No. 2008-1-49
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Received: March 25, 2010 Revised: May 12, 2010
Accepted: May 19, 2010
Published online: August 21, 2010

Abstract

AIM: To evaluate the capacity of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) for detecting multiple primary cancer of upper gastrointestinal (UGI) tract.

METHODS: Fifteen patients (12 without cancer histories and 3 with histories of upper GI tract cancer) were investigated due to the suspicion of primary cancer of UGI tract on X-ray barium meal and CT scan. Subsequent whole body ^{18}F-FDG PET/CT scan was carried out for initial staging or restaging. All the patients were finally confirmed by endoscopic biopsy or surgery. The detection rate of multiple primary malignant cancers was calculated based on ^{18}F-FDG PET/CT and endoscopic examinations.

RESULTS: ^{18}F-FDG PET/CT scan was positive in 32 suspicious lesions, 30/32 were true positive primary lesions, and 2/32 were false positive. In 15 suspicious lesions with negative ^{18}F-FDG PET/CT scan, 12/15 were true negative and 3/15 were false negative. Among the 15 patients, 12 patients had 29 primary synchronous tumors confirmed by pathology, including 8 cases of esophageal cancers accompanied with gastric cancer and 4 of hypopharynx cancers with esophageal cancer. The other 3 patients had 4 new primary metachronous tumors, which were multiple primary esophageal cancers. PET/CT imaging detected local lymph node metastases in 11 patients. Both local lymph node metastases and distant metastases were detected in 4 patients. On a per-primary lesion basis, the sensitivity, specificity, accuracy, negative predictive value and positive predictive value of ^{18}F-FDG PET/CT for detecting multiple primary cancer of UGI tract were 90.9%, 85.7%, 89.4%, 80% and 93.7%, respectively.

CONCLUSION: The whole body ^{18}F-FDG PET/CT may play an important role in evaluating the multiple primary malignant tumors of UGI tract cancer.

Key words: Upper gastrointestinal tract cancer; Esophageal cancer; Gastric cancer; Positron emission tomography/computed tomography; ^{18}F-fluorodeoxyglucose

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Sun L, Wan Y, Lin Q, Sun YH, Zhao L, Luo ZM, Wu H. Multiple primary malignant tumors of upper gastrointestinal tract: A novel role of ^{18}F-FDG PET/CT. *World J Gastroenterol* 2010; 16(31): 3964-3969 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3964.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3964

INTRODUCTION

Metachronous or synchronous esophageal cancer has been identified in patients with head and neck cancer, gastric cancer or colon cancer[1]. Alcohol drinking and tobacco smoking are the major risk factors for upper aerodigestive tract cancers, accounting for a large proportion of cases in developed countries[2,3]. The occurrence of multiple primary cancers in the aerodigestive tract has been explained by the concept of field carcinogenesis. Combined exposure to alcohol and tobacco has a multiplicative effect on carcinogenesis of upper aerodigestive tract[4,5].

X-ray barium meal examination, endoscopic examination, computed tomography (CT) are the main techniques used for the staging and follow-up of upper gastrointestinal (UGI) tract cancer[6,7]. However, the reported increase in sensitivity of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (PET/CT) over CT has been attributed to the capacity of ^{18}F-FDG PET/CT to detect metabolic abnormalities that precede the morphological changes seen by CT. This study was undertaken to further define the value of ^{18}F-FDG PET/CT in evaluating multiple primary metachronous or synchronous cancer of UGI.

MATERIALS AND METHODS

Patients

Fifteen patients with multiple UGI tract cancer (13 males and 2 female, aged 49-78 years with a mean age of 61 years) were selected for a retrospective review from our electronic database, who were imaged by ^{18}F-FDG PET/CT between January 2007 and January 2010 because of the suspicious findings for multiple UGI tract cancer by the X-ray barium meal and endoscopic examinations. All the patients were finally confirmed by endoscopic biopsy or surgery.

^{18}FDG PET/CT technique

The patients were asked to fast for at least 4 h before undergoing ^{18}F-FDG PET/CT. Their blood glucose level should be within the normal range (70-120 mg/dL) prior to intravenous injection of ^{18}F-FDG. The patients received an intravenous injection of 370-666 MBq (10-18 mCi) of ^{18}F-FDG. Data acquisition by an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA) was performed within 60 min after injection. The procedure of data acquisition was as follows: CT scanning was performed first, from the head to the pelvic floor, with 110 kV, 110 mA, a tube rotation time of 0.5 s, a 3.3-mm section thickness, which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan covering the identical transverse field of view was obtained. Acquisition time was 3 min per table position. PET image data sets were reconstructed iteratively by applying the CT data for attenuation correction, and coregistered images were displayed on a workstation.

PET/CT image interpretation

The ^{18}F-FDG PET/CT images were prospectively interpreted by two experienced nuclear physicians. One had 21 years of experience in both nuclear medicine and radiology, and the other had six years of experience in both nuclear medicine and radiology, who read the ^{18}F-FDG PET/CT images on a high-resolution computer screen, and reached a consensus in cases of discrepancy.

Based the knowledge of the normal biodistribution of ^{18}F-FDG, lesions were identified as foci with increased tracer accumulation relative to that in comparable normal contralateral structures and surrounding soft tissues. The lesions were qualitatively graded as definitely or probably abnormal (categorized as representing a tumor) if the accumulation of ^{18}F-FDG was markedly to moderately increased. Diffuse and mildly increased activity or no increased activity (in the case of an abnormality identified on CT, but no corresponding abnormality was present on PET) was considered to be a normal or benign disease.

Statistical analysis

The results of PET/CT were quantified using the following definitions: accuracy [(true positive) + (true negative)/(total patients)], sensitivity [(true positive)/(true positive + false negative)], specificity [(true negative)/(true negative + false positive)], positive predictive value [(true positive)/(true positive +false positive)], and negative predictive value [(true negative)/(true negative + false negative)].

RESULTS

Clinical presentation

At the time of multiple primary lesions of suspected UGI cancer, the mean age of the patients was 61 years with a predominant tendency in men. Twelve men and one woman had heavy tobacco and alcohol consumption histories in this group of patients.

PET/CT is able to demonstrate the entire UGI tract in almost all patients, even following incomplete endoscopic examination due to obstructing upper esophageal tumors in 4 patients (Figure 1). PET/CT imaging is helpful to guide accurately localization of lower esophageal lesions in the remaining patients.

PET/CT diagnosis

^{18}F-FDG PET/CT scan was positive in 32 suspicious lesions, of which 30 were true positive primary lesions and 2 were false positive. Among 15 suspicious lesions with negative ^{18}F-FDG PET/CT scan, 12 were true negative and 3 were false negative.

On a per-primary lesion basis, sensitivity, specificity, accuracy, negative predictive value and positive predictive value of ^{18}F-FDG PET/CT in detecting multiple primary cancer of UGI tract were 90.9%, 85.7%, 89.4%, 80% and 93.7%, respectively.

Primary tumor, *local lymph node metastases and distant metastases*

Of the 15 patients, 12 patients had 29 primary synchro-

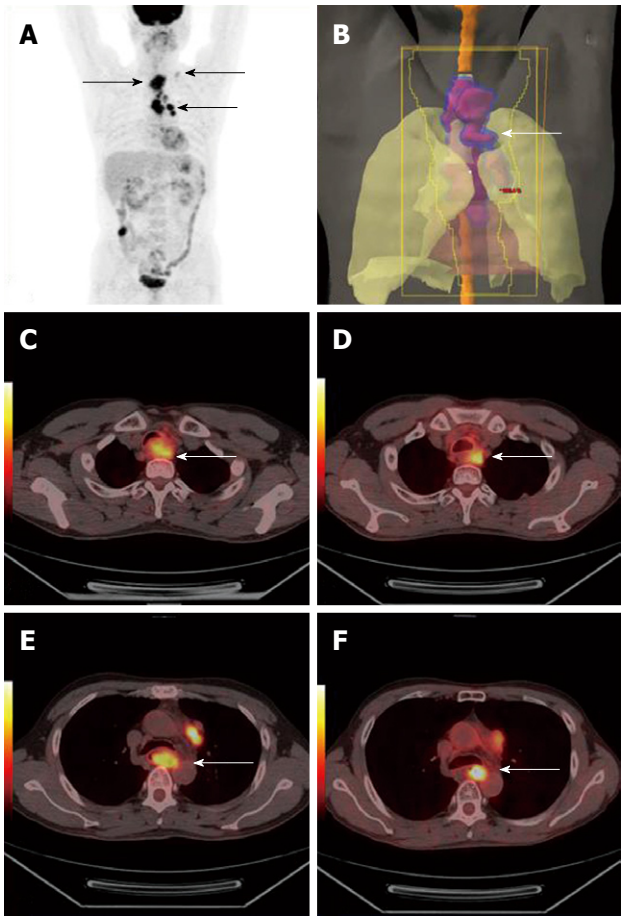

Figure 1 A 52-year-old man with synchronous multiple esophageal cancers. The first positron emission tomography/computed tomography (PET/CT) images revealed multiple hypermetabolic esophageal lesions and hypermetabolic supraclavicular, mediastinal lymph nodes (arrows in A, C, E). PET/CT imaging guided radiation plan (arrows in B). The second PET/CT images after 50Gy radiation treatment demonstrated shrinkage of lesions and decrease of fluorodeoxyglucose uptake (arrows D, F).

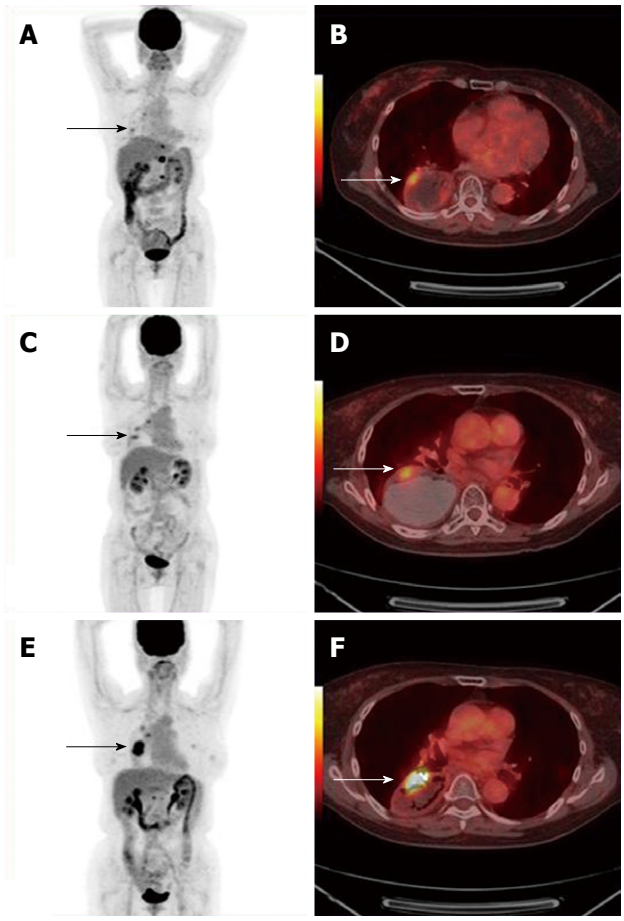

Figure 2 A 56-year-old woman with metachronous gastric cancer after esophageal cancer resection 5 years ago. Positron emission tomography/computed tomography (PET/CT) displayed multi-retroperitoneal lymph node recurrence (arrow in A), which was later verified as esophageal cancer metastasis (squamous cell carcinoma). PET revealed a small lesion at gastric tube, but endoscopy was negative (arrows in A, B, C, D). Third PET/CT showed an enlarged gastric lesion, and endoscopic biopsy verified gastric tube cancer (adenocarcinoma) (arrows in E, F).

nous tumors confirmed by pathology (Table 1), including 8 cases of esophageal cancer combined with gastric cancer and 4 had hypopharynx cancer combined with esophageal cancer. The other 3 patients had 4 new primary metachronous tumors (Table 2 and Figure 2).

PET/CT imaging detected local lymph node metastases in 11 patients (Figure 3). Both local lymph node metastases and distant metastases were detected in 4 patients.

Impact on clinical management

Clinical treatment plans were changed in 11 (73.3%) patients after PET/CT examination. ^{18}F-FDG PET/CT imaging-guided radiotherapy was performed in 11 patients.

DISCUSSION

Synchronous cancers were predominantly located in the aerodigestive tract, primarily in the lung, head and neck and esophagus[8]. Kumagai *et al*[9] reported half of the patients with multiple upper aerodigestive tract squamous cell carcinomas are initially seen with synchronous tumors. It is also believed that these second primary cancers arise independently following exposure to a common carcinogen by a process that has been called field cancerization[10]. At least 75% of head and neck cancers are attributable to a combination of cigarette smoking and alcohol drinking[11]. In our cases, 13 patients (86.7%) had heavy tobacco and alcohol consumption histories. Synchronous tumors of UGI tract were detected in 12 patients. Esophageal cancers combined with gastric cancer (53.3%) were most frequently seen in our study.

Patients with primary head and neck malignancies have a 3%-7% yearly incidence of second primary cancers. Most metachronous squamous cell carcinomas become manifest within 3 years[12]. Detection of second primaries has an important impact on therapy. For the patients treated for advanced squamous cell carcinoma in the oral cavity or oropharynx during the first year after completion of their curative treatment, routine surveillance for detecting early locoregional recurrence, distant metastases, and second metachronous primary tumors is very important[13,14]. Due to the poor prognosis of head

Table 1 Characteristics of synchronous tumor in 12 patients

Sex/age (yr)	PET/CT findings				Endoscopic results
	T	T	N	M	
F/61	EPC (lower)	Cardiac	Gastrohepatic		EPC (lower), cardiac
M/60	Hypopharynx	EPC (mid, lower)	Mediastinal		Hypopharynx
M/62	EPC (upper)	EPC (lower)	Supraclavicular		EPC (upper)
M/59	EPC (lower)	Cardiac	Mediastinal, gastrohepatic		EPC (lower), cardiac
M/61	Hypopharynx	EPC (mid)	Right cervical		Hypopharynx
M/62	EPC (mid)	Cardiac	Gastrohepatic		EPC (mid), cardiac
M/78	EPC (lower)	Pylorus	Mediastinal, gastrohepatic	Lung	EPC (lower), pylorus
M/57	EPC (mid, lower)	Cardiac	Mediastinal, gastrohepatic	Lung	EPC (mid, lower)
M/52	EPC (upper)	EPC (mid, lower)	Mediastinal, supraclavicular		EPC (upper, mid)
M/62	EPC (mid, lower)	Cardiac	Mediastinal, gastrohepatic	Liver	EPC (mid, lower)
M/53	Hypopharynx	EPC (mid, lower)	Cervical, mediastinal, retroperitoneal		Hypopharynx
M/60	EPC (upper)	EPC (mid)	Supraclavicular, mediastinal		EPC (upper, mid)

PET/CT: Positron emission tomography/computed tomography; EPC: Esophageal cancer.

Table 2 Characteristics of metachronous tumor in 3 patients

Sex/age (yr)	PET/CT findings				Endoscopic results
	Primary	Secondary	N	M	
M/68	EPC resection 10 yr ago	Hypopharynx	Cervical		Hypopharynx
M/49	GC resection 25 yr ago	EPC (lower), Cardiac	Supraclavicular	Lung	EPC (lower), cardiac
F/63	EPC resection 5 yr ago	Gastric tube cancer	Retroperitoneal		Gastric tube cancer

PET/CT: Positron emission tomography/computed tomography; EPC: Esophageal cancer; GC: Gastric cancer.

and neck cancer, the data of metachronous cancer of UGI tract were limited. In this study, 3 patients had 4 primary metachronous tumors. The interval from first tumor to second tumor ranged from 5 to 10 years.

In the majority of follow-up protocols, radiologic and endoscopic evaluation has been proven to be useful in the early detection of metachronous and recurrent neoplasms in the follow-up of patients with previously treated carcinomas of the ear, nose, and throat. Adequate staging of UGI cancer including CT and endoscopic ultrasonography has been considered to be helpful for avoiding useless surgery[15,16]. However, more than 30% of the distant metastases have been reported to be radiographically occult with conventional diagnostic strategy and surgery. It has been still performed in a considerable number of patients with distant metastases. Moreover, the overall survival after curative resection does not exceed 25%, with an overall median disease-free survival of only 12 mo.

Conventional staging methods consisting of both CT and EUS evaluate local unresectability or metastatic diseases based on the anatomic alterations. Their low sensitivity and low specificity were related to the low accuracy in determining a curative surgery, indicating the demand for a different approach[17,18]. In contrast to conventional anatomic imaging, PET can reveal metabolic alterations in tumor tissues. Most malignant tumors present a high uptake of ^{18}F-FDG due to an increased anaerobic glycolysis[19]. In our previous studies, ^{18}F-FDG PET/CT was found to be valuable in detecting previously unknown metastases in esophageal cancer. Routinely performed ^{18}F-FDG PET/CT in the preoperative work-up of these tumors may therefore reduce the number of unnecessary surgical procedures[20].

There have been several investigations into the utility of ^{18}F-FDG PET or PET/CT in relation to multiple primary cancers detection. Major advantages of the whole body ^{18}F-FDG PET/CT are the capability to perform full-body scan with the potential to detect local and distant metastases in one single examination and the possibility of distinguishing new active disease from scar or necrotic tissues[21,22] since tumors with increased ^{18}F-FDG uptake are more metabolically active and biologically aggressive[23,24].

The accurate staging of multiple primary cancer of UGI tract is essential to select appropriate treatment and to anticipate disease progression. Conventional imaging methods that rely on detection of the structural changes caused by tumors usually have limitations in determining the extent of UGI, especially lymph node metastasis[25,26]. PET/CT is a fundamentally different imaging technique that identifies focal areas of increased metabolism associated with malignancies. PET/CT is more sensitive than regular CT scan in determining regional and distant lymph node involvement in the squamous cell carcinoma as well as adenocarcinoma of the esophagus. PET can play an important role in evaluating the pretreatment staging of esophageal cancer[27,28]. A noteworthy finding in the present study is the high incidence of 100% (13/13) of local lymph node metastases and distant metastases

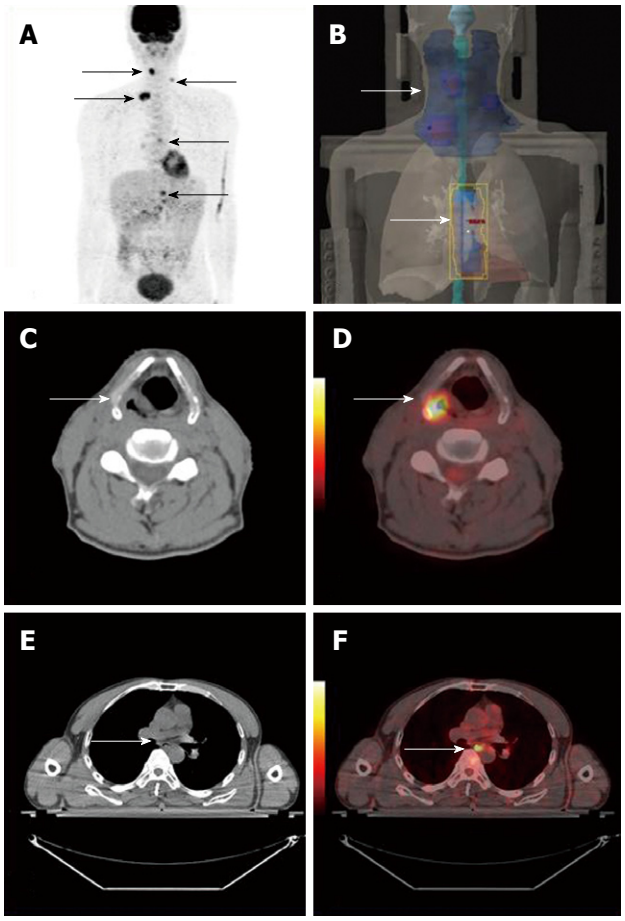

Figure 3 A 53-year-old woman with synchronous hypopharynx cancer combined with esophageal cancer. Positron emission tomography/computed tomography (PET/CT) images revealed hypermetabolic hypopharynx and esophageal lesions, and multiple hypermetabolic supraclavicular, mediastinal lymph nodes (arrows in A). PET/CT imaging guided radiation plan (arrows in B). CT and PET/CT fused images showed hypermetabolic hypopharynx and esophageal lesions (arrows in C, D, E, F).

detected by ^{18}F-FDG PET/CT. Furthermore, clinical decisions of treatment were changed in 11 patients after PET/CT examinations.

There are several limitations in this study. First, due to the retrospective feature of the study, we did not get all of the medical details for some of the patients. There are also some disadvantages associated with PET/CT imaging. For example, small tumors might be undetected because partial-volume effects result in a falsely low measurement of true ^{18}F-FDG activity[29]. Another drawback of PET/CT is that ^{18}F-FDG frequently accumulates in areas of inflammation. Variable physiologic FDG uptake patterns and benign pathological causes of ^{18}F-FDG uptake can be specifically recognized and properly categorized in other instances[30].

The choice of diagnostic techniques must be based on the site and histologic characteristics of the synchronous tumors. Although ^{18}F-FDG PET/CT may be the preferred technique for staging UGI cancer, it can not replace other techniques, such as Lugol chromoendoscopy, for detecting synchronous UGI cancer in high-risk populations[31].

Our results suggested that ^{18}F-FDG PET/CT may be useful in evaluating the multiple primary malignant tumors of UGI cancer. It may play an important role in the initial staging of multiple synchronous or metachronous UGI tract cancers.

COMMENTS

Background

Multiple primary malignancies in a single patient are relatively rare but have increased in frequency in recent decades. Metachronous or synchronous esophageal cancer has been identified in patients with head and neck cancer, gastric cancer or colon cancer. Alcohol drinking and tobacco smoking are the major risk factors for upper aerodigestive tract cancers, accounting for a large proportion of cases in developed countries. The occurrence of multiple primary cancers in the aerodigestive tract also has been explained by the concept of field carcinogenesis. Combined exposure to alcohol and tobacco has a multiplicative effect on carcinogenesis of upper aerodigestive tract.

Research frontiers

Positron emission tomography/computed tomography (PET/CT) provides anatomic landmarks for better characterization of increased ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake. PET/CT is a widely accepted imaging method in the management of a wide variety of cancers. The reported increase in sensitivity of PET/CT over conventional techniques has been attributed to the ability of PET/CT to detect metabolic abnormalities that precede the morphologic changes seen by CT. However, the usefulness and limitations of ^{18}F-FDG PET/CT in evaluating multiple primary malignant tumors of upper gastrointestinal tract still need further clinical evaluations.

Innovations and breakthroughs

The early detection of multiple primary malignant tumors of upper gastrointestinal (UGI) cancer will enable prompt management and will increase the cure rate of the disease. Whole body ^{18}F-FDG PET/CT scan could provide valuable information for early detection and might guide salvage treatment for multiple primary malignant tumors of UGI cancer.

Applications

^{18}F-FDG PET/CT may be useful in evaluating the multiple primary malignant tumors of UGI cancer. It may play an important role in the initial staging of multiple synchronous or metachronous UGI tract cancers.

Peer review

The manuscript is very well written and should be accepted for publication.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3970

World J Gastroenterol 2010 August 21; 16(31): 3970-3978
ISSN 1007-9327 (print)

BRIEF ARTICLE

Use of pre-, pro- and synbiotics in patients with acute pancreatitis: A meta-analysis

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Supported by The Grant for the Research Projects of Sichuan Province, China. No. 07FG002-032
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Received: April 14, 2010 Revised: May 25, 2010
Accepted: June 1, 2010
Published online: August 21, 2010

Abstract

AIM: To assess the clinical outcomes of pre-, pro- and synbiotics therapy in patients with acute pancreatitis.

METHODS: The databases including Medline, Embase, the Cochrane Library, Web of Science and Chinese Biomedicine Database were searched for all relevant randomized controlled trials that studied the effects of pre-, pro- or synbiotics in patients with acute pancreatitis. Main outcome measures were postoperative infections, pancreatic infections, multiple organ failure (MOF), systemic inflammatory response syndrome (SIRS), length of hospital stay, antibiotic therapy and mortality.

RESULTS: Seven randomized studies with 559 acute pancreatic patients were included. Pre-, pro- or synbiotics treatment showed no influence on the incidence of postoperative infections [odds ratios (OR) 0.30, 95% confidence interval (CI): 0.09-1.02, P = 0.05], pancreatic infection (OR 0.50, 95% CI: 0.12-2.17, P = 0.36), MOF (OR 0.88, 95% CI: 0.35-2.21, P = 0.79) and SIRS (OR 0.78, 95% CI: 0.20-2.98, P = 0.71). There were also no significant differences in the length of antibiotic therapy (OR 0.75, 95% CI: 0.50-1.14, P = 0.18) and the mortality (OR 0.75, 95% CI: 0.25-2.24, P = 0.61). However, Pre-, pro- or synbiotics treatment was associated with a reduced length of hospital stay (OR -3.87, 95% CI: -6.20 to -1.54, P = 0.001). When stratifying for the severity of acute pancreatitis, the main results were similar.

CONCLUSION: Pre-, pro- or synbiotics treatment shows no significant influence on patients with acute pancreatitis. There is a lack of evidence to support the use of probiotics/synbiotics in this area.

Key words: Probiotics; Synbiotics; Prebiotics; Nutrition support; Acute pancreatitis

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Zhang MM, Cheng JQ, Lu YR, Yi ZH, Yang P, Wu XT. Use of pre-, pro- and synbiotics in patients with acute pancreatitis: A meta-analysis. *World J Gastroenterol* 2010; 16(31): 3970-3978 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3970.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3970

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process

in mild to severe forms with a high mortality rate, frequently associated with necrosis of the gland[1]. The infected pancreatic necrosis is a principal late complication and a major cause of morbidity and mortality in patients with severe acute pancreatitis. In view of the deleterious effects of infected necrotizing pancreatitis, prophylactic antibiotics have been widely used. However, some randomized controlled trials (RCTs) and meta-analysis have not demonstrated significant benefits of prophylactic antibiotics on patients with necrotizing AP[2-4]. It is also not recommended for the routine use in sterile pancreatic necrosis[5].

A new anti-infectious strategy is needed. The administration of probiotics/synbiotics modulating the intestinal microbiota may be a valuable treatment option. Lilly and Stillwell first defined the term "probiotics" as ingestible microorganisms that benefited the host by improving intestinal microbial balance[6]. Previous studies showed the clinical benefits of probiotics (including *Lactobacilli* and *Bifidobacteria*), such as inhibiting proliferation of harmful bacteria, protecting the intestinal barrier, preventing gut bacterial translocation to blood and distant sites, and modulating the immune function[7,8], all of which contributed to the reduction of the incidence of nosocomial infections related to intestinal microbial imbalance. Prebiotics are also beneficial to enhancing the effects of enteral nutrition and probiotics[9-11]. For example, as one of the most important prebiotics, fibers can selectively stimulate growth or activity of certain colonic bacteria[12]. In addition, fibers are broken down by the probiotic bacteria to produce a whole series of nutrients including short-chain fatty acids which can stimulate mucosal cell growth, reduce translocation, and enhance the intestinal immune function in colon. Combined use of probiotics and prebiotics, which are called synbiotics, have been shown to enhance immunomodulating ability, balance gut microbiota, inhibit bacterial translocation and reduce the incidence of nosocomial infections in clinically surgical patients.

Evidences showed that the use of pro- or synbiotics might reduce postoperative infections after abdominal surgery[13-15]. Several RCTs have also been performed demonstrating a therapeutic and preventive effect of pre-, pro- or synbiotics treatment in patients with acute pancreatitis[16-18]. However, these studies were small in size, and have been underpowered. Recently, a multi-center RCTs reported some unexpected results contradicting the previous studies[19]. This trial was controversial for some shortcomings. Overall, the magnitude of the therapeutic effect remains unknown. We therefore performed this meta-analysis to assess the potential effects of pre-, pro- or synbiotics treatment in patients with acute pancreatitis by reviewing the current literature, and synthesizing the available data.

MATERIALS AND METHODS

Literature search strategy

A systematic review of the literature was performed to identify all RCTs assessing the effects of pre-, pro- or synbiotics treatment in acute pancreatitis. Two authors independently searched the database including Medline (1966 to March 2010), Embase (1980 to March 2010), Web of Science, Chinese Biomedicine Database (1979 to March 2010) and the Cochrane Library (2010, issue 1) with no language restriction using the following terms: "(prebiotic* OR probiotic* OR synbiotic* OR lactobacillus OR Bifidobacterium OR Lactobacilli) AND (acute pancreatitis)". We identified relevant studies initially by title, abstract, and finally by full text. The reference lists of all selected RCTs and previous systematic reviews were also searched by hand. If duplicate article was published by the same author using the same case series, the data from the most recent manuscript publications was included.

Inclusion and exclusion criteria

Only RCTs evaluating the use of pre-, pro- or synbiotics in patients with acute pancreatitis were included in this review. The trials should have at lease one of the followings as a primary outcome variable: number of infections and pancreatic infectious complications, number of multiple organ failure (MOF) and systemic inflammatory response syndrome (SIRS), surgical interventions, length of hospital stay, and mortality. Major reasons for exclusion of studies were (1) animal studies; (2) duplicate publication; and (3) no usable data reported.

Data extraction

Data was abstracted independently by two reviewers according to the following selection criteria: study design and period, population, intervention, and outcome variables listed above. Disagreement was resolved by discussion.

Methodological quality

We assessed the quality of the studies based on the random method, allocation concealment, blinding and follow-up. The methodological quality of the studies included in the meta-analysis was also scored with the Jadad scale[20], which was a 5-point quality scale defining low quality studies as having a score of < 3 and high-quality studies as having a score of $\geqslant 3$.

Statistical analysis

The statistical analysis was performed using the free software Review Manager (Version 5.0; The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark, 2008). Dichotomous data was presented as odds ratio (OR) with 95% confidence intervals (CI). Statistical heterogeneity was measured using the Chi-square test and the inconsistency index (I^2). A χ^2 P value < 0.05 was considered to indicate statistically significant heterogeneity. Fixed effects model was used when there was no heterogeneity of the results. Otherwise, the random effects model was used. Subgroup analyses stratified by the severity of acute pancreatitis were performed. Sensitivity analyses were performed only in high quality trials to avoid errors caused by poor quality studies. Visual

Table 1 Methodological characteristics of the clinical trials included in this meta-analysis

Trial	Patients (synbiotics/control)	Characteristics of patients (synbiotics/control)	Intervention	Control group	Length of treatment (d)
Oláh *et al*[16] (UK, 2002)	45 (22/23)	Mean Glasgow score (2.5/2.8), mean CRP (206.5/188.7) mg/L	10^9 *L. plantarum* 299 + EN + 10 g oat fiber	EN + heat-killed *L. plantarum* 299 + oat fiber	7
Oláh *et al*[17] (UK, 2007)	62 (33/29)	Mean Imrie score (2.9/3.1), mean CRP (216.7/191.2) mg/L	Four LAB: 10^{10} *P. pentosaceus*, *Leuconostoc mesenteroides*, *L. paracasei* and *L. plantarum* + four bioactive fiber (Synbiotics 2000) + EN	EN + four bioactive fiber	7
Karakan *et al*[25] (Turkey, 2007)	30 (15/15)	Mean APACHE Ⅱ score (9.4/9.6), mean CRP (232/244) mg/L	24 g multi-fibers including soluble fibers and insoluble fibers + EN	EN	6-13
Qin *et al*[18] (China, 2008)	74 (36/38)	Mean APACHE Ⅱ score (8.8/8.9), mean CRP (125/136) mg/L	10^8 *L. plantarum* + EN + PN	PN	7
Besselink *et al*[19] (Netherlands, 2008)	296 (152/144)	Mean APACHE Ⅱ score (8.6/8.4), mean Imrie score (3.3/3.4), mean CRP (268/270) mg/L	Six LAB: 10^{10} *L. acidophilus*, *L. casei*, *L. salivarius*, *L.lactis*, *B. bifidum*, and *B. lactis* (Ecologic 641) + cornstarch + maltodextrins + fiber-riched EN	Cornstarch + maltodextrins + fiber-riched EN	28
Li *et al*[26] (China, 2007)	25 (14/11)	APACHE Ⅱ score 8-20	Three LAB:7.2 × 10^7 *B. longum*, *L. bulgaricus* and *S. thermophilus* (Golden Bifid)	Water	7
Wu *et al*[27] (China, 2009)	27 (14/13)	APACHE Ⅱ score 8-20	Three strains: > 6 × 10^4 *L. lactis* + *L. acidophilus* and *S. lactis*	N/A	7

N/A: Not applicable; APACHE Ⅱ: Score of the second acute physiology and chronic health evaluation; CRP: C-reactive protein; LAB: Lactic acid bacteria; *L.*: *Lactobacillus*; *B.*: *Bifidobacterium*; *E.*: *Enterococcus*; *P.*: *Pediococcus*; *S.*: *Streptococcus*; EN: Enteral nutrition; PN: Parenteral nutrition.

inspection of asymmetry in funnel plots was conducted to assess the potential for publication bias.

RESULTS

Main characteristics of the studies

A total of 48 papers relevant to the searching words were identified through the bibliographic search. After initial eligibility screening, 41 of these papers were excluded, of which 24 were not randomized controlled studies, 10 were not conducted in humans, 3 did not report usable data, and 4 were duplicate publications[21-24]. Only 7 RCTs involving 559 patients met the inclusion criteria and were included in the meta-analysis[16-19,25-27]. The flow chart of study selection is summarized in Figure 1.

The main characteristics of the included patients between two groups were well matched in all RCTs (including age and gender). Five studies compared the score of the second acute physiology and chronic health evaluation (APACHE Ⅱ)[18,19,25-27], and five studies compared C-reactive protein[16-19,25]. Two of the studies tested a probiotics[16,18], one of the studies tested a prebiotics[25], while the remaining four studies tested a synbiotics (probiotics plus prebiotics)[17,19,26,27]. Five studies recruited patients with severe acute pancreatitis[17,19,25-27], and two studies recruited patients with mild, moderate and severe degrees of pancreatitis[16,18]. Patients with biliary tract diseases were excluded in one of the studies[16]. Only three studies reported adverse effects associated with administration of pre-, pro- or synbiotics, which included bowel ischemia, catheter-related sepsis, tube intolerance and reintube. The study details are summarized in Table 1. The surgical outcomes from the RCTs included in this meta-analysis are presented in Table 2.

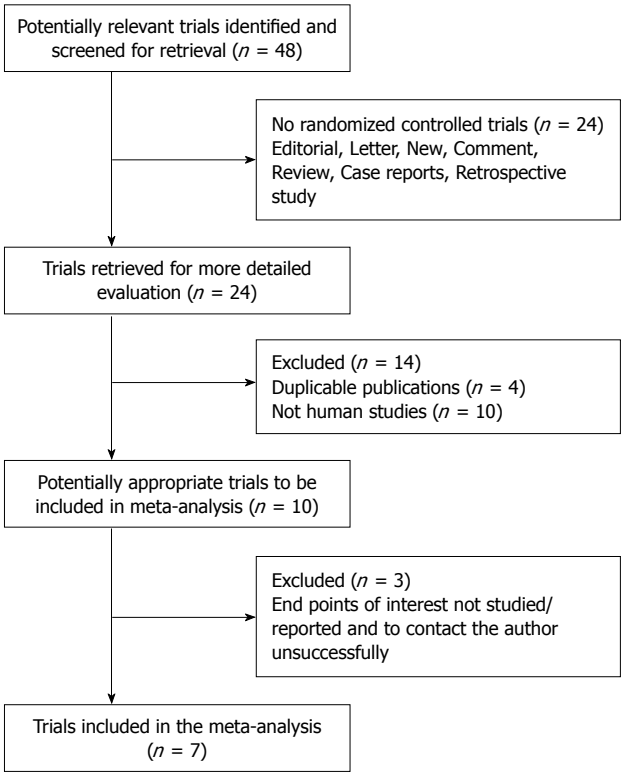


Figure 1 Flow chart showing the study selection procedure.

Quality assessment

Four of these studies were double blind[16,17,19,25]. Allocation concealment was adequate in 3 studies[17,19,25], and unclear in 4 studies[16,18,26,27]. The Jadad score of the studies was evaluated and ranged from 1 to 5 (mean 2.9). Most of the studies were small in size (85.7% had 100 or less

Table 2 Surgical outcomes from randomized studies included in this meta-analysis

Trial	No. of patients	Septic morbidity (%)	MOF (%)	SIRS (%)	Pancreatic infections (%)	Surgical interventions (%)	Hospital stay (d)	Mortality (%)
Oláh *et al*[16]	22/23	5 (22.7)/ 20 (87)	2 (9.1)/ 2 (8.7)	11 (50)/ 6 (26.1)	1 (4.5)/ 7 (30.4)	1 (4.5)/ 7 (30.4)	13.7/21.4 (median)	1 (4.5)/2 (8.7)
Oláh *et al*[17]	33/29	9 (27.3)/ 15 (51.7)	5 (15.1)/ 9 (31)	3 (9)/ 5 (17.2)	4 (12.1)/ 8 (27.6)	4 (12.1)/ 7 (24.1)	14.9/19.7 (median)	2 (6.1)/6 (20.7)
Karakan *et al*[25]	15/15	2 (13.3)/ 2 (13.3)	1 (6.7)/ 2 (13.3)	N/A	N/A	N/A	10 ± 4/15 ± 6	2 (13.3)/4 (26.7)
Qin *et al*[18]	36/38	11 (30.6)/ 29 (76.3)	4 (11.1)/ 7 (18.4)	6 (16.7)/ 14 (36.8)	N/A	N/A	20.9/24.2 (median)	0 (0)/0 (0)
Besselink *et al*[19]	152/144	46 (30.3)/ 41 (28.47)	33 (22)/ 15 (10)	N/A	21 (14)/ 14 (10)	28 (18)/ 14 (10)	28.9 ± 41.5/23.5 ± 25.9	24 (16)/9 (6)
Li *et al*[26]	14/11	N/A	N/A	N/A	N/A	N/A	42 ± 5/49 ± 6.8	N/A
Wu *et al*[27]	14/13	N/A	N/A	N/A	N/A	N/A	34 ± 6/40 ± 6	5 (35.7)/6 (46.2)

N/A: Not applicable; MOF: Multi-organ failure; SIRS: Systemic inflammatory response syndrome.

Table 3 Quality assessment of the included randomized trials

Trial	Generation of random	Allocation concealment	Blinding	Follow-up	Baseline similarity	Jadad score
Oláh *et al*[16]	Unclear	Unclear	Double blinded	No	Similar	4
Oláh *et al*[17]	Unclear	Adequate	Double blinded	No	Similar	4
Karakan *et al*[25]	Clear	Adequate	Double blinded	No	Similar	3
Qin *et al*[18]	Clear	Unclear	No blinding	Yes	Similar	3
Besselink *et al*[19]	Clear	Adequate	Double blinded	Yes	Similar	5
Li *et al*[26]	Unclear	Unclear	No blinding	No	Similar	1
Wu *et al*[27]	Unclear	Unclear	No blinding	No	Similar	1

participants). Follow-up was only reported in two studies[18,19]. The quality assessment of the studies is presented in Table 3.

Infectious complications and pancreatic infection

The incidence of infectious complications was reported in all 7 RCTs in the meta-analysis. The type of infections included pancreatic abscess, infected pancreatic necrosis, pneumonia, catheter-related septic complication, urinary tract infections, wound infections, and sepsis or bacteremia. Overall, there were no significant differences of incidence of total infections in pancreatic patients between the probiotics/synbiotics group and the control group (OR = 0.3, 95% CI: 0.09-1.02, P = 0.05) (Figure 2A). There was significant heterogeneity between studies (I^2 = 84%, P < 0.0001).

Only three studies reported pancreatic infections[16,17,19]. Pancreatic infections included pancreatic abscess and infected pancreatic necrosis. There was no significant difference in the pancreatic infections between the probiotics/synbiotics group and the control group (OR 0.50, 95% CI: 0.12-2.17, P = 0.36) (Figure 2B). The test result for heterogeneity was also significant (I^2 = 73%, P = 0.03).

MOF and SIRS

There were no significant differences in the incidence of MOF and SIRS between the probiotics/synbiotics group and the control group. The odds ratio and 95% CI: were 0.88 (0.35-2.21, P = 0.79) in MOF based on five studies[16-19,25], and 0.78 (0.20-2.98, P = 0.71) in SIRS based on three studies[16-18]. Combination analysis of MOF and SIRS also showed no significant differences between the two groups (OR 0.75, 95% CI: 0.23-2.50, P = 0.64) (Figure 2C). The significant heterogeneity was present among the studies (I^2 = 83%, P < 0.0001).

Length of antibiotic therapy, and hospital stay

Four studies involved in the meta-analysis provided applicable data on length of hospital stay[19,25-27]. The length of hospital stay was significantly shorter in the probiotics/synbiotics group (OR -3.87, 95% CI: -6.20 to -1.54, P = 0.001) (Figure 2D). However, there was no significant difference in the length of antibiotic therapy (three RCTs, OR 0.75, 95% CI: 0.50-1.14, P = 0.18) between the probiotics/synbiotics group and the control group[16,18,19]. The test result for heterogeneity was not significant (P = 0.08 in hospital stay, P = 0.33 in antibiotic therapy).

Surgical intervention and mortality

Three studies reported significant difference in surgical intervention (OR 0.59, 95% CI: 0.11-3.07). Mortality was reported in six of seven RCTs[16-19,25,27]. There was no significant difference in the mortality between the probiotics/synbiotics group and the control group (OR 0.75, 95% CI: 0.25-2.24, P = 0.61) (Figure 2E). The significant heterogeneity was present between studies (P = 0.009 in surgical intervention; P = 0.04 in mortality).

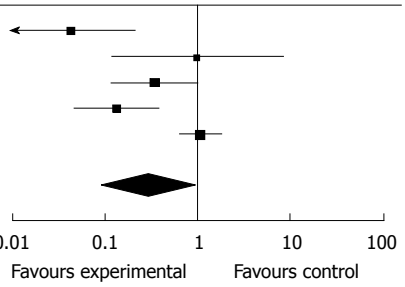
Stratification, sensitivity analysis and publication bias

We analyzed the surgical outcomes stratified by the se-

A

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
Oláh, 2002	5	22	20	23	17.9	0.04 (0.01, 0.21)
Karakan, 2007	2	15	2	15	14.5	1.00 (0.12, 8.21)
Oláh, 2007	9	33	15	29	21.4	0.35 (0.12, 1.01)
Qin, 2008	11	36	29	38	21.6	0.14 (0.05, 0.38)
Besselink, 2008	46	152	41	144	24.6	1.09 (0.66, 1.80)
Toatal (95% CI)		258		249	100.0	0.30 (0.09, 1.02)
Toatal events	73		107			

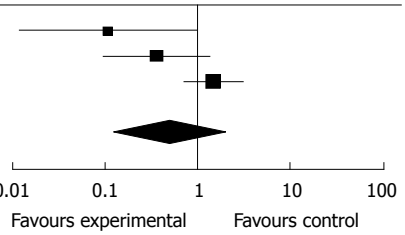
Heterogeneity: Tau^2 = 1.49, χ^2 = 25.18, *df* = 4 (*P* < 0.0001), I^2 = 84%
Test for overall effect: *Z* = 1.93 (*P* = 0.05)



B

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
Oláh, 2002	1	22	7	23	23.0	0.11 (0.01, 0.98)
Oláh, 2007	4	33	8	29	34.3	0.36 (0.10, 1.36)
Besselink, 2008	21	152	14	144	42.8	1.49 (0.73, 3.05)
Toatal (95% CI)		207		196	100.0	0.50 (0.12, 2.17)
Toatal events	26		29			

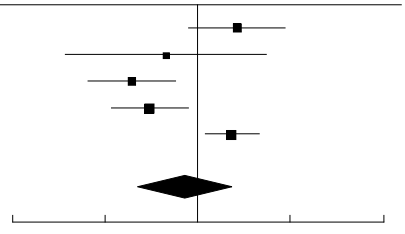
Heterogeneity: Tau^2 = 1.16, χ^2 = 7.34, *df* = 2 (*P* = 0.03), I^2 = 73%
Test for overall effect: *Z* = 0.92 (*P* = 0.36)



C

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
Oláh, 2002	13	22	8	23	20.5	2.71 (0.81, 9.06)
Karakan, 2007	1	15	2	15	12.1	0.46 (0.04, 5.75)
Oláh, 2007	9	33	19	29	21.3	0.20 (0.07, 0.58)
Qin, 2008	10	36	21	38	22.1	0.31 (0.12, 0.82)
Besselink, 2008	33	152	15	144	24.0	2.38 (1.23, 4.61)
Toatal (95% CI)		258		249	100.0	0.75 (0.23, 2.50)
Toatal events	66		65			

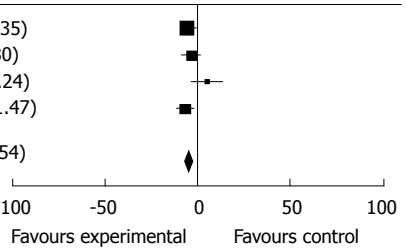
Heterogeneity: Tau^2 = 1.45, χ^2 = 23.83, *df* = 4 (*P* < 0.0001), I^2 = 83%
Test for overall effect: *Z* = 0.46 (*P* = 0.64)



D

Study or subgroup	Favours experimental mean	SD	Total	Control mean	SD	Total	Weight (%)	mean difference IV, fixed, 95% CI
Karakan, 2007	10	4	15	15	6	15	40.9	-5.00 (-8.65, -1.35)
Li, 2007	42	5	14	45	6.8	11	23.7	-3.00 (-7.80, 1.80)
Besselink, 2008	28.9	41.5	152	23.5	25.9	144	8.9	5.40 (-2.44, 13.24)
Wu, 2009	34	6	14	40	6	13	26.6	-6.00 (-10.53, -1.47)
Toatal (95% CI)			195			183	100.0	-3.87 (-6.20, -1.54)

Heterogeneity: χ^2 = 6.72, *df* = 3 (*P* = 0.08), I^2 = 55%
Test for overall effect: *Z* = 3.25 (*P* = 0.001)



E

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
Oláh, 2002	1	22	2	23	17.6	0.50 (0.04, 5.94)
Oláh, 2007	2	33	6	29	24.9	0.25 (0.05, 1.34)
Karakan, 2007	2	15	4	15	22.9	0.42 (0.06, 2.77)
Qin, 2008	0	36	0	38		Not estimate
Besselink, 2008	24	152	9	144	34.6	2.81 (1.26, 6.28)
Toatal (95% CI)		258		249	100.0	0.73 (0.18, 2.98)
Toatal events	29		21			

Heterogeneity: Tau^2 = 1.31, χ^2 = 9.17, *df* = 3 (*P* = 0.03), I^2 = 67%
Test for overall effect: *Z* = 0.43 (*P* = 0.66)

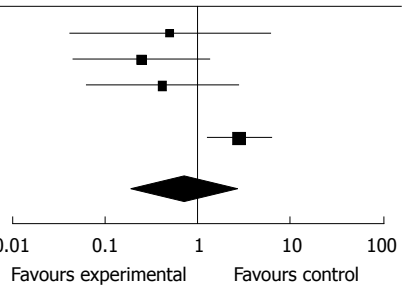


Figure 2 Forest plot for the effects of probiotics/synbiotics in patients with acute pancreatitis. A: Infectious morbidity; B: Pancreatic infections; C: Multiple organ failure (MOF) and systemic inflammatory response syndrome (SIRS); D: Length of hospital stay; E: Mortality.

Table 4 Surgical outcomes stratified by severity of pancreatitis in this meta-analysis

	No. of studies	Synbiotic	Control	OR (95% CI)	*P* for effect size	*P* for heterogeneity
Septic morbidity	3	57/200	58/188	0.89 (0.57, 1.37)	0.58	0.16
Pancreatic infections	2	25/185	22/173	1.06 (0.58, 1.96)	0.84	0.07
Surgical intervention	2	32/185	21/173	1.07 (0.23, 4.92)	0.94	0.04
MOF and SIRS	3	43/200	36/188	0.65 (0.09, 4.44)	0.66	0.0005
Mortality	4	33/214	25/201	0.77 (0.22, 2.72)	0.69	0.02

OR: Odds ratio; CI: Confidence interval; MOF: Multi-organ failure; SIRS: Systemic inflammatory response syndrome.

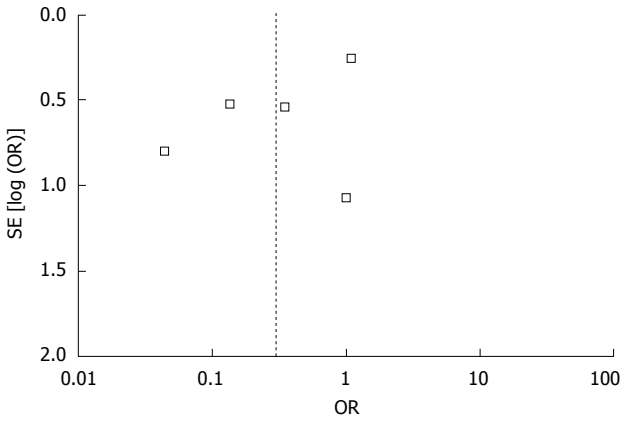


Figure 3 Funnel plot of publication bias.

verity of acute pancreatitis. The use of pre-, pro- or synbiotics had no significant influence on the main surgical outcomes including septic morbidity, pancreatic infections, surgical intervention, mortality, MOF and SIRS in severe acute pancreatitis (Table 4).

Allocation concealment and blinding were unclear in three of included studies[18,26,27]. The results of the sensitivity analysis based on the remaining four studies, after excluding trials of low quality, are similar to the previous results, indicating that the results of meta-analysis are relatively credible. Review of funnel plots is present in Figure 3, which can not rule out the potential for publication bias in all analyses.

DISCUSSION

The microbiota colonizing the gastrointestinal tract has been considered as the main causes of the pathogenesis of the nosocomial infections[28,29]. Surgical trauma, especially surgical trauma of the digestive tract, may result in physical injury and atrophy of the gastrointestinal mucosa, increased intestinal permeability, microbial imbalance, and intraluminal bacterial or bacterial products translocation cross the intestinal barrier to local lymph nodes and distant organs, and ultimately induce an increased morbidity of infectious complications and sepsis[30,31]. At present, probiotics and prebiotics have been administrated in surgical trauma[28,32-34], cancer[30,35,36], active ulcerative colitis[37], and critically ill patients[31,38,39].

Up to now, nine RCTs including 733 patients have been published reporting the effects of pre-, pro- or synbiotics treatment in patients undergoing abdominal surgery. Six studies showed a significantly positive effect of probiotics with or without prebiotics in postoperative septic complications, and the remaining three studies showed no effects. A meta-analysis based on these studies demonstrated that the use of pre-, pro- or synbiotics might reduce the incidence of postoperative infections, duration of hospital stay and length of antibiotic therapy after abdominal surgery[40]. Another meta-analysis based on eight randomized studies reported that the use of pre-, pro- or synbiotics in critically ill adult patients showed no statistically significant benefit in the length of ICU stay, hospital mortality and the incidence of nosocomial infections[41]. However, this meta-analysis did not include postoperative patients and trauma patients admitted to ICU, most of them benefited from probiotics treatment[29-31]. From our meta-analysis, we found that the use of pre-, pro- or synbiotics had no influence on the main outcomes including postoperative infections, pancreatic infections, MOF, SIRS and mortality in patients with acute pancreatitis. In the subgroup analysis and sensitivity analysis, a similar result was observed, after the stratification was conducted by the severity of acute pancreatitis and the trials of low quality were excluded.

There were controversies on the use of pre-, pro- or synbiotics in patients with acute pancreatitis among the recent studies. Three studies suggested that probiotics administration was effective in preventing complications of the experimental acute pancreatitis by decreasing bacterial translocation[42-44]. The first clinical trial was performed by Oláh *et al*[16] to evaluate the use of probiotics and fiber in clinical patients with acute pancreatitis. The study demonstrated that a dose of 10^9 of *L. plantarum* 299 together with oat fiber significantly reduced infected pancreatic necrosis and the number of surgical interventions. Subsequently, several studies reported similarly positive effects of probiotics with or without prebiotics[17,18,25-27]. However, the sample size in these studies was small and the conclusion from them was inconclusive. A large multi-center, randomized double-blinded controlled trial by Besselink *et al*[19] was designed to see whether probiotics could reduce the incidence of infectious complications in patients with severe acute pancreatitis. The trial involved 296 patients in 15 hospitals, and compared the use of a multi-species probiotics preparation with a placebo. The results showed that infectious complications occurred in 30% of the pa-

tients in the probiotics group and in 28% of the placebo group. Nine patients developed bowel ischaemia (8 died) in the probiotics group, whereas none developed this complication in the placebo group. Multiple organ failure occurred in 22% of the patients in the probiotics group and in 10% in the placebo group. In all, 16% patients in the probiotics group and 6% in the placebo group died. The results were opposite to previous evidences, and raised safety question on the use of pre-, pro- or synbiotics. Although Dutch's study was criticized for its design, approval, and conduct, this trial should still be carefully considered for its large sample size[45]. The previous studies have demonstrated that probiotics are safe for use in healthy persons, but should be used with caution in patients with underlying immune compromise, chronic disease, or debilitation because of the risk of sepsis[46]. However, bowel ischemia has never been reported in the previous studies including acute pancreatitis, trauma, critical illness and elective abdominal surgery, and the high incidence of MOF and mortality associated with probiotics treatment were only reported in Besselink's study. Besselink *et al*[19] explained that it was probably not the combination of probiotics but the administration of the combined probiotics together with the severity of the disease that was largely responsible for the effects obtained. This explanation may be one of potential reasons, but not the only one.

There are three major differences between Besselink's study and the other studies in the meta-analysis: the patients in the former received a higher number and more strains of probiotic organisms (six strains of probiotics *vs* 1-4 strains of probiotics in other studies); patients received probiotics administration for a longer period in Besselink's study (28 d *vs* 7 d in the other studies), and the pressor agents were administrated during probiotics feeding in some patients. Little work has been performed and no accepted recommendation was reported on the therapeutic strategy (including the optimal dose and time) of probiotics. The dose of probiotics administered was usually based on the previous studies showing clinical benefit from probiotics. Furthermore, the dose of probiotics varies considerably among different studies. In this meta-analysis, three of the studies tested a probiotics or prebiotics[16,18,25] and showed preventive effects. The remaining four studies tested different doses of synbiotics (probiotics plus prebiotics)[17,19,26,27], 3 of which showed positive results[17,26,27]. Combination of several strains of probiotics was regarded as more powerful in previous opinion. This study indicates that the inappropriate combination of probiotic strains may be harmful, while a single use of probiotics or prebiotics may be safer. Evidences suggest that intestinal blood flow at the mucosal level is generally reduced in acute pancreatitis and critically ill patients[47]. The reduced blood flow and oxygen supply in intestinal mucosa might be further compromised by the administration of enteral nutrition and probiotic bacteria[48,49]. On the other hand, feeding probiotics might cause local mucosal damage with an inflammatory response of the small bowel wall. Multispecies probiotics mixture may strongly increase the concentration of IL-10, and reduce the concentration of IL-6[50]. Experimental studies demonstrated that the increasing IL-10 is able to induce intestinal damage during intestinal ischemia/reperfusion[51], whereas IL-6 is able to protect enterocytes[52]. One may speculate that a long duration of probiotics treatment and a high number of bacterial organisms may lead to probiotics overload in the small bowel, which aggravate the disorder of inflammatory response and reduction of intestinal blood flow and oxygen supply at the phase of acute stage of severe acute pancreatitis, and ultimately lead to bowel ischemia.

The unexpected deleterious effects may refer to more biologic actions of probiotics. It is clear that different strains of probiotics can have different effects. Moreover, their effects may vary in healthy people and patients, in different disease states, and among different age groups. Thus, clinical effects of one probiotics strain in one population cannot be automatically generalized to other strains or to other populations[46]. Although the individual strains used in this study are credible on the basis of their capacity to inhibit growth of pathogenic bacteria and to modulate immune responses, the benefits, disadvantages and interaction of different probiotics strains and their mechanisms of action in special illness is still unclear, and the combination of several strains should be thoroughly evaluated for safety and clinical benefits.

Limitations of study

This meta-analysis has several limitations. The included studies were significantly heterogeneous, so the results should be interpreted with caution. Since these studies include diverse patient groups with diverse severity of disease, the effects of pre- pro- or synbiotics may differ among them. The type and the concentration of the probiotics vary considerably in these reviewed studies, which may explain some of the differences in results between studies. In addition, some strains are effective and some may be harmful, and the interaction of several stains of probiotics as a mixture may lead to the deleterious effects in some special illness. Most of these studies are small in scale and two of them are of low quality, which may influence the analysis. There were also differences in the study design, the population and study team among these studies. Finally, meta-analysis remains retrospective that is subject to the methodological deficiencies of the studies, it is therefore possible that studies with different results may be unpublished, which leads to publication bias.

In conclusion, there are a few recommendations can be offered and further studies are required. In view of the questions raised in safety and efficacy, use of pre- pro- or synbiotics should be cautious in critically ill patients, especially in patients with severe acute pancreatitis. Unless new and strong evidence is presented, probiotics use can no longer be considered risk-free. However, we can not draw a conclusion that the use of pre- pro- or synbiotics is dangerous, and it is too early and inappropriate to deny the beneficial effects of all of pre- pro- or synbiotics in critically ill patients and severe acute pancreatitis. After

all, a single large trial is not sufficient to draw a definitive conclusion. Well-designed randomized controlled trials are needed to further explore the mechanistic issues and probiotic interactions, and assess the effect and safety of pre-, pro- or synbiotics.

COMMENTS

Background

The administration of pre-, pro- or synbiotics has been shown to enhance immunomodulating ability, modulate the intestinal microbiota, inhibit bacterial translocation and reduce the incidence of nosocomial infections in clinically surgical patients, and may be a new anti-infectious strategy for patients with acute pancreatitis.

Research frontiers

Some randomized controlled studies (RCTs) demonstrated a therapeutic and preventive effect of pre-, pro- or synbiotics in patients with acute pancreatitis. However, a multicenter RCTs reported some unexpected results. These studies were controversial for their shortcomings. Overall, the magnitude of the therapeutic effect remains unknown. This meta-analysis was performed to assess the potential effect and safety of pre-, pro- or synbiotics in patients with acute pancreatitis.

Innovations and breakthroughs

Currently, there is still a lack of evidence to support the use of pre-, pro- or synbiotics in patients with acute pancreatitis.

Applications

Pre-, pro- or synbiotics treatment shows no statistically significant benefit in the main outcome of patients with acute pancreatitis. In view of the questions in safety and efficacy, use of pre- pro- or synbiotics should be cautious in critically ill patients, especially in patients with severe acute pancreatitis. Further studies are needed to explore mechanistic issues and probiotic interactions in critical illnesses.

Terminology

Probiotics, is defined as ingestible microorganisms that benefit the host by improving intestinal microbial balance.

Peer review

In this meta-analysis, the use of pre- pro- and synbiotics was compared to placebo in patients with acute pancreatitis. In general, I find the study interesting and clinically relevant. However, the manuscript needs to undergo major revision.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3979

World J Gastroenterol 2010 August 21; 16(31): 3979-3983
ISSN 1007-9327 (print)

BRIEF ARTICLE

Living donor liver transplantation using dual grafts: Ultrasonographic evaluation

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Received: April 12, 2010 Revised: May 27, 2010
Accepted: June 3, 2010
Published online: August 21, 2010

Abstract

AIM: To evaluate the dual-graft living donor liver transplantation (LDLT) with ultrasonography, with special emphasis on the postoperative complications.

METHODS: From January 2002 to August 2007, 110 adult-to-adult LDLTs were performed in West China Hospital of Sichuan University. Among them, dual-graft implantations were performed in six patients. Sonographic findings of the patients were retrospectively reviewed.

RESULTS: All the six recipients survived the dual-graft adult-to-adult LDLT surgery. All had pleural effusion. Four patients had episodes of postoperative abdominal complications, including fluid collection between the grafts in three patients, intrahepatic biliary dilatation in two, hepatofugal portal flow of the left lobe in two, and atrophy of the left lobe in one.

CONCLUSION: Although dual-graft LDLT takes more efforts and is technically complicated, it is safely feasible. Postoperative sonographic monitoring of the recipient is important.

Key words: Liver transplantation; Dual grafts; Complications; Ultrasonography

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Lu Q, Wu H, Yan LN, Chen ZY, Fan YT, Luo Y. Living donor liver transplantation using dual grafts: Ultrasonographic evaluation. *World J Gastroenterol* 2010; 16(31): 3979-3983 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3979.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3979

INTRODUCTION

Adult-to-adult living donor liver transplantation (A-ALDLT) inevitably implies two potential risks including a small-for-size (SFS) graft for the recipient and a small remnant liver for the donor[1]. Problems related to SFS graft and small remnant livers have gradually come to light with the expansion of A-ALDLT[2-4]. The transplantation of SFS grafts may cause an imbalance between hepatic volume and demand of liver function, which may lead to a severe graft dysfunction known as small-for-size syndrome (SFSS)[5]. To overcome this problem and minimize the overall risk of the family, as an alternative, dual grafts from two living donors into one recipient can make up for graft size insufficiency and secure the recipient and donors' safety. Because of the complexity of this procedure, intensive postoperative monitoring of the recipient is required. To our knowledge, there have been few reports about the sonographic findings of dual-graft A-ALDLT. Therefore, in this study we describe our experience of sonographic evaluation of A-ALDLT using dual grafts.

MATERIALS AND METHODS

Clinical data

From January 2002 to August 2007, 110 A-ALDLTs

Table 1 Data of recipients who received dual grafts

No.	Gender/age (yr)	Diseases	Child-Pugh classification	Body weight (kg)	SLV (mL)
1	M/35	Hepatitis B-related cirrhosis, portal hypertension	C	70	1334
2	M/37	Hepatitis B cirrhosis with hepatocellular carcinoma	A	58	1187
3	M/42	Hepatitis-related cirrhosis, portal hypertension	C	75	1037
4	M/34	Hepatitis B-related cirrhosis, portal hypertension	C	71	1310
5	M/38	Hepatitis C cirrhosis with hepatocellular carcinoma	C	65	1210
6[1]	M/28	Hepatitis B cirrhosis with hepatocellular carcinoma	C	59	1290

[1]Received dual left lobe living donor liver transplantation. SLV: Standard liver volume.

Table 2 Data of donors

	Right side graft				Left side graft			Dual-graft GRWR (%)
No.	Gender/age (yr)	Relation	Weight (g)	GRWR (%)	Gender/age (yr)	Relation	Weight (g)	
1	F/56	Mother	630[1]	0.90	M/27	Cadaveric	230	1.22
2	M/35	Brother in law	410	0.71	F/52	Brother	193	1.04
3	F/35	Wife	400	0.53	M/55	Uncle in law	280	0.91
4	F/29	Wife	470	0.66	M/45	Sister	197	0.94
5	F/39	Wife	614[1]	0.94	M/42	Brother	225	1.29
6	F/34[2]	Sister	310	0.53	F/31	Sister	300	1.03

[1]Macrosteatosis of their livers ranged from 20% to 25%; [2]Both donors donated their left lobes. GRWR: The graft to recipient body weight ratio.

were performed in West China Hospital of Sichuan University. Among them, dual-graft implantations were performed in 6 male patients (aged 28-42 years, mean 34.7 years). Clinical data of the recipients is shown in Table 1.

Dual grafts selection

This study was conducted under the approval of the Ethics Committee of our hospital. All donors volunteered to donate their livers. Based on the previous experience, graft recipient weight ratio (GRWR) should be 0.8% or greater to achieve a graft and patient survival of 90%[6]. If GRWR is less than 0.8%, dual grafts should be considered. Furthermore, if macrosteatosis of the liver ranged from 10% to 30% and the grafts with a GRWR less than 1.0%, dual-graft liver transplantation should also be taken into account. Donor candidates who have diabetes, hypertension, or any other significant medical diseases were excluded from the right lobe donation. In order to ensure the safety of the donor, the middle hepatic vein was not harvested in the right lobe graft, and the remnant liver volume was more than 35%[7].

Materials of donors

There were 11 living donors (7 women and 4 men, aged 29-56 years, mean 41.5 years) and one cadaveric donor in this group. The clinical data of the donors is shown in Table 2.

In case 1 and case 5, whose GRWR was 0.90% and 0.94% respectively, dual-graft LDLTs were performed because macrosteatosis of their livers ranged from 20% to 25%. Cadaveric donor liver was split into two parts. The left lobe was used as one portion of dual grafts, and the right lobe including the middle hepatic vein was used in another adult liver transplantation[8].

Ultrasound examination

Ultrasound examinations of the recipients were retrospectively analyzed. Ultrasound scans were performed using a HDI 5000 scanner (Philips Medical Systems, Bothell, WA) with a 2-5-MHz wide band convex transducer or a Sequoia 512 (Acuson, Mountain View, CA, USA) with a 2-6-MHz wide band convex transducer. Patients were examined by ultrasound daily until postoperative day 14 and once a week thereafter until hospital discharge. Intercostal views of the right upper quadrant of the abdomen were obtained to examine the right graft and subcostal views were used to evaluate the left graft. Gray scale and color flow images as well as Doppler spectrums were detected. Angle-corrected velocities were examined with the Doppler angle less than 60°. Parenchyma echogenicity, patency of the hepatic vessels, biliary system and the fluid collection of the thorax and abdomen were documented.

RESULTS

Recipients

All recipients survived the dual-graft A-ALDLT surgery. All recipients (6/6) had bilateral pleural effusion and 2 patients underwent ultrasound-guided drainage. Four patients had episodes of postoperative abdominal complications, including fluid collection between the grafts in 3 patients (Figure 1), and 2 had intrahepatic biliary dilatation (Figure 2). The left-sided graft shrank in size in one patient in whom hepatofugal flow of the left portal vein

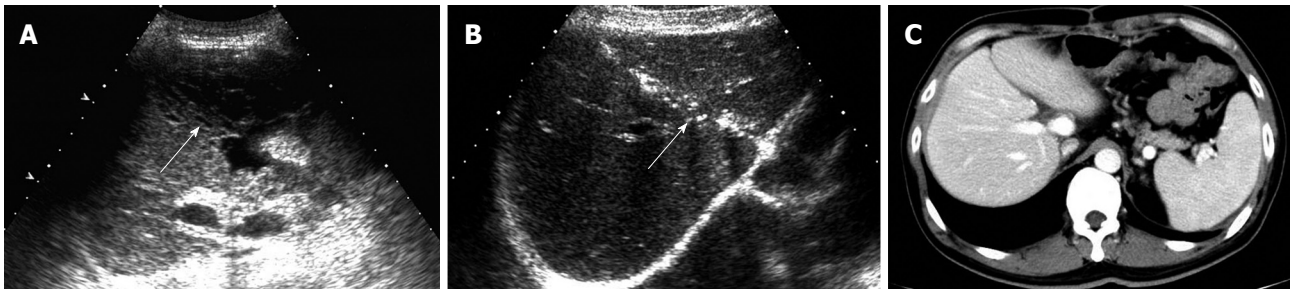

Figure 1 A 42-year-old man with fluid collection between the grafts after dual-graft liver transplantation. A: Gray scale sonogram shows the focal fluid collection with septa inside (arrow); B and C: The grafts joint visibly on follow-up sonogram (arrow) and computed tomography.

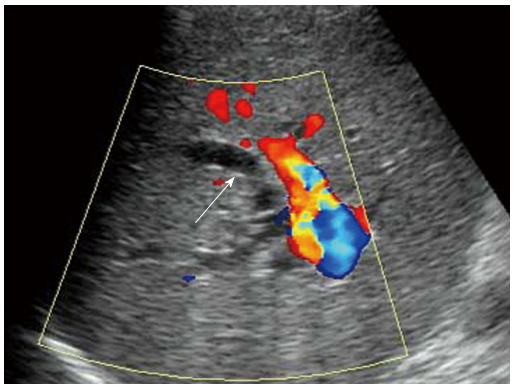

Figure 2 Moderate intrahepatic biliary dilatation (arrow) was shown by the follow-up ultrasound in a 34-year-old man four mos after dual-graft liver transplantation.

was detected 1 mo after surgery (Figure 3). To-and-fro portal venous flow of the left portal vein was found in another case at a routine follow-up 2 years after surgery with a normal size of the left graft. One patient died of tumor recurrence.

Donors

All donors recovered with an uneventful process.

DISCUSSION

The significant gap between available cadaveric grafts and the number of patients on the waiting list for liver transplant triggered the rapid adoption of the right lobe liver transplantation[9-11]. Although the surgical technique of A-ALDLT achieved great progress, SFS grafts still remain a problem[12]. Dual-graft liver transplantation provides an option when the largest single graft available is still small for the recipient.

Dual-graft A-ALDLT is a complicated procedure, therefore, recipients should be monitored intensively after surgery. Ultrasonography has been regarded as the initial imaging modality of choice allowing bedside assessment for detection and follow-up of early and delayed complications, and facilitating interventional procedures[13].

Postoperative pleural effusion is common in liver transplantation recipients. Most of the cases need no special intervention with a small amount of fluid. When the amount of the fluid was large, which may result in atelectasis and higher risk of pulmonary infection and further affected the pulmonary function, ultrasound-guided drainage of the pleural effusion may help resolve the problem.

Hepatofugal portal venous flow was regarded as a serious prognostic sign of critical liver damage after liver transplantation[14,15], but the situation is different in dual-graft A-ALDLT. In our 2 cases, hepatofugal portal venous flow of the left graft was detected, while the hepatic function tests remain normal. This phenomenon could be explained by a compensatory enlargement of the right-sided graft which could meet the metabolic demand of the body even with a compromised left-sided graft. Persistent monophasic Doppler spectrum of the left hepatic vein was obtained during quiet breathing, stenosis of the left hepatic vein was suggested according to Meir and Ko's research[16,17], which may lead to an increased sinusoidal pressure, then the portal vein served as a drainage vein and finally resulted in the atrophy of the graft.

A dual-graft A-ALDLT also has a problem of postoperative biliary complications. Ultrasound detected intrahepatic biliary dilation in 2 patients, but failed to locate the stenosis site. Compared with magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiography, ultrasonography is less sensitive in detecting biliary strictures and ischemic type biliary lesions after liver transplantation[18,19], but ultrasonography is reported to have a high negative predictive value of 95% in the diagnosis of biliary tract complications[20]. Therefore, ultrasound could be used as a screening technique in the aspect of biliary complications.

Fluid collection between the grafts was common in dual-graft LDLT, which occurred in 3 recipients, because a drainage tube was not routinely placed between the grafts. Besides detection, ultrasound facilitates the fluid aspiration procedure which is important to the differential diagnosis of bile leakage or ascites and beneficial to the recovery of patients.

Indeed, some other techniques including splenic artery embolization or ligation, permanent or temporary portacaval shunts play an important role in preventing SFSS under a specific condition that GRWR ratio is near 0.8%[2].

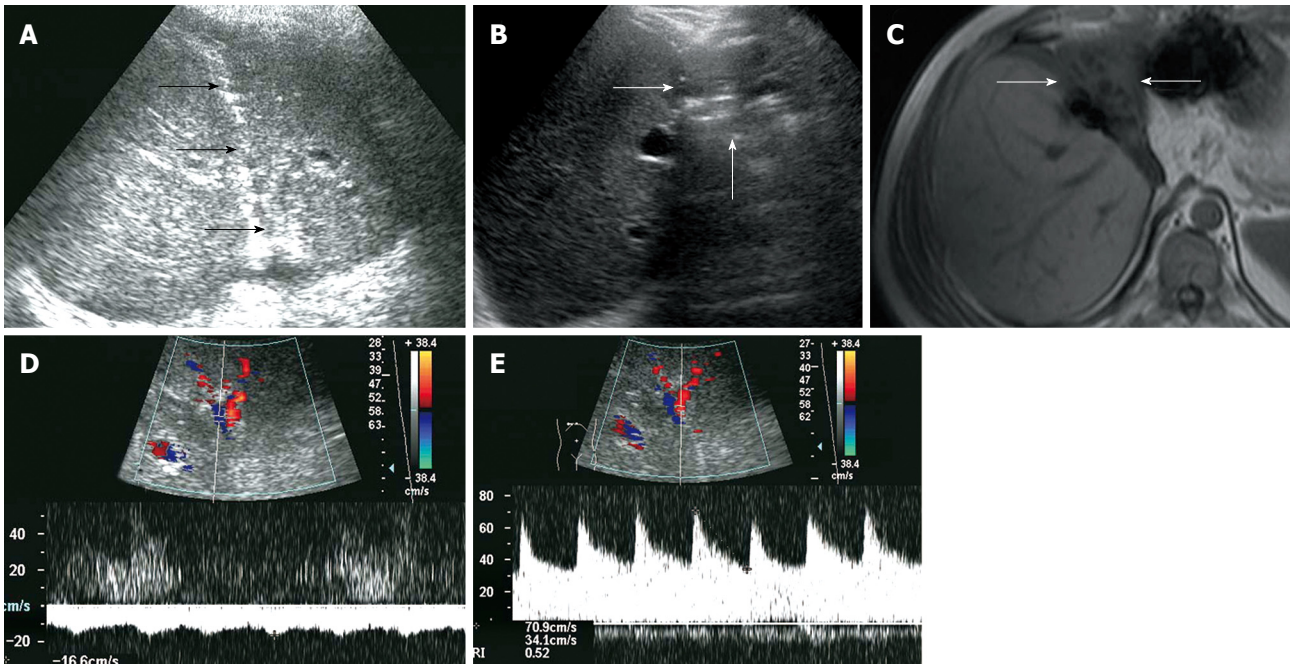

Figure 3 A 37-year-old man with a shranken left graft after dual-graft liver transplantation. A: Gray scale sonogram shows the left graft (arrows) on postoperative day one; B and C: Size of the left graft decreased on follow-up sonogram and magnetic resonance imaging (arrows); D: Doppler spectrum shows the hepatofugal flow of the portal vein of left graft; E: Doppler spectrum shows the normal left hepatic artery.

When the graft is too small, LDLT poses a great danger for the recipients. During the follow-up of 11 donors and 6 recipients over 18 mo, SFSS was not found in the recipients. No liver failure occurred in donors and recipients. Only one patient died of tumor recurrence. Therefore, dual-graft LDLT should be regarded as a feasible method to avoid SFSS if GRWR of the recipients with portal hypertension or acute liver failure was less than 0.8%. However, the number is small and more clinical experience is required.

In conclusion, although A-ALDLT using dual grafts takes more efforts and is technically complicated, it is safely feasible and can increase the donor pool. Whenever a right lobectomy appears to be critical for the donor, the possibility of dual-graft A-ALDLT should be evaluated and discussed to minimize the combined family risk. Postoperative sonographic monitoring of the recipient is important for the early detection and intervention of vascular and non-vascular complications, which is critical to the recovery of recipients.

COMMENTS

Background

Dual-graft adult-to-adult living donor liver transplantation (A-ALDLT) was designed to overcome the problem of small-for-size graft and reduce the overall family risk. Because of the complexity of this procedure, intensive postoperative monitoring of the recipient is required. There have been few reports about the sonographic findings of dual-graft A-ALDLT to date.

Research frontiers

Dual-graft A-ALDLT was evaluated with ultrasonography, with special emphasis on the postoperative complications.

Innovations and breakthroughs

The authors reported six patients who received dual-graft LDLT. Normal and abnormal sonographic findings were discussed. Postoperative sonographic monitoring of the recipient is important for the early detection and intervention of vascular and non-vascular complications, which is critical to the recovery of recipients.

Applications

Ultrasound is important in the diagnosis of postoperative complications and helpful in some interventions, such as ultrasound-guided drainage of fluid. This report adds some evidences to the safety and feasibility of dual-graft liver transplantation by following up the recipients and donors.

Terminology

Living donor liver transplantation: It is the replacement of a diseased liver using partial healthy liver allograft donated by members of the family.

Peer review

This is a nice study, and can be published after minor revisions. Patient number for the dual-graft LDLT procedure appears small (n = 6), but is still the largest published to date. Some editing of the language is needed throughout.

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S- Editor Wang YR **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i31.3984

World J Gastroenterol 2010 August 21; 16(31): 3984-3986
ISSN 1007-9327 (print)

CASE REPORT

Multiple bowel intussusceptions from metastatic localized malignant pleural mesothelioma: A case report

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Received: April 14, 2010 Revised: May 20, 2010
Accepted: May 27, 2010
Published online: August 21, 2010

Abstract

Localized malignant pleural mesothelioma (LMPM) is a rare occurrence, and gastrointestinal intra-luminal metastases have not previously been reported. Herein, we report a patient with LMPM who presented with a local recurrence 10 mo after initial *en bloc* surgical resection. Abdominal computed tomography was performed for intractable, vague abdominal pain with episodic vomiting, which showed a "target sign" over the left lower quadrant. Laparotomy revealed several intra-luminal metastatic tumors in the small intestine and colon and a segmental resection of metastatic lesions was performed. Unfortunately, the patient died of sepsis despite successful surgical intervention. Though local recurrence is more frequent in LMPM, the possibility of distant metastasis should not be ignored in patients with non-specific abdominal pain.

Key words: Localized malignant pleural mesothelioma; Intussusception; Distant metastasis

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Liu H, Cheng YJ, Chen HP, Hwang JC, Chang PC. Multiple bowel intussusceptions from metastatic localized malignant pleural mesothelioma: A case report. *World J Gastroenterol* 2010; 16(31): 3984-3986 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i31/3984.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i31.3984

INTRODUCTION

Localized malignant pleural mesothelioma (LMPM) is a rare occurrence, and only a few cases have been reported in the literature[1,2]. Local recurrence is not uncommon; however, distant metastasis with gastrointestinal involvement, especially bowel intussusception (BI), has not been previously reported. Herein, we report a patient with LMPM who experienced local recurrence and metastases to the bowel with multiple BI. The patient died of sepsis despite successful relief of intestinal obstruction.

CASE REPORT

A 57-year-old male complained of right chest pain and presented to our department with a large, localized chest wall tumor in April 2008. He underwent *en bloc* chest wall resection with an adequate tumor free margin and the pathological examination revealed LMPM, sarcomatoid type. A full course of adjuvant chemotherapy with cisplatin and pemetrexed was administered; however, local recurrence was diagnosed 10 mo after the initial surgical

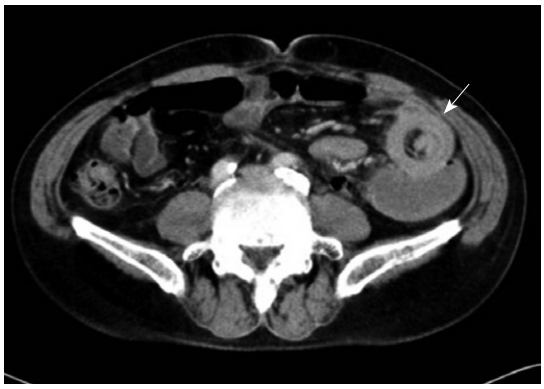

Figure 1 Abdominal computed tomography discloses a typical "target sign" over the small intestine (arrow).

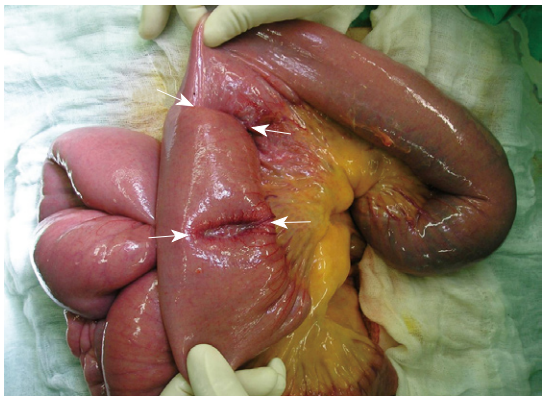

Figure 2 Intraoperative photo showing intussusceptions in the small intestine (arrows).

intervention. At the time of diagnosis, he complained of dizziness, poor appetite, and vague abdominal pain with episodic vomiting. His stool was negative for occult blood and laboratory studies revealed normochromic normocytic anemia (Hgb, 5.9 g/dL). Abdominal computed tomography (CT) revealed a "target sign" in the left lower quadrant (Figure 1).

An emergent laparotomy was performed and 3 intussusceptions with concomitant leading points of intra-luminal, polypoid tumors were found in the small intestine (Figure 2). No peritoneal seeding or extra-luminal invasion was discovered. After a detailed examination of the whole bowel, 7 tumors were found in the jejunum, ascending colon, and transverse colon (Figure 3). Segmental resection of small bowel with end-to-end anastomosis and radical right hemicolectomy with ileocolostomy, and regional lymph node sampling were performed.

Grossly, the surgical specimen consisted of jejunum, ileum, ascending colon and descending colon, 98 cm in length, with 7 polypoid, intra-luminal tumors. Four tumors were in the small bowel, and the others were in the ascending and descending colon. The tumors measured from 2.5 to 8.0 cm in diameter, and the largest, located in the small bowel, was 8.0 cm × 4.0 cm × 3.0 cm in size. When cut, all demonstrated a pinkish-white cut surface without necrosis or hemorrhage.

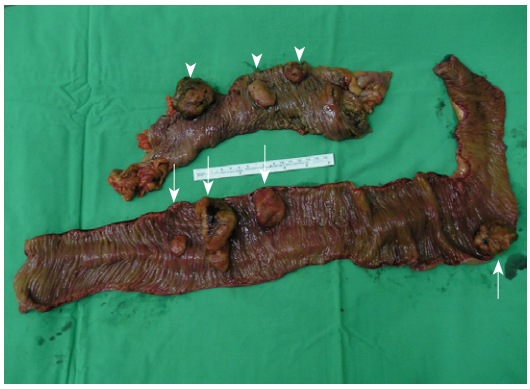

Figure 3 Surgical specimen reveals metastatic localized malignant pleural mesothelioma in the small intestine (arrows) and colon (arrow heads).

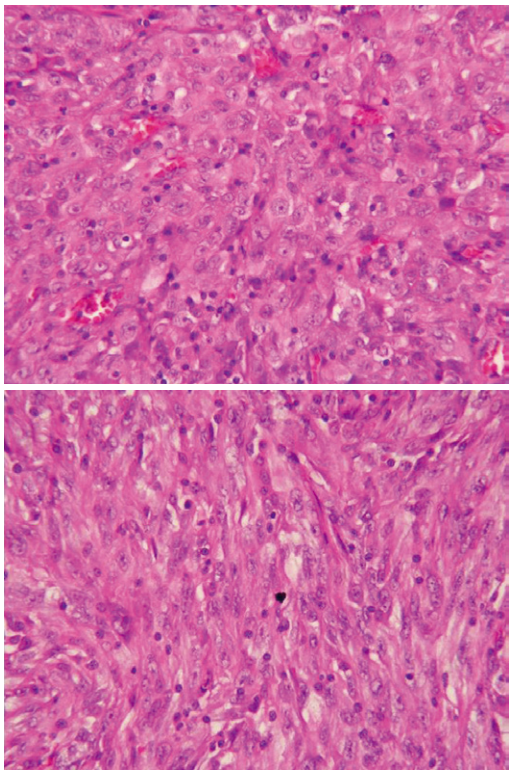

Figure 4 Microscopic examination reveals the tumor to consist of sheets of spindle to epithelioid cells with abundant eosinophilic cytoplasm and vesicular nuclear chromatin with prominent nucleoli (HE stain, × 400).

Microscopically, the 7 tumors were composed of sheets of epithelioid cells and spindle cells which had vesicular nuclei with prominent nucleoli, mixed with inflammatory cells (Figure 4). Mucosal and submucosal involvement was noted. Moreover, tumor metastasis was present in the regional lymph nodes. Immunohistochemical studies showed the tumor cells were negative for calretinin, thyroid transcription factor-1, and cytokeratin 20, and positive for vimentin and cytokeratin. The results were identical to those of the LMPM from the right chest wall.

The patient resumed enteral feeding on the 13th postoperative day. Unfortunately, the patient died of fungemia 32 d after the operation.

DISCUSSION

BI is rare in adults, accounting for 5% of all cases with intussusceptions and almost 5% of bowel obstructions[3]. Secondary BI usually results from organic lesions, such as polyps, inflammatory bowel disease, postoperative adhesions or neoplasms, and manifests with a single leading point. It is reported that malignant tumors are the most common causes of BI in adult patients, accounting for 47% of cases[3]. Nevertheless, distant metastases with multiple BIs are extremely rare, and only few case reports are found in the literature; these have been reported to be associated with melanoma, renal cell carcinoma, or lung cancer[4-6].

LMPMs are extremely rare solitary tumors, and identical to diffuse malignant pleural mesotheliomas microscopically and immunohistochemically. When an extensive review of published English-language literature was carried out, there were found to be a total of 53 LMPMs derived from parietal pleura[1,2,7-9]. Among these reported cases, LMPMs usually presented with local recurrence near prior surgical sites (16 cases, 30%), and there were 2 cases with contralateral recurrence during follow-up. Distant metastasis was less common compared with local recurrence, and only 8 cases were found (2 with brain metastases, 2 with diffuse dissemination, 2 with vertebral metastases, 1 with skin metastasis, and 1 with mediastinal metastasis). No case of intestinal intra-luminal metastases from LMPM has been previously reported. To the best of our knowledge, this is the first case report of multiple BIs from metastatic LMPM despite an initial *en bloc* chest wall resection and adjuvant chemotherapy.

In the case reported herein, the patient had the symptoms of vague abdominal pain and vomiting, and normocytic anemia. Generally, BIs from distant metastasis or malignancy have diverse manifestations. Most patients present with non-specific symptoms consistent with bowel obstruction, such as nausea, emesis, abdominal pain, melena, or weight loss[3]. However, Goh *et al*[10] have stated that anemia and colon intussusception are the predictive determinants for adult BIs resulting from malignancy. Moreover, abdominal CT is the most useful imaging modality and carries an acceptable diagnostic accuracy for adult BIs[3,11]. Early application of CT scanning will facilitate definitive diagnosis and prompt treatment.

Compared with pediatric intussusception, hydrostatic reduction is not mandatory, and aggressive surgical treatment is the gold standard for adult intussusception, though long-term survival remains unsatisfactory in patients with BI from metastases. Complete surgical resection of viable tumors is worthwhile in a select group of patients for the purpose of nutritional support from enteral feeding and metastasectomy with tumor eradication.

In conclusion, this case highlights two important issues. Firstly, clinicians should take gastrointestinal involvement, even malignancy-related intussusception, into consideration when treating patients with a past history of malignancy who are experiencing frequent abdominal pain and have unexplainable anemia. Secondly, though distant metastasis is infrequent compared with local recurrence in patients with LMPM, the possibility should be considered in patients with non-specific symptoms.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2010 August 21; 16(31): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 August 21; 16(31): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

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International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
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October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Case-Based Approach to the Management of Inflammatory Bowel Disease

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World J Gastroenterol 2010 August 21; 16(31): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

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English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and EMBASE/Excerpta Medica. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 32
August 28, 2010
World J Gastroenterol
2010 August 28; 16(32): 3987-4114

Online Submissions
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 32 August 28, 2010

EDITORIAL

3987 Colorectal cancer screening: The role of CT colonography

Laghi A, Iafrate F, Rengo M, Hassan C

3995 Essential role of monocytes and macrophages in the progression of acute pancreatitis

Shrivastava P, Bhatia M

FRONTIER

4003 Lymphangiogenesis: A new player in cancer progression

Nagahashi M, Ramachandran S, Rashid OM, Takabe K

TOPIC HIGHLIGHT

4013 Health related quality of life after surgery for colonic diverticular disease

Angriman I, Scarpa M, Ruffolo C

REVIEW

4019 Gene therapy for liver regeneration: Experimental studies and prospects for clinical trials

Atta HM

ORIGINAL ARTICLE

4031 Extracellular matrices for gastrointestinal surgery: *Ex vivo* testing and current applications

Hoeppner J, Marjanovic G, Helwig P, Hopt UT, Keck T

4039 Curcumin suppresses gastric NF-κB activation and macromolecular leakage in *Helicobacter pylori*-infected rats

Sintara K, Thong-Ngam D, Patumraj S, Klaikeaw N, Chatsuwan T

4047 High expression of ErbB2 contributes to cholangiocarcinoma cell invasion and proliferation through AKT/p70S6K

Treekitkarnmongkol W, Suthiphongchai T

BRIEF ARTICLE

4055 Relationship between alcohol intake and dietary pattern: Findings from NHANES Ⅲ

Liangpunsakul S

4061 Hepatocellular carcinoma in patients with chronic hepatitis C virus infection without cirrhosis
Nash KL, Woodall T, Brown ASM, Davies SE, Alexander GJM

4066 Quality of life assessment in patients with chronic pancreatitis receiving antioxidant therapy
Shah NS, Makin AJ, Sheen AJ, Siriwardena AK

4072 Diagnostic value of cancer-testis antigen mRNA in peripheral blood from hepatocellular carcinoma patients
Zhao L, Mou DC, Peng JR, Huang L, Wu ZA, Leng XS

4079 Effects of moxibustion on dynorphin and endomorphin in rats with chronic visceral hyperalgesia
Liu HR, Qi L, Wu LY, Ma XP, Qin XD, Huang WY, Dong M, Wu HG

4084 Serological diagnostic factors for liver metastasis in patients with colorectal cancer
Wu XZ, Ma F, Wang XL

4089 Altered expression of MUC2 and MUC5AC in progression of colorectal carcinoma
Bu XD, Li N, Tian XQ, Li L, Wang JS, Yu XJ, Huang PL

4095 Efficacy of telbivudine in HBeAg-positive chronic hepatitis B patients with high baseline ALT levels
Lv GC, Ma WJ, Ying LJ, Jin X, Zheng L, Yang YD

4100 Specific shRNA targeting of *FAK* influenced collagen metabolism in rat hepatic stellate cells
Dun ZN, Zhang XL, An JY, Zheng LB, Barrett R, Xie SR

CASE REPORT

4107 Development of fatal acute liver failure in HIV-HBV coinfected patients
Anderson AM, Mosunjac MB, Palmore MP, Osborn MK, Muir AJ

4112 Successful recanalization of acute superior mesenteric artery thrombotic occlusion with primary aspiration thrombectomy
Yang HJ, Cho YK, Jo YJ, Jung YY, Choi SA, Lee SH

World Journal of Gastroenterology
Volume 16 Number 32 August 28, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHING
Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Bulding,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: 00852-3115-8812
Telephone: 00852-5804-2046
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
August 28, 2010

CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

EDITORIAL OFFICE
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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http://www.wjgnet.com

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm. If you do not have web access please contact the editorial office.

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.3987

World J Gastroenterol 2010 August 28; 16(32): 3987-3994
ISSN 1007-9327 (print)

EDITORIAL

Colorectal cancer screening: The role of CT colonography

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Received: April 7, 2010 Revised: May 17, 2010
Accepted: May 24, 2010
Published online: August 28, 2010

Abstract

Computed tomography colonography (CTC) in colorectal cancer (CRC) screening has two roles: one present and the other potential. The present role is, without any further discussion, the integration into established screening programs as a replacement for barium enema in the case of incomplete colonoscopy. The potential role is the use of CTC as a first-line screening method together with Fecal Occult Blood Test, sigmoidoscopy and colonoscopy. However, despite the fact that CTC has been officially endorsed for CRC screening of average-risk individuals by different scientific societies including the American Cancer Society, the American College of Radiology, and the US Multisociety Task Force on Colorectal Cancer, other entities, such as the US Preventive Services Task Force, have considered the evidence insufficient to justify its use as a mass screening method. Medicare has also recently denied reimbursement for CTC as a screening test. Nevertheless, multiple advantages exist for using CTC as a CRC screening test: high accuracy, full evaluation of the colon in virtually all patients, non-invasiveness, safety, patient comfort, detection of extracolonic findings and cost-effectiveness. The main potential drawback of a CTC screening is the exposure to ionizing radiation. However, this is not a major issue, since low-dose protocols are now routinely implemented, delivering a dose comparable or slightly superior to the annual radiation exposure of any individual. Indirect evidence exists that such a radiation exposure does not induce additional cancers.

Key words: Computed tomography colonography; Colon neoplasms; Colon polyps; Colorectal cancer screening; Computed tomography colonography safety; Computed tomography colonography accuracy; Computed tomography colonography radiation exposure; Computed tomography colonography cost-effectiveness

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Laghi A, Iafrate F, Rengo M, Hassan C. Colorectal cancer screening: The role of CT colonography. *World J Gastroenterol* 2010; 16(32): 3987-3994 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/3987.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.3987

INTRODUCTION

It is now 16 years since Vining *et al*[1] presented the first virtual images of the colon at the 1994 meeting of the Society of Gastrointestinal Radiologists. This marked the birth of "virtual colonoscopy" (VC), an intriguing name useful for marketing to patients, providers and the public, or "CT colonography" (CTC), the name most radiologists prefer.

When discussing the role of CTC in colorectal cancer (CRC) screening, it is necessary to separate the present and existent role from the potential one.

The present role is, without any further discussion, the integration into established screening programs as a replacement for barium enema (BE) in the case of incomplete colonoscopy (CC). In fact, since 2006 the American Gastroenterological Association (AGA) Clinical Practice and Economics Committee has endorsed CTC as the method of choice for colon investigation in cases of incomplete colonoscopy[2] and numerous evidence exists in the literature showing a clear superiority of CTC over BE in the detection of CRC and polyps[3-5]. It is also clear that performing CTC in patients with a positive Fecal Occult Blood Test (FOBT) would not be an efficient triage technique in terms of cost-effectiveness, due to the high prevalence of clinically relevant colonic lesions[6,7].

The potential role of CTC is to act as a first-line CRC screening modality. In this regard, it was in March 2008 that CTC obtained its major success: the American Cancer Society (ACS), the US Multi-Society Task Force on Colorectal Cancer and the American College of Radiology (ACR) released consensus guidelines on CRC screening for average-risk individuals. These guidelines distinguished diagnostic tests into two groups: those able to detect CRC, thus potentially reducing mortality (FOBT; Fecal Immunochemical stool Testing, FIT; and stool DNA testing), and those able to detect both polyps and cancer, thus potentially reducing both the incidence of and the mortality from CRC. This latter group of tests includes CC, sigmoidoscopy, BE and - for the first time - also CTC, with the recommendation that it be performed every 5 years starting at 50 years of age[8]. Unfortunately, this position did not remain unequivocal: in fact, the US Preventive Services Task Force (USPSTF) considered the evidence insufficient because of the unknown impact of both extra-colonic findings and radiation exposure, the poor data on cost and cost-effectiveness, and the still unsolved problem of ideal bowel preparation[9]. Other associations, such as the Asia Pacific Working Group on Colorectal Cancer[10] and the American College of Gastroenterology[11], consider CTC a second-line screening test for those unwilling or unable to undergo CC and for those in whom CC was incomplete. Furthermore, in 2008 in the USA, the Centers for Medicare and Medicaid Services denied the reimbursement of screening exams done with CTC[12].

These facts mean on one hand that CTC is considered useful by clinicians, but, on the other hand, that radiologists have much to do until this imaging method is implemented for screening, as testified by a survey conducted among US primary care physicians (PCP)[13]. When asked which diagnostic tests they perceive as being very effective in reducing CRC mortality, 22% answered CTC *vs* 95% CC; and when asked which test they would recommend for CRC screening, most respondents indicated CC (95%) and FOBT (80%), but only about 5% answered CTC.

Keeping in mind these facts, the potential role of CTC in CRC screening will be discussed in the next paragraphs, taking into consideration that an ideal screening test is not yet available and that any screening test is a compromise among four major variables: efficacy, compliance, safety and cost.

EFFICACY

The issue of diagnostic accuracy of CTC for CRC and polyps has been debated for a long time, because of the conflicting results in some of the papers published in the literature[14-16]. This has been recently confirmed by a meta-analysis showing that "CTC is highly specific for the detection of colorectal polyps and tumors" and that "some studies reported high sensitivities, but the results of the studies were highly heterogeneous, while the studied variables explained only part of this discrepancy"[17].

These results led researchers to design three important studies: two large, multicenter trials testing the performance of CTC in comparison with CC in respectively asymptomatic subjects at average risk, i.e. a typical screening population [the American College of Radiology Imaging Network (ACRIN) trial performed in the USA][18] and in a mixed population of asymptomatic subjects at risk higher-than-average and in patients referred for a positive FOBT [Italian Multicenter Polyps Accuracy CTC study (IMPACT) trial][19]; and one multicenter trial [Special Interest Group in Gastrointestinal and Abdominal Radiology (SIGGAR) trial run in the UK] conducted on symptomatic patients with the aim to detect CRC[20]. In particular, the ACRIN trial tried to minimize the variables possibly affecting CTC performance. For this reason only $\geqslant$ 16-row MDCT scanners were used, patients were administered oral contrast agent for stool tagging together with cathartic agent and training of the radiologists observing the images was an important component of the study. In particular, CTC readers were obligated to have read at least 500 cases, or to have attended a 1.5-d training course, and all had to pass a certified exam in which they detected at least 90% of adenomas 1 cm or larger in 50 cases. More than half of the readers had to undergo additional training in order to pass the certified exam initially and, with additional training, all the readers eventually passed.

Results from ACRIN and IMPACT have been recently published[18,19], whereas those from SIGGAR[20] are still under data analysis. Both the ACRIN and IMPACT trials reported per-patient sensitivity of 90% for polyps > 10 mm and 78%-84% for polyps larger than 6 mm; per-patient specificity was extremely high as well, over 85% independently of lesion size (Table 1). The major drawback of ACRIN was represented by the poor positive predictive value (PPV) (23% for polyps $\leqslant$ 10 mm), which might negatively affect a screening program, leading to useless CC, with patient discomfort, embarrassment of radiologists, potential risk of complications and increased costs. Unfortunately there is no explanation for these data, unless one would claim a psychological attitude to overcall in order to reach the threshold of 90% for clinically significant polyps despite a loss in specificity. A definitely better PPV was documented in the IMPACT trial (62% for lesions larger than 6 mm) as well as in studies obtained

Table 1 Results from major trials on a per-patient basis: sensitivity, specificity, positive and negative predictive values

Multicenter trials	All polyp size	Polyps (≥ 5 mm)	Polyps (≥ 6 mm)	Polyps (≥ 7 mm)	Polyps (≥ 8 mm)	Polyps (≥ 9 mm)	Polyps (≥ 10 mm)
Per patient sensitivity							
ACRIN	N/A	65%	78%	84%	87%	90%	90%
IMPACT	N/A	N/A	85%	86%	88%	91%	91%
Munich	84%	91%	N/A	N/A	N/A	N/A	92%
Per patient specificity							
ACRIN	N/A	89%	88%	87%	87%	86%	86%
IMPACT	N/A	N/A	88%	87%	86%	85%	85%
Munich	47%	93%	N/A	N/A	N/A	N/A	98%
Per patient PPV							
ACRIN	N/A	45%	40%	35%	31%	25%	23%
IMPACT	N/A	N/A	62%	N/A	N/A	N/A	N/A
Munich	48%	N/A	70%	N/A	N/A	N/A	79%
Per patient NPV							
ACRIN	N/A	95%	98%	99%	99%	99%	99%
IMPACT	N/A	N/A	96%	N/A	N/A	N/A	N/A
Munich	84%	N/A	98%	N/A	N/A	N/A	99%

Results are categorized according to polyp size. PPV: Positive predictive value; NPV: Negative predictive value; N/A: Not assessable.

in high-experience centers, the University of Wisconsin (PPV, 91.5%) and a group of Korean hospitals (PPV, 69% for lesions > 6 mm and 92% for those > 10 mm)[21,22].

However, the negative predictive values in both the ACRIN and the IMPACT trials was rather high, approaching 100%; this is extremely important in order to reassure negative patients about the significance of the examination.

Excellent results were also obtained in the Munich Colorectal Cancer Prevention Trial[23], a single-center study where around 300 asymptomatic subjects underwent low-dose CTC in comparison with other screening tests (CC, sigmoidoscopy and FOBT).

It is noteworthy to mention that in a screening project offered by the University of Wisconsin[24], after 2 years of recruitment over 3000 subjects of two different, non-randomized groups underwent CTC and CC. The detection rate for advanced adenomas was 3.2% for CTC and 3.4% for CC (difference not statistically significant), with the advantage of a large reduction in the number of polypectomies in the CTC group without any complication as opposed to seven perforations which occurred in the CC group.

Despite the good results there are still some open issues under debate within the radiological as well as the gastroenterological communities. These are the significance of diminutive (< 6 mm) polyps, the management of intermediate (6-9 mm) lesions, the detection rate for non-polypoid, flat lesions and the impact of the extra-colonic findings.

According to a very recent systematic review[25] of published studies reporting the distribution of advanced adenomas in asymptomatic screening cohorts, diminutive polyps have a minimal clinical impact. In fact, the frequency of advanced lesions among patients whose largest polyp was ≤ 5 mm, 6-9 mm, < 10 mm, and > 10 mm in size was 0.9%, 4.9%, 1.7%, and 73.5%, respectively (Figure 1). As a consequence, a 6-mm polyp size threshold for polypectomy referral would identify over 95% of subjects with advanced adenomas, whereas a 10-mm threshold would identify 88% of cases. From a cost-effectiveness point of view, detection and removal of all polyps including those smaller than 5 mm, would be very inefficient, with a cost per year of life gained > $460 000[26], absolutely unacceptable in terms of cost-effectiveness. It is also true that this approach, not removing diminutive polyps, necessitates an extensive education of patients and PCP. In fact, according to a recently published survey[27], the majority of patients, PCP and gastroenterologists would not choose to follow up small polyps identified by CTC with CC because of the fear of missing precancerous lesions.

The management of intermediate (6 to 9 mm) lesions is also under debate, despite the fact that today any polyp 6 mm or larger should be preferably referred for CC and polypectomy, also according to ACS CRC screening guidelines[2]. However, evidence does exist from cost-effectiveness[28] as well as follow-up[29] studies indicating that, for the future, polyp follow-up might be an alternative to referral for CC and polypectomy. These data have been recently reinforced by studies conducted with CC and subsequent polypectomy, where the rate of advanced adenoma in 6-9 mm polyps was demonstrated to be 6.6%[30].

A potential disadvantage of CTC would be the possible impaired ability to detect non-polypoid, flat lesions (Figure 2). This issue deserves some consideration. First of all, flat lesions represent a subset of sessile polyps, and according to a recent publication[31] the overall prevalence in a screening population is around 5.8% (if flat lesions are defined as those with a height not exceeding 0.5 of the diameter[32]). However, within this definition, slightly elevated lesions are also included, which, in some cases, may be classified as sessile. For this reason, an elevation not higher than 3 mm is often used, especially for small lesions not larger than 2-3 cm. The other important remarks are that "completely flat lesions are exceedingly

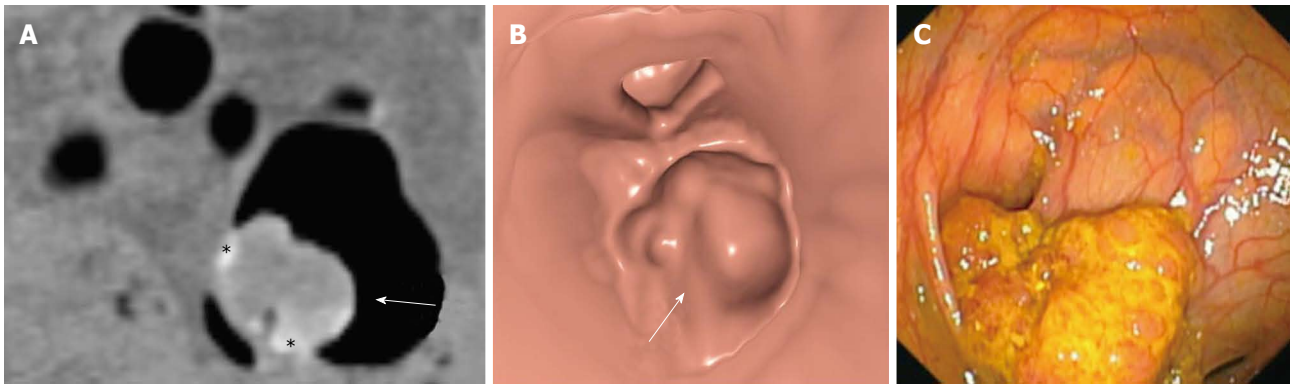

Figure 1 Sessile polyp: adenoma with low-grade dysplasia. A: Coronal reformatted image showing a polypoid lesion (arrow), partly surrounded by tagged fluid (asterisks); B: The same lesion as shown on 3D endoluminal view (arrow); C: Conventional colonoscopy.

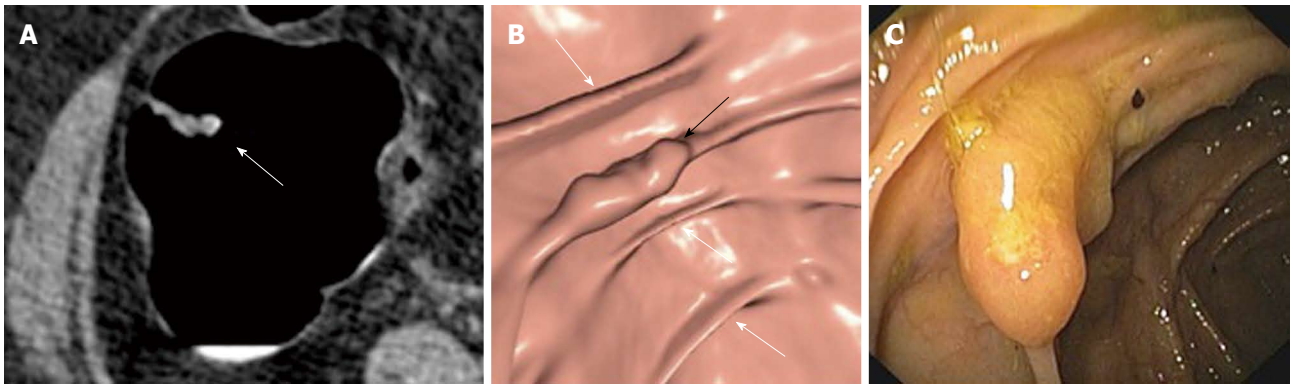

Figure 2 Non-polypoid (flat) lesion: advanced adenoma. A: On 2D axial computed tomography image an irregularly thickened fold (arrow) is detected; B: On 3D endoluminal image the lesion is better appreciated (black arrow), in particular if compared with the normal adjacent colonic folds (white arrows); C: The same lesion at colonoscopy before removal.

rare" and that depressed lesions are less than 1% of all colorectal lesions and only a quarter of those are observed in a screening population. This means that the majority of non-polypoid lesions are at least slightly elevated and this works in favor of the possible detection with CTC. Unfortunately, at the moment only few and conflicting data about the sensitivity of CTC for flat lesions are available. At the beginning disappointing results were published[33,34], because of technical limitations as well as readers' experience. More recently, better results were reported, with sensitivity in the range of 80%-90% for flat adenocarcinomas[32,35]. These results seem to be confirmed by a recent retrospective analysis of the data from the ACRIN trial showing a sensitivity of 89% for flat adenomas ≤ 6 mm (at the prospective analysis, sensitivity was 68%) (Fidler J, presented at ACRIN meeting, October 1, 2009).

The impact of extra-colonic findings will be discussed in the paragraph concerning costs.

COMPLIANCE (ADHESION TO A SCREENING PROGRAM)

Compliance means adhesion of healthy individuals to a screening program. This is a very complex issue, long debated in the literature and presently without a solution, since virtually in any Western country a high proportion of adults 50 years of age or older have not undergone any CRC screening test[36]. Colonoscopy, considered to be the most effective screening method, suffers from a very low participation rate. Very recent data from Italy[37] underline the difficulties and barriers in implementing a CC population screening, at least through primary care. Reported participation in a CC screening arm was extremely low in Southern Italy (2.8%), and higher, but still disappointing, in North-Central Italy (12.4%).

To increase screening uptake is extremely important, since the adhesion rate of the invited population directly affect the efficiency of the program. For example, if we consider that the efficacy of CC in reducing the incidence of CRC is around 76%[38], the efficiency with regard to CRC prevention rate, considering an adhesion rate of 20%, drops to 15% (76% × 20% = 15%).

The question is whether CTC would be able to increase screening uptake. The three most common deterrents that patients expressed about receiving CC were bowel preparation, embarrassment and fear of discomfort[39,40]. The advantage of CTC is the use of a gentler preparation or unprepped (laxative-free) examination[41]. Furthermore, the pain related to colon distension by air may be minimized by the use of carbon dioxide delivered

by an electronic pump. The use of carbon dioxide is also associated with a faster absorption, making the patient more comfortable immediately after the examination[42].

Unfortunately, only few data are available regarding adhesion rate and CTC. In a study conducted in Western Australia[43], 2000 people were invited and 28.4% accepted to undergo CTC for screening, with 62% of them preferring CTC over optical colonoscopy. The fact remains that we need data resulting from real screening experiences.

SAFETY

CTC is a safe test, definitely safer than colonoscopy. The results of different surveys show a perforation rate associated with CTC ranging between 0.06% and 0.08%[44,45], even lower in screening[46], compared with 0.1%-0.2% for diagnostic colonoscopy[47]. It should also be noted that the comparison between CTC and CC is very difficult, with the risk of overestimating the clinically significant perforations at CTC, because of the much higher sensitivity of CT in the detection of even tiny air bubbles. In fact, most of the perforations reported in a UK survey[44] were treated conservatively, without surgical intervention. CTC complications in most of the cases are due to technical factors, such as the use of a rigid catheter for bowel distension (now replaced by thin rubber devices), manual distension with air (now minimized by the use of an electronic pump delivering carbon dioxide and able to control pressure and volume), inexpert personnel and incorrect patient selection.

Other complications occasionally reported have been vasovagal reactions due to colonic overdistention[48].

The main potential drawback of screening with CTC is the exposure to ionizing radiation[49] and the consequent theoretical risk of inducing cancer. The risk is theoretical because there are still many uncertainties with regard to the true effects of ionizing radiation at low doses, such as those used in diagnostic radiology[50,51]. According to the Health Physics Society[52], "below 5-10 rem (50-100 mSv) (which includes occupational and environmental exposures), risks of health effects are either too small to be observed or are nonexistent"; and the French Academy report[53] stated that the linear no-threshold (LNT) hypothesis for assessing the risk associated with low doses is not based on scientific evidence. However, in contrast, the International Commission on Radiological Protection (ICRP)[54] and the Biological Effects of Ionizing Radiation (BEIR) Ⅶ report[55] considered LNT as the best method to assess low dose exposures, in order to be more conservative and more protective towards patients because of the multiple uncertainties. Unfortunately even if this hypothesis may be true it cannot be proved since we have little direct evidence of harm below 100 mSv.

Even if the LNT hypothesis is considered the most accurate, the problem of radiation exposure of patients undergoing CTC seems to be minimal. In fact, the mean exposure in the case of a screening examination has been calculated in a recent survey[56] to be around 5-6 mSv, which is twice the normal background radiation exposure in the US (2.5-3.0 mSv per year)[57]. In addition we have to consider, in a screening scenario, that CTC should be repeated every 5 years, not earlier. For a radiation dose of around 5-8 mSv at age 50 years, the lifetime risk of death from cancer varies between 0.02% and 0.03%[58]. If we think about cost/benefit of the examination, this minor risk should be compared with the theoretical risk of CRC in average risk individuals, which is around 5%[59].

A more precise idea about the amount of radiation exposure comes from a comparison with other categories of workers continuously exposed to low dose radiation, such as, for example, airline crews and nuclear workers. As an example, the value of 5-6 mSv, to be received in a screening scenario every 5 years, should be compared with radiation exposure of airline crews, who are submitted to an average of 5 mSv per year every single year of their activity, with a long life exposure close to 80 mSv. A recent survey of airline pilots from eight different European countries has shown no increase in mortality from radiation-induced cancers over a 30-year period of time[60]. Similar data were observed in nuclear workers in two recently published experiences[61,62].

COSTS

Cost analysis is a very difficult task, especially in the absence of real data and based only on mathematical models. Among the studies published in the literature[63-69] 5 out of 7 are in favor of CC and only two were able to demonstrate a better cost-effectiveness of CTC. A recent review of the literature[70] has pointed out the profound differences among the models as well as the weakness of such an approach, where a minimal variation of a single input may completely alter the final results. As an example, if the cost ratio between CTC and CC is ⩽ 0.7, the model is usually in favor of CTC, but if the cost of CTC is higher than 80% of the cost of CC, it will be CC which is the most cost effective method. Another very important issue, adhesion rate, is never taken into account in the models, where it is considered to be equal among the different screening tests, although this is probably not the case. In addition, differences in healthcare systems, reimbursement, cost of the equipment and personnel are other important variables affecting the final outcome.

When considering cost, the issue of extra-colonic findings should be taken into account. The detection of extra-colonic findings can be considered a potential advantage of CTC, since previously unknown life-threatening diseases, which are not insignificant[71], can be diagnosed and treated, with a clear impact on patients' life expectancy. However, the major problem is the extra time necessary for reporting these findings and the cost induced by unnecessary investigation of common benign abnormalities, especially because of their high prevalence[72,73]. In a recent publication[74], the mean cost per patient was $31.02 for nonsurgical and $67.54 for surgical work-up procedures. Although extra-colonic findings have been traditionally

regarded as an additional cost, they have been recently considered as a potential benefit (i.e. detection of unsuspected abdominal aortic aneurysm or renal cancer), able to improve CTC cost-effectiveness[75].

CONCLUSION

In conclusion, CTC has a present role in CRC screening programs, i.e. the replacement of BE in the case of incomplete colonoscopy. The potential role is the proposal of CTC as a first-line CRC screening modality. In this setting, CTC has clear advantages, such as accuracy, safety and subject acceptance. Further research should be warranted to clarify, in particular, two aspects: the uptake rate of the general population and the real cost and benefits derived from a CTC screening program.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.3995

World J Gastroenterol 2010 August 28; 16(32): 3995-4002
ISSN 1007-9327 (print)

EDITORIAL

Essential role of monocytes and macrophages in the progression of acute pancreatitis

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Received: April 1, 2010 Revised: May 31, 2010
Accepted: June 7, 2010
Published online: August 28, 2010

Abstract

Acute pancreatitis (AP) is an inflammatory condition of the pancreas caused by an imbalance in factors involved in maintaining cellular homeostasis. Earliest events in AP occur within acinar cells accompanied by other principal contributors to the inflammatory response i.e. the endothelial cells, immunocytes (granulocytes, monocytes/macrophages, lymphocytes) and neutrophils. Monocytes/macrophages are important inflammatory mediators, involved in the pathophysiology of AP, known to reside in the peritoneal cavity (in the vicinity of the pancreas) and in peripancreatic tissue. Recent studies suggested that impaired clearance of injured acini by macrophages is associated with an altered cytokine reaction which may constitute a basis for progression of AP. This review focuses on the role of monocytes/macrophages in progression of AP and discusses findings on the inflammatory process involved.

Key words: Acute pancreatitis; Monocytes; Peritoneal macrophages; Alveolar macrophages; Kupffer cells

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Shrivastava P, Bhatia M. Essential role of monocytes and macrophages in the progression of acute pancreatitis. *World J Gastroenterol* 2010; 16(32): 3995-4002 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/3995.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.3995

INTRODUCTION

Inflammation is a complicated progressive process which is initiated by the body in response to tissue injury or infection. Inflammation proceeds *via* the sequential release of mediators that leads to vasodilatation and increased blood flow, increased vascular permeability, causing the accumulation of a fluid exudates, and the activation of neurosensory pain fibers giving rise to the classical signs of acute inflammation i.e. heat, redness, swelling, and pain[1]. Acute inflammation is associated with high levels of polymorphonuclear cells, particularly neutrophils, whereas chronic or adaptive immune inflammation has higher levels of mononuclear cells, macrophages, T- and B-lymphocytes.

Regulated release of chemokines and expression and activation of cellular adhesion molecules recruits leukocytes at the site of inflammation. Leukocyte recruitment is a critical step in the inflammatory process[2]. Membrane bound vascular cell adhesion molecule-1 (VCAM-1), intracellular cellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 and E-selectin are expressed on endothelial cells, smooth muscle cells, and tissue macrophages. These adhesion molecules in coordination with others, for example, selectins, allow binding of ligands on leukocytes to mediate rolling, firm attachment and transendothelial migration. Endothelial cells being the interface between the tissue and circulation play

an important role in inflammatory and immune-relevant cells[3,4].

Acute pancreatitis (AP) is an inflammatory condition of the pancreas that involves peripancreatic tissue and remote organs. Excessive systemic inflammatory response syndrome (SIRS) in AP leads to distant organ damage and multiple organ dysfunction syndrome (MODS), which is the primary cause of morbidity and mortality in this condition. Mild AP is self limiting but up to 25% of the patients suffer a severe attack and around 30% of these will die. Approximately half of the deaths in AP occur within the first 2 wk of illness and are generally attributed to organ failure. The rest of the deaths occur weeks to months later, characterized by extensive retroperitoneal pancreatic necrosis and septicemia[5]. AP involves a complex cascade of events initializing in pancreatic acinar cells. An unknown trigger within the pancreas leads to conversion of digestive proenzymes into their active form, initiating auto digestion of the gland causing hemorrhage, necrosis, edema and complete destruction of pancreatic parenchyma. Intrapancreatic activation of trypsinogen by lysosomal hydrolases is an early triggering event in AP[6]. Interestingly both pharmacological and genetic deletion of lysosomal hydrolases like cathepsin B can reduce the severity of pancreatitis[7]. Other pharmacological agents which block trypsinogen activation can also modulate the outcome of AP[8,9].

Immune cells involved in elaborating the inflammatory mediators in AP are the pancreatic acinar cells, endothelial cells, neutrophils, lymphocytes, monocytes and macrophages. Inflammatory mediators believed to participate in the pathophysiology of this condition include: tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1β), interleukin-6 (IL-6), platelet activating factor (PAF), ICAM-1, IL-8, growth related oncogene-a/cytokine-induced neutrophils chemo attractant (GRO-α/CINC), monocyte chemotactic protein-1 (MCP-1), IL-10, complement component C5a, substance P (SP), hydrogen sulfide (H_2S), and neutral endopeptidase (NEP)[10]. In recent years, it has become clear that the signaling molecule nuclear factor κB (NF-κB) plays a central role in the initiation and progression of AP[11]. The emerging body of evidence suggest that blocking NF-κB activation can markedly reduce the severity of AP[12,13]. These findings have opened a window of opportunity for the use of selective NF-κB inhibitors in regulating the inflammatory process in AP. The expression levels of various proinflammatory mediators like TNF-α and IL-1β in AP are positively regulated by NF-κB[14,15]. Systemic amplification of AP is associated with excessive release of these inflammatory mediators from local tissue and systemically. This systemic amplification is responsible for most of the mortality associated with AP[16]. Studies indicate that both pancreatic and extra pancreatic (lung, liver, monocytes, macrophages and endothelial cells) activation of NF-κB is associated with development of MODS in AP[17,18].

In this review we discuss the recent advances, till date, pointing towards the fundamental role of different monocyte/macrophage populations in the progression of AP.

MONOCYTE/MACROPHAGE SYSTEM

Macrophages are among the most versatile cells of the body. Compelling evidence has emerged in recent years with respect to their roles in innate immunity, inflammation, and tumor progression. Macrophages are released from bone marrow into the bloodstream as promonocytes which develop into monocytes and migrate to tissue and undergo final differentiation into specific types of tissue resident macrophages[19]. The phenotypes of these tissue resident macrophages vary markedly within tissues, including Kupffer cells in liver, alveolar macrophages in lungs, osteoclasts in bone, microglial cells in brain, Langerhans cells in skin.

Monocyte and macrophage in AP

Since macrophages orchestrate both the initiation and the resolution of inflammation, they are an interesting target for designing a therapeutic strategy focused on the control of systemic effects of AP. Macrophage activation constitutes a key component of the immune response and several proinflammatory cytokines and bacterial products participate actively in the triggering of this process.

The degree of macrophage activation might be one of the important factors that determine the severity of AP[40]. Besides acinar cells, monocytes/macrophages are the main inflammatory cell population involved in the pathogenesis of AP (Figure 1). In AP, the inflammatory process starts with the migration of monocytes and neutrophils in the circulation into the pancreatic interstitial space, mediated by adhesion molecules on leukocytes. These infiltrating cells assist the production of different cytokines and various inflammatory mediators. As a result, amplification of non-infectious inflammation initiated in the pancreas to specific distant organs such as the lung, liver and spleen occurs[20]. The CC chemokines such as MCP-1, macrophage inflammatory protein (MIP)-1α and RANTES (CCL5) are believed to activate primarily monocytes, whereas the CXC chemokines, such as IL-8, tend to preferentially activate neutrophils. Bhatia *et al*[21] have shown that administration of bindarit, a blocker of MCP-1 synthesis (prophylactically or therapeutically), significantly reduces the severity of AP suggesting that MCP-1 may be an early inflammatory mediator in AP.

CD14, is a glycosyl-phosphatidylinositol anchored cell surface molecule expressed on the cell surface of immune effector cells of myeloid and monocytes hematopoietic lineage, which acts in concert with mammalian Toll-like receptors (TLR). It exists in 2 forms - soluble and membrane bound - and the soluble form has both immune stimulatory and inhibiting activities. Increased soluble CD14 expression and expansion of the proinflammatory CD14/CD16 monocytes subset has been reported in AP, suggesting that soluble CD14 receptor may serve as a key mediator of the systemic endothelial response, and its targeted disruption with anti-CD14 monoclonal antibodies may afford some therapeutic benefit in preventing the development of MODS and septic complications associated with AP[22]. Li *et al*[23] demonstrated that during early stages of

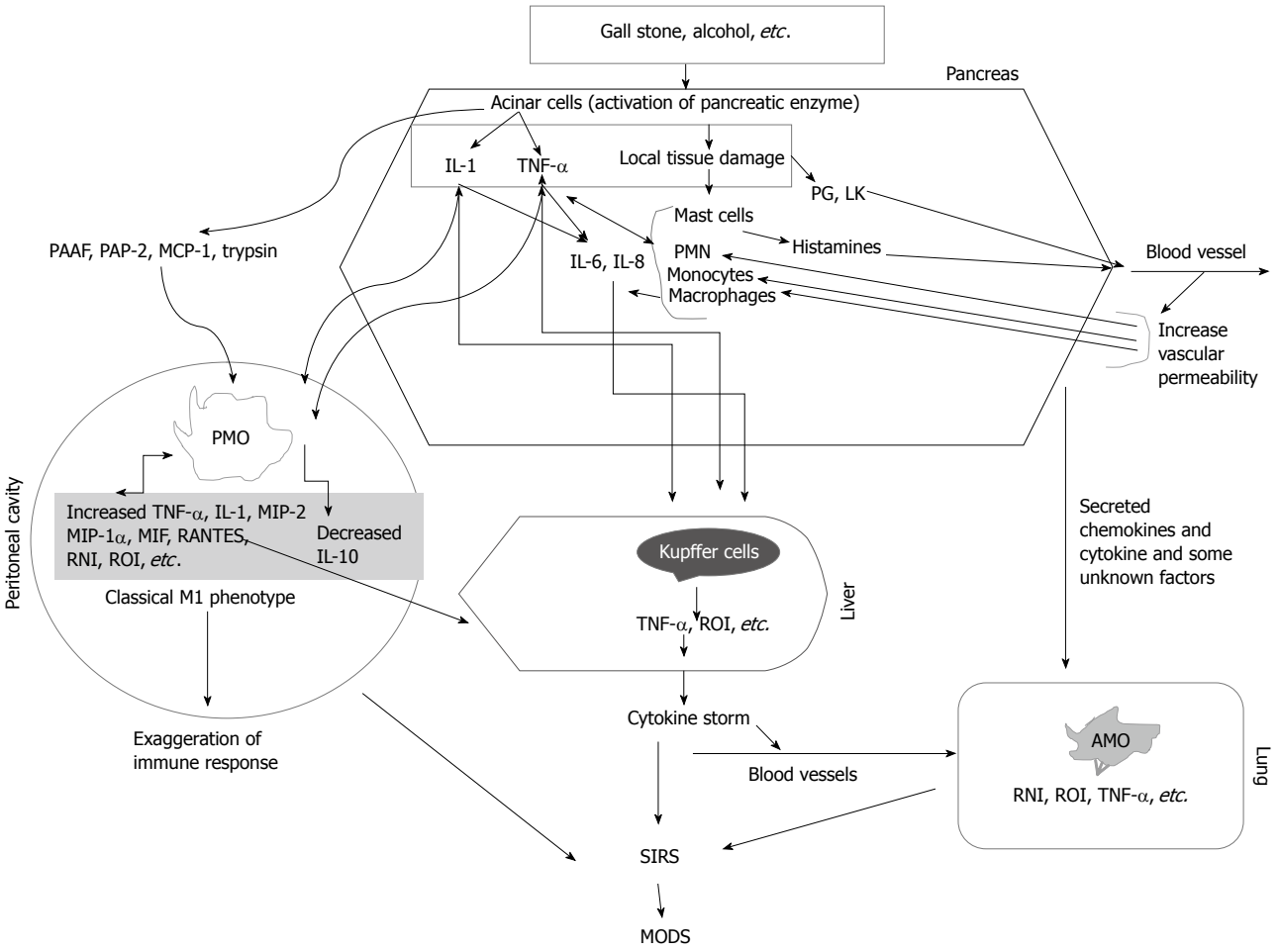

Figure 1 Our current understanding of the role of monocytes and macrophages in the pathogenesis of acute pancreatitis. Local inflammation in the pancreas leads to secretion of pro-inflammatory cytokines and some unknown factors. These inflammatory mediators activate peritoneal macrophages (PMO), Kupffer cells, and alveolar macrophages (AMO), which if uncontrolled can cause multiple organ dysfunction syndrome (MODS). PMO display a classical M1 type activation in acute pancreatitis thus supporting the inflammatory process. TNF: Tumor necrosis factor; RNI: Reactive nitrogen intermediates; ROI: Reactive oxygen intermediates; PAAF: Pancreatitis associated ascitic fluid; PAP: Pancreatitis-associated protein; MCP: Monocyte chemotactic protein-1; PG: Prostaglandins; LK: Leukotrienes; SIRS: Systemic inflammatory response syndrome; PMN: Polymorphonuclear leukocytes; MIP: Macrophage inflammatory protein.

AP, expression of TLR4 is upregulated in peripheral blood mononuclear cells (PBMC) with a simultaneous increase in TNF-α expression suggesting an important role of TLR-4 in pathogenesis of AP. TNF-α and IL-1β are regarded as most prominent "first-line" cytokines. Studies have indicated that during the course of pancreatitis, initially IL-1β and TNF-α are produced in the pancreas by acinar cells, in a process mediated by leukocytes, and organs such as liver, lungs and spleen which have large populations of macrophages, serve as subsequent sources of production of pro-inflammatory cytokines[24]. The pancreas itself contains an unknown proportion of resident macrophages. The liver and lung are susceptible to injury and organ failure during AP. Peritoneal macrophages, Kupffer cells and alveolar macrophages play a pivotal role in controlling the progression of AP, due to their ability to generate pro- and anti-inflammatory mediators.

Peritoneal macrophages

Peritoneal inflammatory cells play an important role in the production of chemical mediators and in the defense against infection of the abdominal cavity. Macrophages are the major resident immune cells within the pancreas and in the peritoneal cavity. Severe AP (SAP) and early multi system organ failure are associated with the sustained release of both pro- and anti-inflammatory cytokines in the ascitic fluid, the thoracic lymph and the systemic circulation. The peritoneal compartment which is in the close vicinity to the site of pancreatic inflammation and necrosis is the site for net proinflammatory reaction. Release of IL-1β and TNF-α by peritoneal macrophages in early stages of AP induces a cascade of other inflammatory cytokines, activation of neutrophils, and induction of the pro-inflammatory response whereas the anti-inflammatory response is mainly systemic in nature. Preventing the activity of both cytokines concurrently has no additional effect on the degree of pancreatitis but does attenuate the systemic stress response and is associated with an additional but modest decrease in mortality[10]. Thus, in AP, peritoneal macrophages act as a principal contributor to the acute systemic inflammatory response that in turn determines the severity of disease. Peritoneal macrophages isolated from rats with SAP showed over production of nitric oxide. Increased nitric oxide secretion is implicated in progression of AP[25].

Abnormal trypsin activation in the pancreas contributes to the pathogenesis of AP. Lundberg *et al*[26] showed that trypsin stimulates the production of cytokines in peritoneal macrophages and that injection of trypsin into the peritoneal cavity induces lung injury. Hori *et al*[27] demonstrated that TGF-β produced by peritoneal macrophages induces hepatocellular injury *via* apoptosis in the rat SAP model.

Studies demonstrate that pancreatitis associated ascitic fluid (PAAF) of SAP affects peritoneal macrophage functions thereby contributing to the pathologic course of disease. They showed that incubation of peritoneal macrophages with PAAF leads to rapid and prolonged activation of NF-κB and TNF-α production[28]. The major site of TNF-α gene transcription in AP is the pancreatic activated macrophages[29]. Their deactivation in the early course of AP increases survival and decreases the severity of disease. Liu *et al*[30] suggested that activation of NF-κB and p38 MAPK in monocytes/macrophages from animals with AP might play a role in transcription and biosynthesis of TNF-α and IL-6. Animal experiments indicated that sterile ascites without cytokines from AP can stimulate production of cytokines from macrophages derived from spleen and lung *in vitro* and can induce cytokine production systemically *in vivo*[31].

Mikami *et al*[32,33], by depleting peritoneal macrophages using liposome encapsulated dichloromethylene biphosphonate, suggested that peritoneal macrophages extend inflammation from the pancreas to the peritoneal cavity and subsequently induce lung injury leading to SIRS and multiple organ failure opening the possibility that therapeutic modification of peritoneal macrophages may be a new therapeutic approach in patients with AP. The immune response in AP depends to a larger extent on macrophages as they represent about a third of infiltrating mononuclear cells. Macrophages are the main source for the production of inflammatory mediators; their presence in pancreatitis might contribute to the amplification of the immune response during disease progression.

With the progression of AP, there is a change in number and ratio of CD4+ and CD8+ lymphocytes indicating the possible involvement of T-lymphocytes. Observation by Demlos *et al*[34] demonstrated that CD4+ T cell depletion reduces the severity of pancreatitis. CD4+ T cells act as a costimulator for macrophage activation *via* antigen presentation and proinflammatory cytokine release[35].

Activation and trafficking of inflammatory cells involves chemokines and their receptors. Interestingly deletion of the receptor for MIP-1α and RANTES, the CCR1 receptor, was associated with protection from pulmonary inflammation but did not reduce the severity of cerulein-induced pancreatitis. This protection from lung injury is associated with decreased levels of TNF-α in a temporal sequence indicating the critical role of CCR1 receptor in the extension of pancreatic injury to the systemic response. The authors underline these findings by suggesting that CCR1 may be activated on either peritoneal or lung macrophages, leading to an autocrine process whereby increased levels of TNF-α drive further induction of both α and β chemokines, resulting in recruitment of inflammatory cells to the pancreas and lung[36]. However, in the same model, inhibition of CXCL2 (MIP-2) protected partially against both pancreatic and lung injury[37]. Attenuation of pancreatic injury in this study may be due to the fact that CXCL2 (MIP-2), in contrast to CCL5 (RANTES) and CCL3 (MIP-α), also attracts neutrophils in addition to monocytes. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine released by macrophages and lymphocytes, is emerging as an important player in the pathogenesis of AP. Pre-treatment with anti-MIF antibodies improved the survival in rats with AP[38]. Recently, work by Sorli *et al*[39] suggested that peritoneal macrophages showed a classical M1 phenotype in AP, characterized by high expression of TNF-α and lack of changes in the mannose receptor. TNF-α can in turn activate macrophages for release of other macrophage derived inflammatory cytokines thus exaggerating the inflammatory response. Their study also indicates that peritoneal macrophages could be reprogrammed *in vitro* to reparative alternatively activated (M2) macrophages by IL-4 and IL-13 thus opening the possibility that therapeutic modulation of macrophage activation can help in the treatment of AP[39].

In any immune response dying cells are normally phagocytosed by macrophages; this elimination of dying cells is associated with anti-inflammatory cytokine switching. Recent observations by Liang *et al*[40] have suggested that there is a close relation between modes of pancreatic acinar cell death, the release of cell contents and the inflammatory reaction of peritoneal inflammatory cells.

In recent years, neuropeptide SP has gained considerable attention as a mediator of neurogenic regulation of inflammation. Earlier work by Sun *et al*[41] has suggested that neuropeptide SP is a pro-inflammatory mediator involved in pancreatitis and during acute inflammation, SP induces chemokine release from macrophages infiltrating into local and distant damaged tissues. Mice lacking NK-1Rs which bind to SP are protected against cerulein induced pancreatitis and associated lung injury. Macrophages have receptors for SP. Recently we have also shown the effect of SP on the murine macrophage cell line RAW264.7, as well as isolated primary macrophages, indicating that SP, at nanomolar concentrations, elicited selective chemokine production from murine macrophages[42]. Indeed, unpublished observations from our lab indicate that macrophages isolated from mice lacking NK-1R receptors show M2 phenotype displaying an anti-inflammatory cytokine switch. These observations led us to conclude that SP, acting *via* NK-1R on macrophages, plays an important role in regulating the severity of pancreatitis and associated lung injury.

Pancreatitis-associated proteins (PAP) are members of the *Reg* gene family; these 14-17 kDa proteins have been shown to be strongly induced during AP[43]. Although originally identified during AP, they have been reported in other inflamed pathologic organ systems including Crohn's disease, inflammatory bowel disease, liver injury, neuronal, ovarian, and cardiac tissue damage[44,45]. Recent investigation suggests that PAP2 is a potential mediator of early

inflammation in AP, its act specifically by orchestrating the macrophage inflammatory response, and may do so by working in concert with other PAP isoforms[46]. They also demonstrated that macrophages may express a potential PAP2 receptor[46].

Kupffer cells

Liver macrophages or Kupffer cells are the most abundant mononuclear phagocytes in the body. They are a predominant source of inflammatory cytokine released in the systemic circulation. Cytokine release from the liver represents about 50% of total cytokine release from the body[47]. Recent studies have shown the involvement of the liver in complex networks of events triggering the multiorgan dysfunction associated with AP. During AP the inflamed pancreas generates soluble inflammatory mediators. Once pancreatic mediators reach the liver, they strongly activate Kupffer cells (resident macrophages), they then greatly amplify the release of cytokines into the blood stream and thus contribute to the systemic manifestation of AP. Activated Kupffer cells release different inflammatory mediators, immunoregulatory and inflammatory cytokines, reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), hydrogen peroxide, *etc.* which play a significant role in progression of pancreatic inflammation into a systemic process[48]. TNF-α released from the pancreas triggers the early events in AP, as pancreatitis progresses TNF-α reaches the liver, further manifesting the disease outcome by causing a cytokine storm. This cytokine is considered as one of the most important mediators of the systemic complications associated with AP[49]. The observation that in native pancreatitis the systemic manifestation of AP is more severe as compared to graft pancreatitis (as the mediators released by pancreas in graft pancreatitis are sent directly through the iliac vein) suggested that the liver plays an active role in the development of lung injury secondary to AP[50]. Gloor *et al*[51,52] further confirm this observation by preventing the passage of blood coming from pancreas to liver. Interestingly it has been shown that blocking the activity of Kupffer cells by gadolinium chloride in a sodium taurocholate AP model significantly reduces the increased TNF-α in serum during pancreatitis to its control levels, followed by reducing the inflammatory response in the lung, and in turn reducing the systemic complications associated with AP and improving the survival rate in mice. The authors confirm their observation *in vitro*, by using cultured Kupffer cells[53,54]. Additionally, inhibiting the Kupffer cell activity before induction of AP significantly diminished the associated lung injury with a decrease in NF-κB activity[55]. Altogether, these results show a link between the local inflammatory process in the pancreas and its manifestation by liver cells, especially Kupffer cells, causing the appearance of systemic organ dysfunction secondary to AP.

Alveolar macrophages

Patients with AP may develop acute lung injury, manifest clinically as the adult respiratory distress syndrome (ARDS). Most patients who die during the early stages of SAP die either with or as a result of this lung injury. However, the events that link AP to acute lung injury are not fully understood. It has been postulated that alveolar macrophages (AM) are involved in development of acute local disorders as a consequence of extra pulmonary stimuli like pancreatitis, peritonitis, or trauma. AM have the capacity to secrete a very large number of inflammatory mediators, including lipid mediators, chemokines, cytokines, growth factors and reactive oxygen and nitrogen species. They may therefore play multiple roles in the respiratory tract and may be pro-inflammatory or anti-inflammatory. They may be activated by several stimuli, including cigarette smoke, pro-inflammatory cytokines, endotoxin and immune stimuli. Their capacity to release multiple chemokines leads to the recruitment of several cell types from the circulation, including monocytes, neutrophils and T lymphocytes. Polymorphonuclear neutrophils play an important role in the development of ARDS and activation of the complement system, and generation of AM derived factors promotes neutrophil aggregation into lungs. The capacity of AM to mobilize a large amount of leukocyte and to release secretory products such as RNI and ROI suggests that these cells can be involved in lung damage associated with AP. The activation of AM seems to be regulated by cytokines and inflammatory mediators, which are generated during the course of AP. Sailai *et al*[56] suggested that the inhibition of NF-κB activation may reverse the lung injury of acute necrotizing pancreatitis (ANP) by inhibiting the release of inflammatory mediators by AM. Nitric oxide (NO) is an important marker of oxidative injury in the lung. High TNF-α and NO released by activated AM in ANP suggest a role for AM in inducing lung injury associated with ANP[57]. Studies have suggested that, during the early phase of AP, AM-derived NO contributes to lung injury. Administration of the NOS inhibitor L-NMMA prevented lung injury in this model. In conclusion, this study shows that lung damage induced by experimental AP develops with AM activation. The liver plays an active role in the activation of AM in this experimental model[58]. In addition, neutrophil recruitment into the lungs during AP seems to be mediated by chemotactic mediators (TNF-α and MIP-2) released by activated AM.

In AP, endothelial cells, polymorphonuclear neutrophils and macrophages release PAF, which has been implicated as a key mediator in the progression of AP, leading to complications and unacceptably high mortality rates. PAF participates in the occurrence and development of AP and administration of platelet-activating factor receptor antagonists (PAF-RAs) could significantly reduce local and systemic events after AP[59]. BN52021 (PAF-RA) prevented the labilization of the lysosomal membrane of AM thus suggesting that complications of AP could be dependent on the stabilization of AM lysosomes[60]. Another PAF antagonist (TCV-309) prevented hyperactivity of AM by reducing cytokine induced neutrophil chemoattractant expression by AM[61]. These studies further point to the use of PAF antagonist in reducing the secondary complications associated with AP.

Hsp72 induction is associated with the early stages of

lung neutrophil infiltration. In a study using a 5% sodium taurocholate model of AP it was found that Hsp72-was over expressed in bronchial epithelium, alveolar macrophages, infiltrating neutrophils, blood vessels and blocking of P-selectin activity diminished the expression of Hsp72 in lungs thus suggesting that Hsp72 induction is mediated by neutrophil infiltration into the lungs[62].

Macrophages generate ROI and therefore contribute to the increased oxidative stress. Increased levels of pancreatic phospholipase A2 (PLA2) are detected in the systemic circulation and bronchoalveolar lavage fluid from patients with lung injury in AP. Reports indicate that PLA2 regulates the cytokine production of monocyte/macrophages and the phagocytosis and superoxide (O2-) generation of neutrophils[63]. AMs are activated by PLA2 and produce a large amount of NO that contributes to lung injury in AP. Blocking of PLA2 activity prevented the lung injury associated with pancreatitis[64].

The secretion of multiple inflammatory proteins by AM is largely a result of increased expression of inflammatory genes orchestrated by proinflammatory transcription factors, such as NF-κB and AP-1. Sphingosine-1-phosphate (S1P), a biological active lipid generated by numerous cell types, significantly reduced the NF-κB activation of the AM, ameliorating pulmonary injury[65], thus suggesting that AM could be a therapeutic target of S1P for combating pulmonary injury associated with pancreatitis.

CONCLUSION

Though the role of monocytes/macrophages has long been considered as evidence for a host response against the inflammatory process, recent findings suggest that monocytes/macrophages are active players in the progression of AP. Over the past few years understanding of molecular mechanisms underlying the progression of AP has improved considerably. A better understanding of regulation and function of different macrophage populations involved in AP will help to establish more therapeutically effective novel therapies for AP management. Strategies targeting molecules (NF-κB and STAT-3) and mechanisms supporting M2 type macrophages will further help in restraining the inflammatory process.

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S- Editor Wang JL **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4003

World J Gastroenterol 2010 August 28; 16(32): 4003-4012
ISSN 1007-9327 (print)

FRONTIER

Lymphangiogenesis: A new player in cancer progression

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Author contributions: Nagahashi M, Ramachandran S, Rashid OM and Takabe K all generated the ideas and contributed to the writing of this paper.
Supported by SUMITOMO Life Social Welfare Services Foundation (to Nagahashi M); Virginia Commonwealth University Grant BIRCWH K12HD055881, and Susan G Komen for the Cure Career Catalyst Research Grant KG090510 (to Takabe K)
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Received: May 3, 2010 Revised: June 15, 2010
Accepted: June 22, 2010
Published online: August 28, 2010

Abstract

Lymph node metastasis is the hallmark of colon cancer progression, and is considered one of the most important prognostic factors. Recently, there has been growing evidence that tumor lymphangiogenesis (formation of new lymphatic vessels) plays an important role in this process. Here, we review the latest findings of the role of lymphangiogenesis in colorectal cancer progression, and discuss its clinical application as a biomarker and target for new therapy. Understanding the molecular pathways that regulate lymphangiogenesis is mandatory to pave the way for the development of new therapies for cancer. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-lymphangiogenesis agents will hopefully improve patient outcomes. This progression to the clinic must be guided by new avenues of research, such as the identification of biomarkers that predict response to treatment.

Key words: Colorectal neoplasms; Angiogenesis; Lymphangiogenesis; Lymphatic vessels; Lymphatic metastasis; Vascular endothelial growth factor; Monoclonal antibody D2-40; Therapy-related neoplasms; Biomarkers

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Nagahashi M, Ramachandran S, Rashid OM, Takabe K. Lymphangiogenesis: A new player in cancer progression. *World J Gastroenterol* 2010; 16(32): 4003-4012 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4003.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4003

INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer to develop and to cause death in the United States[1]. Although the pattern of spread of CRC may vary, the initial step involves lymphatic invasion and metastasis to regional lymph nodes[2]. Patients with lymphatic invasion have a less favorable outcome, and lymph node metastasis is one of the most important prognostic factors in CRC[3]. In fact, both the Dukes and TNM staging systems, which have been the most widely used staging systems for CRC, are based on the assessment of lymph node metastasis in addition to the extent of primary tumor and distant metastatic disease[4,5]. Patients with an early stage tumor without evidence of lymph node metastasis (Dukes A, TNM stage Ⅰ) have an excellent post-operative prognosis and a 5-year survival rate of 80%-90%, while patients with advanced tumors with regional lymph node disease (Dukes C, TNM stage Ⅲ) have a 5-year rate of 25%-60%. Furthermore, patients with distant metastatic disease (Dukes D, TNM stage Ⅳ) have a 5-year rate of less than 10%[6-8]. In addition, the number of nodes with metastatic disease has an important impact on the prognosis of patients with CRC. In fact, the influence of lymph node metastatic

disease on prognosis in CRC is so great that there is not only a difference between N1 and N2 (1 to 3 nodes *vs* 4 or more nodes), but based upon separate analyses by the Surveillance Epidemiology and End Results program, there is also a significant prognostic difference within each of these groups. Accordingly, N1 is subdivided into N1a (1 node involvement) and N1b (2-3 node involvement), and N2 is subdivided into N2a (4 to 6 node involvement) and N2b (7 or more). This new TNM staging system lymph node sub-categorization is based on survival[8], which is in further agreement with the importance of lymph node metastatic disease in CRC patient outcome. Moreover, detailed analysis of lymph node status allows for accurate staging, which is now shown to be associated with better outcomes[8].

While local or regional CRC can be controlled with complete surgical resection, combination therapy is required to treat systemic disease. Among patients with newly diagnosed CRC, 25% will first present with metastatic disease[9]. Even among patients who present with localized, resectable disease, 30% will have a recurrence with metastatic disease[9]. There has been remarkable progress in the treatment of metastatic CRC during the last decade in the fields of surgery, radiation, chemotherapy, and targeted therapy[9-11]. Over the last decade, a better understanding of the processes involved in tumorigenesis and cancer metastasis has led to the development of a new category of systemic drugs called targeted therapies. The term targeted therapy refers to drugs that selectively target specific molecular pathways involved in tumorigenesis and/or tumor metastatic progression[11]. In CRC, two targets have been intensively investigated and are currently under Phase Ⅲ clinical trials: the vascular endothelial growth factor (VEGF) pathway that controls angiogenesis, the phenomenon where new blood vessels are formed to feed the enlarging tumor and develop access to the blood stream; and the epidermal growth factor pathway that controls cell survival and proliferation. The former is targeted by the anti-VEGF monoclonal antibody, bevacizumab (Avastin™), and the latter is targeted by the anti-EGFR monoclonal antibodies, cetuximab or panitumumab[9,12]. Recently, there has been growing evidence that not only angiogenesis, but also lymphangiogenesis, the formation of new lymphatic vessels, is important in CRC metastatic progression[2,13-23]. This article will review the latest reports on lymphangiogenesis not only in experimental models, but also in clinical studies, and also review its clinical application as a biomarker and as a new targeted therapy.

WHY IS ANGIOGENESIS IMPORTANT FOR CANCER PROGRESSION AND METASTASIS?

In order for cancer to progress and metastasize, the primary tumor must have access to the systemic circulation, either through blood or lymphatic vessels. Angiogenesis is defined as the process whereby new blood vessels are formed from existing vessels, and as such, is a natural physiological process. Under normal physiologic conditions, angiogenesis only occurs in adults during menstruation, gestation and wound healing. At other times, anti-angiogenic factors maintain the endothelial cells that form blood vessels in a quiescent state[24]. The theory that angiogenesis could support tumor metastatic progression and therefore be a target for cancer therapy was proposed by Folkman *et al*[25,26] in the 1970s. He hypothesized that cancer requires angiogenesis to "feed" the cancer enabling it to grow beyond a certain size, and to allow for systemic spread. After 2 decades of developing this theory, modern molecular and cell biology techniques verified the role of angiogenesis in cancer growth *via* animal tumor models and clinical trials of bevacizumab, a humanized monoclonal antibody that neutralizes VEGF[27,28].

Tumors secrete multiple angiogenic factors and/or down-regulate angiogenesis inhibitors to induce tumor angiogenesis. VEGF-A is one of the key factors responsible for stimulation and maintenance of the disorganized, leaky, and torturous tumor vasculature. Other factors include members of the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor, angiopoietin, and hepatocyte growth factor (HGF) families[29]. Conversely, blockade of VEGF function inhibits angiogenesis and suppresses tumor growth *in vivo*[30]. While the discovery of factors important for angiogenesis has not yet led to a new cure for cancer, understanding that this process is essential for tumor metastasis has revealed several possibilities for targeted therapy. Applying this research approach to lymphangiogenesis can produce new potential targeted therapies.

WHY IS LYMPHANGIOGENESIS IMPORTANT FOR CANCER PROGRESSION AND METASTASIS?

The importance of lymph node metastasis in the progression of CRC has been well established and has a great impact on prognosis[8]. For cancer metastasis to the lymph node to occur, the cancer cells must access the lymphatic vessels to reach the regional lymph nodes. Applying approaches similar to the ones used to understand angiogenesis is expected to identify molecular mechanisms that control the processes related to lymphangiogenesis[31]. Therefore, understanding how lymphatic vessels are formed under physiologic and non-cancerous pathologic conditions can help provide an understanding of lymphangiogenesis in cancer in order to provide new avenues for targeted therapy development.

Although the ancient Greeks had already described aspects of the lymphatic system, the lymphatic vasculature was only properly considered to be a distinct circulatory system in 1622 by Asselli[32]. The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissues and returns it to the blood circulation *via* the thoracic duct. Lymphatic vessels are also known to be an essential part of the body's immune defense. Descrip-

tions of the metastatic spread of cancer can be found as far back as the 14th century[33], and the involvement of the lymphatic system in metastatic progression has been described since the 18th century[34]. The traditional theory was that tumor cells metastasized to lymph nodes by utilizing pre-existing lymphatic vessels, and that lymphatic vessel entry occurred by permeation or embolization, not through the creation of new lymphatic vessels in response to cancer. Although the regeneration of lymphatic vessels was observed by Clark and Clark in 1932, cancer metastasis and the concept of lymphangiogenesis were not linked until the last two decades[35]. Despite an acceptance for centuries of the important role of the lymphatic system as the primary pathway for the metastatic spread of tumor cells to regional lymph nodes, and possibly even also to distant organs, the exact mechanism of this process has remained unclear until recently[36].

Over the past few years, understanding of the cellular and molecular aspects of physiologic lymphangiogenesis and tumor-induced lymphangiogenesis has advanced after the discovery of VEGF-C and its function to promote the growth of lymphatic vessels[37]. Initially, the study of lymphangiogenesis largely focused on the primary site of tumor growth and adjacent tissues, which is known as "tumoral lymphangiogenesis"[38,39]. However, lymphangiogenesis was also observed around regional lymph nodes, in particular the sentinel nodes where tumor cells first metastasize, a phenomenon now known as "lymph node lymphangiogenesis"[6,40]. Lymph node lymphangiogenesis and increased lymph flow through tumor-draining lymph nodes are speculated to actively promote metastasis *via* the lymphatics[41]. Recent evidence indicates that tumor cells can also induce lymph node lymphangiogenesis - even before they metastasize - and that metastatic tumor cells continue to induce lymphatic vessel growth within sentinel lymph nodes, theoretically promoting their further metastatic dissemination[42,43].

As expected, the majority of studies point to a positive correlation between tumor-induced lymphangiogenesis and lymphatic metastasis[6,13-17,44,45]. Because the physiologic role of the lymphatic system is to collect interstitial fluid from peripheral tissues and return it to the systemic blood circulation, it is hypothesized that tumor-induced lymphangiogenesis occurs in order to drain interstitial fluid away from the tumor. Therefore, targeting this process provides a potential avenue for cancer therapy[46]. In fact, experimental inhibition of this process in animal models suggested that lymphangiogenic growth factors facilitate the metastatic spread of tumor cells *via* the lymphatics[14,18-20]. The results highlight the key role that lymphangiogenic growth factors and new lymphatic vessels play in tumor metastatic progression. These early studies indicate that targeting lymphangiogenic growth factors in tumors could be a strategy for restricting the metastatic spread of cancer[31].

IN VIVO AND *IN VITRO* MODELS OF LYMPHANGIOGENESIS

The recent discovery of the key lymphangiogenic factors

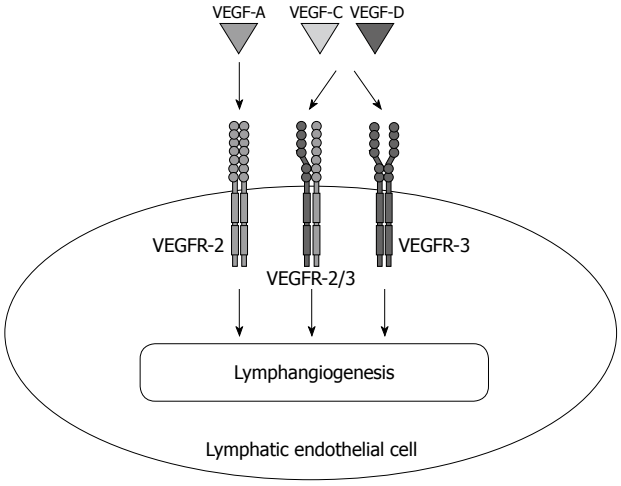

Figure 1 Lymphangiogenic growth factors and their receptors expressed by lymphatic endothelium. Vascular endothelial growth factor (VEGF) receptor (VEGFR)-3 is a member of the fms-like tyrosine kinase family and specifically binds VEGF-C and VEGF-D, but not VEGF-A. Recent studies also indicate an important role for the VEGF-A/VEGFR-2 signaling pathway in lymphangiogenesis.

VEGF-C and VEGF-D, other proteins related to these factors, and their receptor VEGF receptor (VEGFR)-3, have provided novel insights into how the lymphatic vessels and blood vessels coordinately grow and affect human disease[47]. In fact, these factors are associated with a number of human tumor types[31]. These secreted glycoproteins largely signal *via* the cell surface tyrosine kinase receptor VEGFR-3/Flt4 present on the surface of lymphatic endothelial cells (LECs), and VEGFR-3 activation promotes LEC proliferation, migration, and survival, which result in lymphatic vessel proliferation *in vitro* and *in vivo*[6]. Furthermore, recent studies indicate that the VEGF-A/VEGFR-2 signaling pathway plays a major role not only in angiogenesis, but also in lymphangiogenesis[48,49] (Figure 1).

In vitro techniques to study lymphangiogenesis have evolved with the development of methods to isolate and culture LECs. LECs have been isolated from lymphatic vessels or skin followed by enzymatic digestion and flow cytometric cell sorting using markers specific to LECs. Several LEC markers have been recently identified, including: lymphatic vessel endothelial hyaluronan receptor-1[50]; glomerular podocyte membrane mucoprotein, podoplanin (D2-40)[51]; the homeobox gene product, Prox-1[52,53]; and VEGFR-3[54,55]. Although these markers have aided in the purification of LECs, the limited quantity of cells obtained and the reduced growth potential of these cells have posed a challenge. To address the challenge of only having a limited quantity of cells after the purification of LECs, immortalization with SV40 large T antigen[56] or transformation with human telomerase reverse transcriptase[57] have been utilized to extend the life span of LECs, and transgenic mice have been developed to harvest immortalized LECs[58]. Protocols for the isolation of LECs from microlymphatic vessels in different tissues in rats have recently been established[59,60]. In most experimental assays, LECs are seeded as monolayers on culture plates or onto the surface of matrix-coated plates. While 2-D cul-

tures cannot undergo all of the steps of lymphatic vessel formation, these culture systems allow the analysis of each step individually, using various assays of cell activity (e.g. gene expression profiling), cell proliferation, apoptosis, adhesion, migration (wound scratch assay, Boyden chamber assay), and morphogenesis (tubulogenesis)[61].

Numerous *in vivo* models to study the growth of lymphatic vessels have utilized the same techniques as those used for blood vessel growth. The growth of vessels into the avascular cornea in response to specific factors or inflammation has been historically utilized as a model to study lymphangiogenesis[62-64]. Another extensively used model is the development of lymphedema and lymphangiomas. Lymphedema is swelling due to the failure of fluid drainage by the lymphatics which occurs as a result of obstruction or secondary changes impairing lymph flow. Several mouse models carrying mutations or chromosomal aberrations recapitulate this phenotype[65,66], and surgical ablation of lymphatic vessels can induce lymphedema and subsequently lymphangiogenesis[67,68]. Lymphangiomas, characterized as benign malformations of the lymphatic system, have been induced by injection of incomplete Freund's adjuvant, either into the mouse ear[69] or intraperitoneally[70], causing lymphatic vessel hyperplasia leading to inflammation and lymphangiogenesis[71].

Breast, gastric or CRC cells over-expressing VEGF-C implanted into transgenic mice induced tumor-associated lymphangiogenesis in orthotopic mouse models[13,72,73]. Skin carcinogenesis models in transgenic mice over-expressing VEGF-A or C showed that tumors in these mice were significantly more likely to metastasize[40,74]. As compared to VEGF-A, VEGF-C did not increase the size of the primary tumors, but induced the expansion of metastatic networks within the lymph nodes and promoted metastasis to distant sites such as distant lymph nodes and the lungs[74]. In addition to VEGF, PDGF-BB, FGF-2, HGF and angiopoietin enhance lymphangiogenesis[75-79]. Sphingosine-1-phosphate (S1P) also stimulates lymphangiogenesis in both *in vitro* and *in vivo* models[80,81]. S1P is generated by the action of two sphingosine kinases, sphingosine kinase 1 and 2[82,83]. Tumor cells, which are characterized by high levels of sphingosine kinase 1 expression, can release S1P into the extracellular space[84], which in turn can lead to paracrine-induced angiogenesis and lymphangiogenesis[81]. Interestingly, LEC-specific deletion of sphingosine kinase 1 in the sphingosine kinase 2 knockout mouse inhibits lymphatic vessel maturation[85].

In vivo models demonstrate that lymphangiogenesis promotes CRC metastasis, suggesting new avenues for the development of targeted therapy and prognostic markers. VEGF-C and VEGF-D, which are up-regulated in CRC, appear to drive tumor lymphangiogenesis through the VEGF-C/VEGF-D/VEGFR-3 pathway, while other growth factors, such as VEGF-A, have modulatory effects on this process[48,49]. VEGF-A expression levels significantly correlate with metastasis to the lymph nodes in CRC[48,49]. Orthotopic implantation of VEGF-C over-expressing DLDA colon cancer cells demonstrated that VEGF-C induced lymphangiogenesis-mediated tumor spread and the formation of metastatic disease in the lymph nodes[13], while the inhibition of VEGF-C expression reduced lymphangiogenesis, the extent of lymph node metastatic disease, and enhanced survival in mice[14]. The inhibition of VEGF-C expression also dramatically suppresses tumor lymphangiogenesis, tumor growth, and regional lymph node metastasis in mice[18]. Inhibition of VEGFR-3 using small interfering RNA also significantly inhibited tumor growth[19,20]. These *in vitro* and *in vivo* mouse data demonstrate the possible clinical application of lymphangiogenesis as a biomarker and/or as a new target for therapy in CRC in humans.

LYMPHANGIOGENESIS IN HUMAN SAMPLES

The role of intra- and peri-tumoral lymphatics in tumor biology and the initial steps of lymphatic metastatic progression, i.e. the invasion of tumor cells into the lymphatic vessels, are just beginning to be elucidated in human samples[44]. Animal studies have demonstrated that intra-tumoral lymphatic vessels are poorly functional due to high intra-tumoral pressure and may not be required for lymphatic metastatic progression. Conversely, lymphatic vessels in the tumor periphery are functional and can drain colloids from the tumor. In several common human tumors, such as cutaneous melanoma[86,87], head and neck squamous cell carcinoma[38,88], transitional cell carcinoma of the bladder[89,90] and non-small cell lung cancer[91], tumoral lymphangiogenesis detected by lymphatic vessel density (LVD) can be readily appreciated and has been shown to be of prognostic significance. In contrast, in breast[92,93], cervical and prostate carcinoma[94] tumors that metastasize to the lymph nodes, there is little evidence of significant tumoral lymphangiogenesis detected by LVD, with most proliferating vessels lying within the peritumoral tissues[6]. Another important factor to consider is the location of the tumor in relationship to the amount of pre-existing lymphatic vessels, such as in biliary cancer, which is very prone to metastasize *via* the lymphatic system[95,96]. Furthermore, mouse models of cecal cancer metastasis to the liver have demonstrated that both VEGF-C and VEGF-D produced less metastatic disease in the liver compared to primary cecal tumors, suggesting the importance of the tumor microenvironment for the production of these lymphangiogenic factors[97]. Taken together, the pattern of tumoral lymphangiogenesis and metastasis to the lymph node varies between tumor histological type and anatomic location of the tumor, involving both the lymphatic system and the microenvironment. Clearly, further studies are awaited to understand this complex process.

Although several studies have reported the discrepancy between LVD measurements and clinical outcome, it should be noted that there is a great deal of variability in their methodologies and consequently also in their results. In addition to tumor characteristics, the discrepancies in terms of the correlation of LVD with metastasis

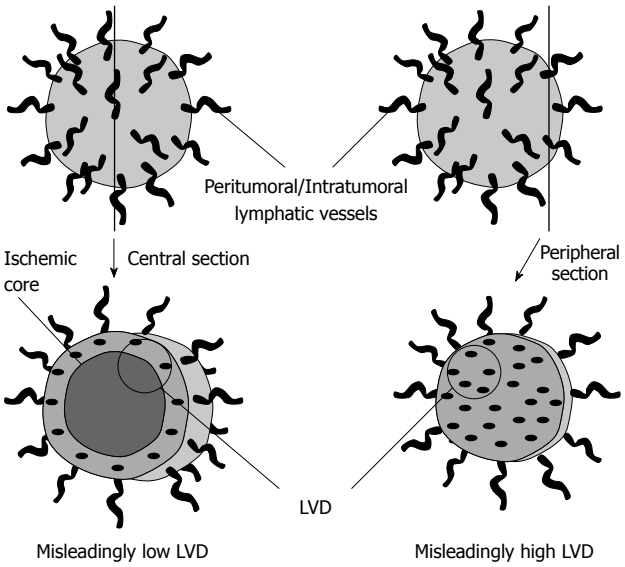

Figure 2 Limitations of lymphatic vessel density estimations. The central section of a tumor with a necrotic central core may estimate a misleadingly low lymphatic vessel density (LVD) (left), while the peripheral section of the same tumor may estimate a misleadingly high LVD (right).

to the lymph nodes and prognosis in these studies can be attributed to the limitations of the methodologies used. The limitations include the different types of tissue preservation, variable immunostaining techniques, different LVD quantification methods employed, and the lack of standardization in the estimation of lymphangiogenesis[98] (Figure 2). To address the limitations of this qualitative analysis methodology, some studies have attempted to quantify the expression of VEGF-C and VEGF-D mRNA or protein in excised primary tumor tissue of patients with various cancers. They frequently report that the levels of these molecules exhibit a strong correlation with parameters associated with poor patient outcome, such as the invasion of lymphatic vessels by tumor cells, the extent of lymph node metastatic disease, and disease-free as well as overall survival[31]. However, even these attempts to quantifiably measure lymphangiogenesis have not been entirely successful. In fact, recent epigenetic studies demonstrated that the analysis of mRNA or protein expression may not reflect actual lymphangiogenesis due to posttranscriptional modifications of proteins. In order to adequately assess the degree of lymphangiogenesis, a better method to accurately quantify the amount of lymphangiogenesis is needed.

LYMPHANGIOGENESIS AS A BIOMARKER FOR CRC PROGRESSION

Although animal models show a strong relationship between lymphangiogenesis and lymph node metastasis and survival, the clinical significance of lymphangiogenesis in CRC remains uncertain, as is the case for other tumors[6]. Parr *et al*[21] showed that the expression of VEGFR-3 receptor, prox-1, 5'-nucleotidase expression, and podoplanin expression in cancer tissue were significantly higher than in the normal background tissue. Jia *et al*[16] showed that the extent of lymph node metastatic disease in VEGF-C-positive patients (81.1%) was significantly higher than that in the negative group (42.1%). Lu *et al*[17] showed that quantitative analysis of podoplanin in CRC specimens correlates with metastasis to regional lymph nodes. Yan *et al*[22] showed that the co-accounting of LVD and microvessel density (MVD) was an independent prognostic factor in CRC. Moehler *et al*[15] showed that the expression of VEGF-D is significantly associated with lymphatic involvement in CRC patients and that cetuximab can block such expression effectively. In addition, the quantification of VEGF-C and VEGF-D in blood samples has the potential to serve as a biomarker to predict the extent of lymph node metastatic disease[23]. Interestingly, Sundlisaeter *et al*[99] showed that LVD was significantly increased in tumor tissue compared with the normal mucosa, but there were no changes in LVD between stage Ⅱ and Ⅲ CRC. This indicates that lymphangiogenesis occurs in CRC, and indeed suggests that it is triggered at an early stage of tumor development. Taken together, these studies suggest that these lymphangiogenesis-related markers indicate an increase in lymphangiogenesis in CRC, and might therefore have prognostic value for CRC patients.

However, other reports failed to find an association between higher LVD, the aggressiveness of tumor behavior and poorer clinicopathological variables. Kazama *et al*[100] revealed that the expression of VEGF-C was significantly correlated with lymphatic involvement, lymph node metastatic disease and tumor size, but not with venous involvement, metastasis to the liver in invasive carcinomas, or overall survival. Miyazaki *et al*[101] showed that an elevated level of plasma VEGF-C correlated with deeper invasion, and more severe venous and lymphatic invasion of the primary tumor, although there was no significant difference in the plasma level between patients with CRC and the healthy controls. Gao *et al*[98] showed that MVD and LVD were higher in the tumor compared with the corresponding normal mucosa, but they were not related to clinicopathological variables and overall survival. However, it should be noted that these studies rely on qualitative analysis methodologies, which are not objectively quantifiable assays. Duff *et al*[102] showed that the balance between the expression of VEGF-C and VEGF-D at the invading tumor edge may enhance lymphatic metastasis by promoting tumor lymphangiogenesis or by activating pre-existing lymphatic vessels. However, no relationship was identified between LVD and clinicopathological variables. Again, it should be noted that these reports rely on qualitative analysis methodologies, which are not objectively quantifiable assays. Taken together, lymphangiogenesis occurs in CRC development, but it has not been clearly linked to CRC patient prognosis. The conflicting reports in the literature regarding the possible correlation of LVD with clinical factors can be attributed to the use of qualitative analysis methodologies. Therefore, the development of a new quantifiable assay that uses standardized metrics is necessary.

In the future, the intra-tumoral expression of specific molecules, e.g. deleted in CRC[103,104] or 18q loss of heterozygosity[104], DNA microsatellite instability[103-105], KRAS mutation[103-105], or thymidylate synthase[103,104] could become biomarkers to predict prognosis or the response to therapy, independently of TNM stage group or histologic grade. It is now clear that there is an interaction between the T and N designations that is likely to rely on the expression of specific molecules within the cancer. In the latest edition of their cancer staging manual, the American Joint Committee on Cancer has stated that they will add molecular profiling information to the TNM classification to enhance the prediction of prognosis and/or even response to therapy[8]. Because lymphatic invasion and metastasis to the lymph nodes have a great impact on patient prognosis, lymphangiogenesis-related molecules are good candidates for the biomarkers that will be included in future editions of the TNM staging system.

LYMPHANGIOGENESIS AS A NEW THERAPEUTIC TARGET FOR CRC

Based upon the importance of angiogenesis and lymphangiogenesis in cancer progression, specific antibodies against angiogenic factors have been developed. The humanized VEGF antibody, known as bevacizumab (Avastin™), has been approved by the United States Food and Drug Administration (FDA) for treating metastatic carcinoma of the colon or rectum, and recurrent or metastatic non-squamous non-small cell lung cancer. Recently, bevacizumab also received accelerated FDA approval for the treatment of metastatic HER2-negative breast cancer[31]. However, the addition of bevacizumab to chemotherapy as adjuvant therapy in CRC did not improve disease-free survival[106]. Bevacizumab is being tested in other clinical settings such as adjuvant therapy, maintenance therapy, and in combination with both cytotoxic chemotherapy and other targeted agents, such as the epidermal growth factor receptor kinase inhibitor, erlotinib[106]. In addition to bevacizumab, other antibody-based therapies targeting the VEGF pathway are being tested. Ramucirumab and IMC-18F1 are monoclonal antibodies that target the VEGF receptors VEGFR-2 and VEGFR-1, respectively.

In addition to anti-angiogenesis therapies, many clinical trials in cancer patients are underway or have been completed with inhibitors that have the potential to suppress tumor-induced lymphangiogenesis. However, analysis of the effects of these treatments on tumor lymphatics is not always explicitly mentioned in the trial descriptions listed by the U.S. National Institutes of Health. There is only one study that has mentioned the role of VEGF-C in tumor progression, with VEGFR-3 being considered a target. This study is a Phase Ⅱ trial of sunitinib for patients with chemo-refractory metastatic gastric cancer[34]. It is hoped that more clinical trials will consistently address the possible effects of novel cancer therapeutics on tumor-induced lymphangiogenesis so that correlative data regarding the possible effects of interfering with tumor lymphatics on patient survival can be generated[34]. It is also important to note that treatment with the VEGFR tyrosine-kinase inhibitors sunitinib and sorafenib is associated with a significant increase in the risk of bleeding[107]. Further assessments need to be performed for treatment with these inhibitors.

Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth by using targeted therapeutic strategies against molecules involved in lymphangiogenic signaling, in addition to the inhibition of angiogenesis. Because VEGF-A has been shown to promote tumor lymphangiogenesis, and because VEGF-C and VEGF-D are also able to activate VEGFR-2, the combined inhibition of VEGFR-2 and VEGFR-3, or of VEGF-A and VEGF-C/D, may result in an even more potent blockade of tumor-induced lymphatic vessel growth. Indeed, a combination of both anti-VEGFR-2 and anti-VEGFR-3 blocking antibodies has been shown to be more efficient in reducing experimental lymph node and distant breast cancer metastatic disease than each antibody alone, and it will be of interest to see whether a recently developed biospecific antibody against VEGFR-2 and VEGFR-3 will also show enhanced activity *in vivo*[42]. On the other hand, several recent trials have shown that the addition of anti-EGFR monoclonal antibodies to bevacizumab and chemotherapy resulted in worse outcomes. This was surprising, given that preclinical and early clinical studies had suggested a benefit in combining anti-VEGF and anti-EGFR antibodies. Taken together, further clinical trials are required to reveal the efficacy of the combination of targeted therapies against lymphangiogenesis with other targeted therapies, and/or other anti-cancer therapies.

Which patients will benefit from anti-angiogenesis and anti-lymphangiogenic therapies? Considering that tumors appear to undergo angiogenesis and lymphangiogenesis at an early stage[99], the anti-lymphangiogenic effect may have an even greater impact on the micrometastatic and/or the in-transit metastatic disease of concern after the resection of early stage malignancies. Therefore, these anti-angiogenesis and anti-lymphangiogenesis therapies may be more effective in patients with early stage CRC. However, the role of adjuvant therapy in stage Ⅱ CRC is still controversial[108]. Although there is a cohort of stage Ⅱ CRC patients who will have recurrent disease even after complete resection, there are no markers to identify this cohort. This subgroup appears to be a good candidate for anti-angiogenesis and anti-lymphangiogenesis therapies, if they could be identified with the appropriate biomarkers. Lymphangiogenesis factors have the potential to be used as biomarkers to predict which patients would benefit from adjuvant therapy with anti-lymphangiogenesis therapies to both prevent recurrence and improve overall survival. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-angiogenesis and anti-lymphangiogenesis agents will hopefully result in better patient outcomes.

In addition to the development of the ideal combina-

tion therapies, the prevention of CRC is also essential to improve patient outcomes. Cancer chemoprevention is a strategy that uses treatments with natural or synthetic agents to inhibit, delay, or reverse the carcinogenesis process even before the development of invasive cancer[109,110]. The rationale for chemopreventive approaches to prevent CRC comes from epidemiologic and observational studies indicating that the long term ingestion of aspirin may reduce mortality in CRC[111]. Recent clinical trial studies demonstrated that celecoxib, a selective COX-2 inhibitor, is equally effective in reducing colorectal adenomas in animal models and patients with familial adenomatous polyposis (FAP), and it is approved by FDA for the chemoprevention of CRC in patients with FAP[112]. Prostaglandin E2 (PGE2) induced by COX-2 exerts several biological properties that may be advantageous for carcinogenesis, including promoting angiogenesis with increased VEGF, bFGF, and PDGF production[112]. Celecoxib enhances tumor cell apoptosis, thereby inhibiting the growth and angiogenesis of tumors by inhibiting COX-2, PGE2 synthesis, and VEGF expression in tumors in a mouse model of human CRC[113]. Interestingly, VEGF-C and COX-2 are coexpressed and are significantly associated with metastasis to the lymph nodes as well as prognosis in human CRC[114]. Moreover, celecoxib inhibits not only angiogenesis, but also lymphangiogenesis by blocking the VEGF pathway in mouse lung cancer models[115,116]. Taken together, lymphangiogenesis appears to play an important part in carcinogenesis in connection with the COX-2 pathway, and to be one of the important targets in chemoprevention, although the role of lymphangiogenesis in CRC within the adenoma-carcinoma sequence is still unknown. Further investigation will be required in this field.

CONCLUSION

Understanding the molecular pathways that regulate lymphangiogenesis is mandatory to pave the way for the development of new targeted therapies for cancer patients. A new quantifiable assay using standardized metrics is required to measure lymphangiogenesis and evaluate its impact on clinical outcome. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-lymphangiogenesis agents will hopefully improve patient outcomes. This progression to the clinic may be guided by new avenues of research such as the identification of biomarkers that predict response to treatment.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4013

World J Gastroenterol 2010 August 28; 16(32): 4013-4018
ISSN 1007-9327 (print)

TOPIC HIGHLIGHT

Marco Scarpa, Dr., PhD, *Series Editor*

Health related quality of life after surgery for colonic diverticular disease

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Received: May 1, 2010 Revised: June 1, 2010
Accepted: June 8, 2010
Published online: August 28, 2010

Abstract

Diverticular disease (DD) of the colon is very common in developed countries and is ranked the fifth most important gastrointestinal disease worldwide. The management of acute diverticulitis without perforation and peritonitis is still debated. Health related quality of life (HRQL), subjectively perceived by patients, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic disease. To date only a few published studies can be found on Medline examining HRQL in patients with DD. The aim of this study was to review the impact of surgery for DD on HRQL. All Medline articles regarding HRQL after surgery for colonic DD, particularly those comparing different surgical approaches, were reviewed. DD has a negative impact on HRQL with lower scores in bowel function and systemic symptoms. Both surgery-related complications and disease activity have a significant impact on patients' HRQL. While no significant differences in HRQL between different operations for DD in non-randomized studies were revealed, the only prospective double-blind randomized study that compared laparoscopic and open colectomy found that patients undergoing laparoscopic colectomy had significantly reduced major postoperative complication rates and subsequently had better HRQL scores. Formal assessment of HRQL could be a good instrument in the selection of appropriate patients for elective surgery as well as in the assessment of surgical outcome.

Key words: Colonic diverticular disease; Health related quality of life; Laparoscopy

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Angriman I, Scarpa M, Ruffolo C. Health related quality of life after surgery for colonic diverticular disease. *World J Gastroenterol* 2010; 16(32): 4013-4018 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4013.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4013

INTRODUCTION

Diverticular disease (DD) of the colon is very common in developed countries and is ranked the fifth most important gastrointestinal disease worldwide. It is probably even more common in the Western world with a prevalence of approximately 33% in persons over 60 years[1-3] placing a substantial burden on inpatient and outpatient resources[2]. In

fact, 10%-25% of these patients will suffer an acute attack with a further 30% developing complicated DD[3,4]. The frequency of perforation associated with DD is increasing, indeed it reached a prevalence of almost 4 cases per 100 000 in 2000[4]. Colonic resection is standard practice in cases of perforation and peritonitis in DD and primary anastomosis with defunctioning stoma seems to be the optimal surgical strategy for fit patients, while Hartmann's procedure is recommended in high risk patients[5].

The management of acute diverticulitis without perforation and peritonitis is still debated. In these cases, medical therapy is usually successful[1,6], but up to 25% of these patients may end up requiring an urgent operation[7], and more than half of these procedures involve a colostomy[8-10]. Elective colectomy is, thus, often recommended to avoid the risks and high mortality rate connected with emergency surgery usually associated with recurrent diverticulitis. The timing of elective surgery is, nevertheless, controversial with most advisory bodies recommending surgery after the second episode[2,11]. Many surgeons advise prophylactic colon resection after a single hospitalization in younger patients because the disease is considered more virulent in these subjects[11-15].

The evolution of laparoscopic colorectal surgery in the past decade has brought immediate short-term benefits to patients, including earlier postoperative pain relief and return of bowel function, shorter hospital stay, and better cosmesis. There is some evidence in the recent literature that immediate postoperative health related quality of life (HRQL) is better after certain laparoscopic procedures than after open surgery[16].

In the case of acute complications such as perforation with peritonitis in an emergency setting, the surgical approach can be in one stage or multiple stages and quality of life assessment is increasingly being recognized as an integral factor in surgical decision-making regarding disease management choices[17].

However, the results of one of our previous studies did not show any long-term advantage in terms of quality of life, symptoms recurrence and risk of surgery in submitting patients to colonic resection for DD without perforation and peritonitis[18]. The decision to electively operate on patients after their recovery from an acute episode of diverticulitis is still debated.

Although the use of objective outcome measures after surgical procedures is an important means of defining a patient's degree of health, the patient's subjective perceptions and expectations need to be factored into that objective assessment to determine the patient's actual quality of life, particularly in the treatment of benign disease such as DD. Moreover, HRQL measures have been shown to be useful in predicting health care expenditures. With the development of well-validated reliable and sensitive non-disease specific (generic) questionnaires such as the Short Form survey 36 (SF-36) or disease specific questionnaires like the gastrointestinal quality of life (GIQLI)[19-26], there is a HRQL measuring tool than can be applied to post-operative quality of life research not only to define the long-term outcome in elective operations, but may also be useful in comparing different surgical approaches and techniques. The aim of this study was to review the impact of surgery for DD on HRQL.

A text word literature review was performed using the PubMed and Medline databases. Although this was not a systematic review, the search terms used were as follows: DD, acute diverticulitis, surgery, elective OR emergency resection OR surgery AND HRQL. The reference lists of identified articles were searched for further relevant publications. Aided by a clinical librarian, the databases were consulted from January 1965 to April 2010. Two researchers (Angriman I and Ruffolo C) independently selected the studies, particularly those comparing different surgical approaches. Whenever there was discordance regarding study inclusion the two researchers negotiated an agreement.

DD HAS AN IMPACT ON QUALITY OF LIFE

In the majority of patients, colonic diverticula (diverticulosis) are asymptomatic[27]; nevertheless, an estimated 20% of affected individuals develop symptoms in their lifetime, such as abdominal pain and/or discomfort, bloating and disturbance of bowel habits. This clinical condition is termed DD[28] and may be symptomatic uncomplicated, recurrent symptomatic, or complicated. DD treatment is aimed at relieving symptoms and preventing major complications. HRQL, subjectively perceived by patients, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic disease where the aim of therapy is to keep patients either symptom-free or to reduce the discomfort caused by the disease. To date only a few published studies can be found on Medline examining HRQL in patients with DD.

Bolster *et al*[29] suggested that DD does affect a person's HRQL. In their study they used a disease specific questionnaire which had been validated for patients with inflammatory bowel disease and consisted of 32 questions which assessed four aspects of patients' lives: gastrointestinal symptoms, systemic symptoms, emotional function and social function. Patients with DD had mean scores well below the optimal scores of the questionnaire in all four categories, and compared with a control group of healthy volunteers, patients with DD had statistically significant lower scores in all categories. The authors concluded that DD has a negative impact on HRQL.

Similar results were observed by Comparato *et al*[30] in 58 patients affected by uncomplicated symptomatic DD. They used the SF-36 questionnaire and clinical evaluation was registered by means of global symptomatic score at baseline and after 6 mo. They concluded that DD has a negative impact on HRQL compared with the normal population and medical therapy improves HRQL if symptoms are relieved.

QUALITY OF LIFE AFTER SURGERY FOR DD

Surgical resection for DD, outside of the emergency setting, is primarily intended to obviate future hospitalization and/or emergency surgery for the patient, even though the risk of any single individual suffering a subsequent acute exacerbation is unpredictable. In contrast, symptomatic DD itself causes considerable ongoing disruption in terms of lifestyle and general "well-being". However, little formal weight is currently given to quality of life consideration *per se*. This is probably because it remains unclear whether surgery can restore the missing HRQL, but outcome may instead represent the most compelling reason to offer surgery to an individual.

Several studies observed a significant improvement in quality of life and social function following elective sigmoid resection in the majority of patients.

Most of those studies compared the HRQL before and after surgery. Forgione *et al*[16] performed a prospective analysis of 46 patients undergoing elective, laparoscopic sigmoidectomy for prior acute diverticulitis demonstrated by CT scans over an 18-mo period. Preoperative measures of HRQL were assessed by the GIQLI questionnaire administered at baseline and then again regularly throughout the first postoperative year. Mean preoperative GIQLI score was 99.5 and postoperative scores were significantly higher at each postoperative time point. The mean GIQLI score 12 mo after operation was 111.5 ($P < 0.05$). Postoperative augmentation of GIQLI was correlated most with improvements in the symptoms domain and was inversely correlated with the preoperative score. The authors concluded that the development of a more disease-specific questionnaire for patients being considered for elective surgery after prior diverticulitis may allow even greater discrimination in preoperative selection.

Roblick *et al*[31] observed a significantly higher HRQL after surgery in patients suffering from DD and only slightly below a validated normal population. In this study, a total of 45 patients who underwent surgery for diverticulitis at stage Ⅰ-Ⅱa (Hinchey classification) were included. HRQL was measured using the SF-36 questionnaire and the follow-up period was at least 2 years.

In one of our previous studies[18], to evaluate the impact of colonic resection for DD on the natural history and long-term quality of life in these patients, HRQL of DD patients undergoing surgery was compared to those on medical treatment. The study was particularly focused on the long-term clinical outcome of non-complicated diverticulitis. HRQL was also assessed in 69 healthy subjects [39 males and 30 females with a mean age of 43 (22-85) years] without gastroenteric symptoms enlisted from hospital employees and their relatives.

For HRQL assessment, the Cleveland Global Quality of Life (CGQL) score[32], which consists of three items (current quality of life, current quality of health, and current energy level), each on a scale of 0 to 10 (0, worst; 10, best), was used. The CGQL was created to assess HRQL in patients affected by ulcerative colitis after restorative proctocolectomy and was then used in HRQL analysis of patients with Crohn's disease[33,34]. No significant differences were observed in the rate and in the timing of readmission and surgical procedures for DD in the two groups. The CGQL total score as well as the two items on current quality of life and current energy level responses were similar in the two groups of patients and in the group of healthy subjects. Only the scoring on the current quality of health was significantly worse in patients who had undergone colonic resection. Similarly, in the Hinchey 1 subgroup, no significant differences in CGQL score, current quality of health, current quality of life and current energy level were observed in patients who had been operated on and those who had been treated conservatively. Those results indicated that there are no long-term advantages to colonic resection for DD if the goal of the surgical treatment is to improve HRQL, and these data seemed to be supported by the analysis of the small group of Hinchey stage 1 patients.

QUALITY OF LIFE AFTER OPEN AND LAPAROSCOPIC SIGMOID COLECTOMY

Long-term outcome and HRQL were evaluated in patients undergoing laparoscopic colectomy (LC) *vs* open colectomy (OC) for benign colorectal diseases, based on standardized, validated measures, in a retrospective study[35]. All consecutive patients who underwent elective LC for uncomplicated diverticulitis in an 8-year time period were evaluated and compared to controls treated with conventional OC in the same period. HRQL was assessed by the SF-36 Physical and Mental Component Summary Scale (PCS; MCS) and by the SF-36 Health Survey. None of the 8 SF-36 Health Survey domains or the PCS and the MCS showed significant differences in HRQL between LC patients and OC patients. The occurrence of postoperative incisional hernias and bowel obstructions, which represented the only surgery-associated long-term complications, was comparable in both groups, as was the patients' HRQL. The two limitations of this study were the small patient cohort and the short follow-up (6-9 mo postoperatively with no long-term available). The lack of statistical difference between LC and OC patients in SF-36 scores related to the development of long-term complications may have been due to these limitations. Surgery-related complications were the only events that had a significant impact on the patients' HRQL, reflecting lower SF-36 scores in certain areas. The laparoscopic colorectal surgery itself had no significant influence on the patients' HRQL in the follow-up of these patients. The authors observed that better immediate postoperative HRQL after laparoscopic procedures may have been related to treatment of the disease *per se* and to favourable parameters such as faster convalescence, shorter hospital stay, and better cosmesis. On the other hand, long-term HRQL seemed to be influenced by chronic sequelae of the surgical procedures. Interestingly, more favourable cosmesis

itself had no impact on either the patients' overall HRQL or the emotional and social domains of the SF-36.

Another comparison of long-term HRQL between patients undergoing LC and OC was performed by Seitz *et al*[36] using the GIQLI questionnaire. Fifty-four patients who underwent sigmoid colectomy for recurrent diverticulitis were included. Patients who underwent LC seemed to feel better after surgery compared with those undergoing OC; however, this trend did not reach statistical significance. Patients' satisfaction regarding the cosmetic result was significantly higher in those undergoing LC than patients treated with OC. All patients had a similar GIQLI postoperatively, independent of the type of surgery. Eypasch's GIQLI did not identify clear differences between LC and OC. The limitation of this study was that only the *status quo* was determined and a comparison before and after surgery was not performed. The missing difference between LC and OC may be secondary to the higher rate of persistent symptoms in the LC group compared with the OC group, which was determined by simply asking the patients whether they felt that the disease had recurred. Thus, the authors commented that possible long-term advantages after LC with regard to HRQL may have been lost owing to this difference in "subjective recurrence".

Recently, a prospective, multicentre, double-blind, randomized controlled trial was designed to compare the impact of LC and OC on postoperative complication rates in patients with symptomatic diverticulitis[37]. Quality of life was assessed using the SF-36 questionnaire measured preoperatively and 6 wk after surgery. One hundred and four consecutive patients who underwent elective surgery for symptomatic diverticulitis of the sigmoid colon were randomized in 5 centres. Fifty-two LC patients were compared to 52 OC patients for gender, age, body mass index, American Society of Anesthesiology classification, prevalence of comorbidities, previous abdominal surgery, preoperative workup, and indication for surgery. SF-36 data showed no preoperative intergroup differences. Postoperative SF-36 data were significantly better in LC patients for role limitations due to physical and emotional problems, social functioning, and pain level. The main finding of this randomized controlled trial was that LC patients had significantly reduced major postoperative complication rates as compared with OC patients for symptomatic diverticulitis. Subjectively, patients who underwent LC scored significantly better than OC patients on the Visual Analogue Scale (VAS) for quality of life-pain score and SF-36 questionnaire. Several items in the latter showed improved role limitations due to physical health, role limitations due to emotional problems, social functioning, and pain.

A multicentre study compared HRQL in patients affected by DD submitted to LC *vs* those who underwent OC during long term follow up, using the Padova Inflammatory Bowel Disease Quality of Life (PIBDQL) score, CGQL score, VAS, Body Image Questionnaires (BIQ) and cosmetic score (CS), ad hoc DD symptom score (DDSS) and Bristol Stool Form Scale[38]. Sixty consecutive patients were included: 20 underwent LC, 15 OC and 25 had only medical therapy. The PIBDQL[35,39,40] was validated for use in patients with DD. Preliminary results showed that the PIBDQL scores were significantly worse in all patients with DD than those obtained from healthy subjects and correlated with the symptoms score. The CGQL was similar in patients who had LC compared to healthy subjects. BIQ scores correlated inversely with the presence of a stoma. The intestinal symptoms item was worse in patients who had LC than in those who had OC. On multivariate regression, the DDSS score was the only independent predictor of the PIBDQL score. No significant difference was observed in VAS for quality of life among the three groups of patients and in total CS in the two groups submitted to surgery. Only the scar score item was significantly better in patients who underwent LC compared to that in patients who had OC. Similarly, no significant difference was observed in the BIQ items and total score in the two groups submitted to surgery. The authors concluded that disease activity is the only independent predictor of the disease-specific quality of life.

QUALITY OF LIFE AFTER STAGED RESECTION FOR COMPLICATED DD

Constantinides *et al*[17] assessed long-term differences in HRQL using the SF-36 questionnaire between single and staged resections, in complicated DD. The authors divided the study population into two groups: one group consisting of patients who underwent primary colonic resection and anastomosis and the other group who underwent staged resections followed by restoration of intestinal continuity (HP). Three subgroups were created for each of the single staged and staged resection groups on the basis of when HRQL was assessed (1 group less than 3 years after primary surgery, 1 group 3-6 years after primary surgery and 1 group more than 6 years after primary surgery). One hundred and fifty-eight patients who underwent single staged resections and 30 patients who underwent staged resections with restoration of intestinal continuity were included. Significant differences were observed between the two groups in patients suffering from major comorbidities. No statistically significant differences were found in any of the eight domains between the single and staged resection groups. No significant differences were found between the two surgical methods across any of the eight SF-36 domains, for any of the time periods. The PF and RP domains were both subject to a significant decrease in mean score with advancing age. The BP domain had a progressive, but not statistically significant, decrease in score with advancing age.

Patients with no postoperative complications had significantly higher scores in the PF domain, the RP domain and the BP domain. According to these authors, in the setting of complicated DD, long-term HRQL tends to be similar between surgical interventions but remains significantly lower in selected domains when compared to the general population norms.

The main limitations of this study were that: (1) the two groups were inhomogeneous in terms of patient comorbidities and therefore, the effect of comorbidity on HRQL as a confounder could not be eliminated; and (2) no patient had a residual stoma and as a result, the study did not assess the impact of a colostomy on quality of life. Furthermore, HRQL was not assessed in the preoperative state.

CONCLUSION

The conclusions that can be drawn from these different studies are that DD has a negative impact on HRQL with lower scores in bowel function and systemic symptoms. Currently, the criteria for selection of DD patients for elective surgery remain debatable. Formal assessment of HRQL could be a good instrument in the selection of appropriate patients for elective surgery as well as in the assessment of surgical outcome. Both surgery-related complications and disease activity have a significant impact on patients' HRQL. While no significant differences in HRQL between different operations for DD in the non-randomized studies were revealed, the only prospective double-blind randomized study that compared LC and OC found that patients undergoing LC had significantly reduced major postoperative complication rates and subsequently had better HRQL scores.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4019

World J Gastroenterol 2010 August 28; 16(32): 4019-4030
ISSN 1007-9327 (print)

REVIEW

Gene therapy for liver regeneration: Experimental studies and prospects for clinical trials

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Received: December 30, 2009 Revised: March 3, 2010
Accepted: March 10, 2010
Published online: August 28, 2010

Abstract

The liver is an exceptional organ, not only because of its unique anatomical and physiological characteristics, but also because of its unlimited regenerative capacity. Unfolding of the molecular mechanisms that govern liver regeneration has allowed researchers to exploit them to augment liver regeneration. Dramatic progress in the field, however, was made by the introduction of the powerful tool of gene therapy. Transfer of genetic materials, such as hepatocyte growth factor, using both viral and non-viral vectors has proved to be successful in augmenting liver regeneration in various animal models. For future clinical studies, ongoing research aims at eliminating toxicity of viral vectors and increasing transduction efficiency of non-viral vectors, which are the main drawbacks of these systems. Another goal of current research is to develop gene therapy that targets specific liver cells using receptors that are unique to and highly expressed by different liver cell types. The outcome of such investigations will, undoubtedly, pave the way for future successful clinical trials.

Key words: Liver regeneration; Gene therapy; Genetic vectors; Growth factors

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Atta HM. Gene therapy for liver regeneration: Experimental studies and prospects for clinical trials. *World J Gastroenterol* 2010; 16(32): 4019-4030 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4019.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4019

INTRODUCTION

The need to enhance the capacity of liver regeneration has long been recognized but only avidly pursued recently. The clinically successful massive liver resection or small-for-size liver transplant carry the risk of liver failure due to impaired regeneration of the remnant/split liver[1]. Liver regeneration is also an integral component of the recovery processes of liver cirrhosis, fibrosis or liver failure[2]. Major advances in understanding the molecular mechanisms that govern liver regeneration have been made over the past few years[3,4]. Identification and molecular characterization of specific growth factors that promote liver regeneration has allowed the development of recombinant growth factors and their use to promote liver regeneration. The success of this strategy was hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously[5-9]. To overcome this problem, investigators successfully used gene transfer technology to transfer the genes that encode these growth factors. The intrinsic production of growth factor proteins following the transfer of their encoding genes enhances liver proliferation in various animal models with partial hepatectomy and/or chemical injury. Despite the success of proof of principle studies of gene therapy to enhance liver regeneration, and the potential of translation into clinical settings, no systematic review of published studies has appeared so far. Therefore, an evaluation of the current literature on gene therapy for liver regeneration is required and a look at future perspectives is

warranted. This article briefly summarizes the current key concepts in liver regeneration and gene therapy as they are related to each other, gives an overview of the published studies, and envisions future progress in the field.

LIVER REGENERATION: BASIC CONSIDERATIONS

Following two-thirds partial hepatectomy, the residual liver lobes enlarge within a week to make up for the mass of the removed lobes. Liver regeneration is carried out by proliferation of all adult liver cells including hepatocytes, sinusoidal endothelial cells, biliary epithelial cells, Kupffer cells and hepatic stellate cells (HSCs)[10]. It has been firmly established that mature hepatocytes are not terminally differentiated and that they have an almost unlimited capacity to proliferate, so that the liver can be entirely repopulated by intact hepatocytes that represent 1% of the hepatocyte population[10-15].

The molecular mechanisms of liver regeneration can be divided into two critical steps: the transition of the quiescent G_0 phase hepatocyte into the cell cycle (priming phase), and progression beyond the restriction point in the G_1 phase of the cycle (progression phase). These phases are under separate control; priming by the cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6), and cell cycle progression by the growth factors hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α[11]. The priming phase does not lead to DNA replication unless the cells can progress through the cell cycle which is accomplished by growth factors. Once hepatocytes pass the G_1 restriction point they are irreversibly committed to replication (Figure 1)[16,17].

The mechanisms that initiate cytokine cascade liver regeneration have not yet been fully identified. It has been proposed that liver injury causes the release of reactive oxygen species and lipopolysaccharide (LPS), which trigger the activation of the complement system. After complement activation, cleavage of C3 or C5 leads to generation of the potent anaphylatoxins C3a and C5a. LPS, C3a and C5a in turn activate the non-parenchymal cells (NPCs) such as Kupffer cells, through the cell surface receptor TLR4 and C3aR and C5aR, which causes activation of the transcription factor nuclear factor (NF)-κB signaling pathway and the production of cytokines such as TNF-α and IL-6[18,19]. Also, the cytokine cascade can be triggered through the binding of TNF to its receptor TNFR1, which leads to activation of the NF-κB in NPCs, with the production of TNF and IL-6. Thus, the released TNF acts on the same NPCs in an autocrine fashion and on hepatocytes by a paracrine mechanism. Released IL-6 binds to its receptor on hepatocytes and leads to activation of the transcription factor STAT3 (signal transduction and activator of transcription), which translocates to the nucleus where it induces transcription of a number of target genes (Figure 2). The precise role played by each cytokines is, however, debatable[3,11]. TNF is not a direct mitogen for hepatocytes. It does, however, enhance the mitogenic effects of direct mitogens such as HGF. For example, it has been shown in stellate cells in culture that TNF and IL-6 activate the transcription factor C/EBPβ (CCAAT/enhancer-binding protein β), which induces HGF mRNA expression[20]. TNF is also involved in the activation of TGF-α[4]. IL-6 has both mitogenic and anti-apoptotic effects on hepatocytes and protects the regenerating liver against ischemic injury[11]. IL-6 has a crucial role in initiating acute phase response in hepatocytes, with the production of many proteins that assist in controlling acute or chronic inflammation[21].

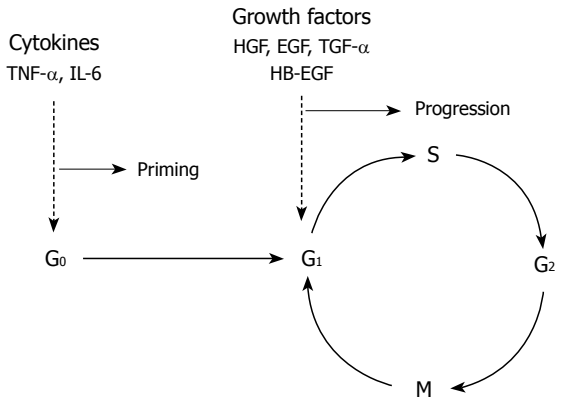

Figure 1 Effect of cytokines and growth factors on hepatocyte cell cycle. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF-α: Transforming growth factor-α; HB-EGF: Heparin-binding EGF-like growth factor.

While cytokines are responsible for the passage of quiescent hepatocytes into the cell cycle (G_0 to G_1), cell cycle progression is then driven by growth factors, which override a restriction point in the late G_1 phase[3]. HGF and ligands of epidermal growth factor receptor (EGFR) are important growth factors that drive cell cycle progression during liver regeneration. Studies have shown that despite the expression of many mitogenic receptors, including receptors for platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), the only mitogens for hepatocytes are HGF and ligands of EGFR. The family of ligands that bind EGFR, in addition to EGF, includes TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR)[3,22]. Stimulation of the tyrosine kinase receptors for HGF and the EGF ligands activates numerous intracellular signaling pathways that regulate transcription factors involved in liver regeneration[3,4]. It is important to mention, with the possible exception of HGF, that complete elimination of a single growth factor does not entirely abrogate liver regeneration.

HGF is the most extensively investigated growth factor for liver regeneration. It stimulates regeneration in normal and injured liver. It is produced by NPCs and stimulates hepatocytes by a paracrine or endocrine mechanism. Following binding to its receptor, cMet, on hepatocytes, it stimulates DNA synthesis. HGF effects are multiple including mitogenic, motogenic, morphogenic and anti-apoptotic effects[4,11,17,23].

EGFR ligands are direct mitogens for hepatocytes. EGF is continually available to the liver through the portal

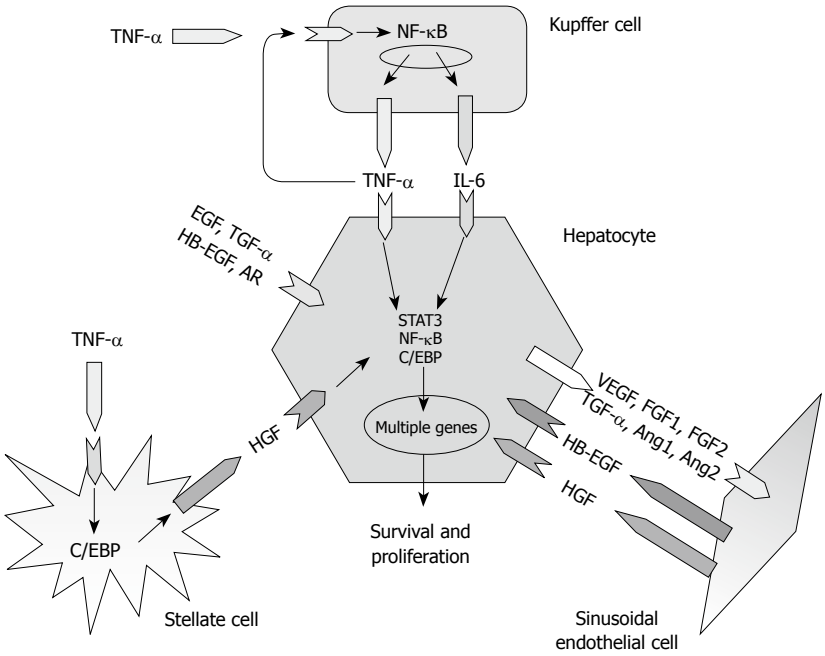

Figure 2 Major cytokine and growth factor signals during liver regeneration. NF-κB: Nuclear factor-κB; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; EGF: Epidermal growth factor; TGF-α: Transforming growth factor α; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; HB-EGF: Heparin-binding EGF-like growth factor; AR: Amphiregulin; C/EBP: CCAAT/enhancer-binding protein; HGF: Hepatocyte growth factor.

vein, and is produced from Brunner's glands of the duodenum. EGF given to intact animals causes hepatocyte proliferation. TGF-α is an autocrine growth factor that is produced by and active on hepatocytes. Transgenic mice that overexpress TGF-α display hepatocyte proliferation and develop tumors[24]. On the other hand, TGF-α knockout mice have no defects in liver regeneration; probably because of the overlap between various ligands of the EGF family. TGF-α is also a mitogen for endothelial cells and bile duct epithelial cells. HB-EGF is produced by endothelial and Kupffer cells and is a key factor for hepatocyte progression through G_1/S transition during liver regeneration[25]. AR also contributes to liver regeneration, because mice deficient in AR have deficient liver regeneration[26]. It is likely that the different growth factors have independent but partially overlapping functions in liver regeneration[3,4].

Cytokine and growth factor pathways interact during different phases of liver regeneration[3,27,28]. For example, TNF activates TGF converting enzyme (TACE) that results in release of TGF-α, activation of EGFR and hepatocyte proliferation[3]. It should be noted at this point that there is significant redundancy between the components of each pathway, such that the lack of a single component generally causes a delay and/or reduction of regeneration. In other words, loss of an individual component gene rarely leads to complete inhibition of liver regeneration[3,23]. In contrast to the large number of hepatocyte growth promoters, very few inhibitors of liver regeneration have been identified. The most potent of these inhibitors is TGF-β[22]. For a more detailed review on molecular mechanisms of liver regeneration, readers should refer to references[1,3,4,23,29-33].

The molecular events involved in liver regeneration are significantly influenced by the extent of resection, as massive (85%-90%) liver resection leads to suppression and delay of liver regeneration, compared to 70% partial hepatectomy (PH), because of suppressed and delayed induction of the regenerative genes TNF-α and IL-6 after 90% PH. Moreover, apoptosis rates are also elevated in 90% PH compared to 70% PH[34]. Several studies have shown that growth factors that promote liver regeneration (HGF and TGF-α) are upregulated in 70% PH, whereas no or only reduced induction occurs after 90% resection. These findings suggest that expression of the factors relevant to the regeneration of liver tissue is influenced by the extent of resection[17,34-36].

A simplified summary of the interactions between cytokines and growth factors and between different cell types during liver regeneration is shown in Figure 2. Hepatocytes are the first to undergo proliferation, based on external stimuli from a variety of sources. HGF is rapidly becoming available to hepatocytes very rapidly through local matrix release and activation induced by urokinase-type plasminogen activator. Stellate and endothelial cells are sources of new HGF, which is synthesized after 3 h following PH. Hepatocytes produce growth factors that are mitogenic for stellate cells (PDGF)[37] and for endothelial cells (VEGF, FGF1, FGF2, stem cell factor, angiopoietins 1 and 2, and TGF-α). Proliferation of endothelial cells aims to restore the network of sinusoids that occurs over a long period of time, from days 3 to 6 after PH. Kupffer cells have not been clearly proven to proliferate during regeneration; however, they do produce TNF and IL-6, which appear to have a contributory role in STAT3 and NF-κB activation during the early stages of liver regeneration. Of note, the original hepatocyte mass is not restored through proliferation of stem cells, but through replication of residual mature hepatocytes. Hepatic stem cells (oval cells) are mobilized and differentiate into hepatocytes, only when proliferation of hepatocytes is totally blocked or when hepatocytes are chronically destroyed[10,23,33,38,39].

Identification and molecular characterization of spe-

Table 1 General characteristics of most commonly used vectors

System	Size of insert (kb)	Infect non-dividing cell	Genomic integration	Duration of expression	Immune response
Adenovirus					
1st generation	5	Yes	No	3-4 wk	High
2nd generation	8	Yes	No	Longer with	High
Gutless	35	Yes	No	Immuno-supression	Less
Adeno-associated virus	< 4.8	Yes	Yes and episomal	Long-term	Low
Herpes simplex virus 1	35	Yes	No	Long	High
Retrovirus	≤ 8	No	Yes, random	Long-term	Low
Lentivirus	≤ 8	Yes	Yes, into active genes	Long-term	Low
Baculovirus	> 20	Yes	No	Transient	Low
Plasmid-naked	Large	Yes	No	Short	Low
Plasmid-polymer	Large	Yes	No	Short	Low
Plasmid-lipid (liposomes)	Large	Yes	No	Short	Low

cific growth factors that promote liver regeneration allow the development of recombinant growth factors and their use to promote liver regeneration[6,40-43]. The success of this strategy is hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously. To overcome this problem, investigators have successfully used gene transfer technology to transfer the genes that encode these growth factors into liver cells.

GENE THERAPY FOR LIVER REGENERATION: KEY CONCEPTS

The strategy of introducing genetic material into liver cells to enhance proliferation or to inhibit apoptosis has been employed in experimental liver research for more than a decade. The transferred genetic material can be a natural gene[44-46], gene segment[47], chimeric gene[48], oligodeoxynucleotides (ODN)[49,50], or siRNAs. To facilitate transfer (transduction) into cells, the foreign gene (transgene) is packaged into construct named vectors. Gene transfer vectors are classified as either viral or non-viral. Viral vectors provide a powerful means for delivering therapeutic genes to targeted cells due to their high transduction efficiency. They are made replication-defective by deletion of viral genes involved in the replication and pathogenesis of the virus. This allows for the inclusion of non-viral genetic material in the viral genome. The general characteristics of most commonly used vectors are shown in Table 1. The most commonly used viral vectors are retrovirus, adenovirus, adeno-associated virus (AAV), herpes simplex virus, lentivirus and baculovirus. For a gene to be expressed inside a cell, its coding DNA sequence should be linked to an appropriate promoter. These regulatory DNA sequences can be categorized as viral (universal) promoters, which allow transgene expression in most transduced cells, housekeeping promoters, or tissue-specific promoters, which drive gene transcription only in selected cell types[51]. Because of their universal activity, viral promoters were components of many first-generation vectors. However, many of the viral promoters, such as the cytomegalovirus (CMV) promoter, are attenuated or completely shut-off in organs such as the liver. In comparison to viral or housekeeping promoters, tissue- or liver-specific promoters direct higher levels of expression *in vivo*. Successful application of gene therapy depends on the choice of relevant therapeutic genes, appropriate promoters, and effective vectors that allow an adequate level and duration of transgene expression[52-54].

Although retroviral vector transfection results in long-term survival of the gene in the transduced cell, its major disadvantage is the risk of insertional mutagenesis as a result of random integration of the virus into the host chromosome. Moreover, the transduction rate after retroviral gene transfer into hepatocytes *in vivo* is disappointingly low. Efficient retrovirus integration into the host-cell genome requires the active proliferation of target cells with DNA replication and nuclear membrane breakdown during mitosis. Under normal physiological conditions at any given time, only 0.005% of hepatocytes divide. For retrovirus liver transduction, hepatocyte proliferation induced by PH must occur on or about the time of retroviral delivery. To increase gene transfer without hepatectomy, mouse hepatocytes have been transduced *in vivo* with a recombinant adenovirus that transiently expressed urokinase[55], or with recombinant HGF[56]. The induced liver regeneration allowed persistent and efficient retroviral-mediated gene transfer in hepatocytes[55,56].

Adenoviral vectors are the most investigated vectors in animal and human gene therapy studies. Adenoviral vectors exhibit several merits that make them suitable for liver regeneration gene therapy. Adenoviruses are highly hepatotropic and it is relatively easy to produce high titers of recombinant adenoviral particles[57]. Unlike retroviruses, adenoviruses transduce dividing and non-dividing cells and do not integrate into the host chromosomes, thereby eliminating the risk of insertional mutagenesis. These merits make adenoviral vectors suitable for proof of principle experimental studies to verify the effect of overexpression of a specific growth factor gene on liver regeneration. The major limitation of adenoviral vectors is their serious and potentially fatal toxicity as exemplified by the death of an 18-year-old man who received 6×10^{11} viral particles/kg of E1/E4-deleted human adenovirus type 5 vector that contained human ornithine transcarbamylase cDNA[58,59]. Moreover, the severe immune response of the host contributes to the limited survival of the adenovirus

DNA in targeted cells and results in transient expression of the therapeutic gene. Until resolved, adenoviral-vector-induced toxicity will limit its application in clinical gene therapy studies. The transient nature of gene expression with adenoviral vectors may be advantageous because the process of liver regeneration is usually completed in approximately 1 wk. However, liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis or cirrhosis requires a longer period of gene expression. Furthermore, transduction efficiency of diseased liver is much lower than that of healthy liver. Garcia-Bañuelos *et al*[60] have demonstrated that adenovirus-mediated gene transfer *via* the iliac vein at 3×10^{11} viral particles per rat resulted in approximate 40% transduction in livers made cirrhotic by chronic intoxication with carbon tetrachloride, compared with approximate 80% in control non-cirrhotic livers. In rats made cirrhotic by bile-duct obstruction only, 10% efficiency of transduction was observed. Yu *et al*[61] have shown that NPCs are transduced with greater frequency than hepatocytes at all adenoviral titers tested, both *in vitro* and *in vivo*. After liver injury, adenoviral transduction is reduced for all liver cell types compared with that for cells from normal livers (at all virus titers). Again, transduction efficiency remains greater in NPCs than in hepatocytes after liver injury.

Non-viral vectors can be divided into two categories: physical and chemical. Physical methods involve the introduction of plasmid DNA into cells using electroporation, ultrasound, or hydrodynamic delivery. Chemical methods use lipid or polymer carriers that complex with DNA to deliver the transgene into cells[62,63]. Several non-viral vectors have been used for *in vivo* liver gene therapy including various liposome preparations, protein-DNA conjugates, nanoparticles, and naked or complexed DNA[57,64,65]. Expression is usually both transient and at low level because the DNA is not stable in cells. Despite these limitations, non-viral vectors offer many advantages including being simple to use, ease of production of large quantities, and absence of host immune response.

A major advance in the intravascular delivery of vectors followed the development of the hydrodynamic injection technique. The technique involves rapid tail vein injection of a large volume of the vector (around 10% of the body weight of a mouse or rat) in a short time period (5-7 s in mice and 15-20 s in rats). The hydrodynamic method results in dramatically higher hepatic transfection efficiency compared to conventional injection. Typically, 10%-15% of hepatocytes are transfected in mouse liver following injection of 10 μg plasmid, but levels up to 40% have been reported[66]. Liver enzymes are transiently elevated and liver histology shows minimal damage that resolves within a week, which is similar to the results obtained from intravascular delivery into liver vessels[66,67]. It has been postulated that increased pressure in the inferior vena cava causes retrovenous blood flow from the central to the portal vein, and the resultant increased intrahepatic vascular pressure promotes massive endocytosis that generates intracellular water movement that facilitates gene entry[68,69]. There are multiple lines of evidence that the species differences in the diameter of sinusoidal fenestrae are a critical determinant of transgene expression after adenoviral transfer. The small diameter of fenestrae in humans should be considered in any rational design of gene transfer technology for hepatocyte-directed transfer. Hydrodynamic gene transfer is highly successful in rodents. The significantly lower efficacy in higher species may also partially be due to species differences in liver architecture[70]. Intrinsic factors, in particular compliance (elasticity) of the liver are likely to be crucial in determining the degree of swelling for a given level of intrahepatic vascular pressure. Liver compliance is likely to be the major reason for the low level of hydrodynamic gene delivery in the pig model, and will influence the effectiveness of the approach in humans, both in general and in different disease states[71].

This procedure has great limitations for application to clinical practice, therefore, a clinically relevant method for regional hydrodynamic delivery of vectors has been developed. The method entails the use of an occlusion balloon catheter into the inferior vena cava and retro dynamically injecting towards the liver and through the hepatic vein, 100 mL of the plasmid in saline solution (20 mg/mL), at a rate of 7.5 mL/s. This retrodynamic hepatic vein gene delivery method has been performed in pigs, and was as well tolerated as in mice and led to liver transgene expression, however, the plasma levels of the transgene protein were four orders of magnitude lower than those reached in the murine model[68,72]. A variety of different modifications have been reported recently[73,74].

Recently, retrograde administration of adenoviruses into the common bile duct has been shown to induce efficient transgene expression in the liver without causing severe adverse effects, thus supporting the feasibility of adenovirus-mediated gene transfer into the liver in clinical settings by means of endoscopic retrograde cholangiography[75-77]. Repeat administration of adenoviruses into the common bile duct is successful in re-expressing the transgene in the liver[78]. This contrasts with the failure of re-expression of transgene following intravenous readministration of an adenoviral vector long after the initial administration[79].

OVERVIEW OF PUBLISHED STUDIES

The general features of the reviewed gene therapy studies for enhancing liver regeneration are summarized in Table 2. Gene therapy investigations that fulfilled the following criteria were selected for review: (1) demonstrated, objectively, enhanced liver cell proliferation and or increased survival as compared with controls; (2) animals and/or livers receiving gene therapy were not genetically modified as they do not directly represent human liver diseases (e.g. liver cirrhosis, fibrosis or failure) in which liver regeneration has a critical role in recovery; and (3) gene therapy was administered *in vivo*. The selection of homogeneous cohort studies based on these criteria allows us to delineate the main characteristics of these studies, and more importantly, envision what needs to be done in fu-

Table 2 Main features of reported gene therapy experiments[39,44-50,80-96]

Vector, Ref.	Dose	Transgene (promoter)	Liver model, animals, route	Measured parameters
Adenovirus vector				
Hogaboam *et al*[80], 1999	1×10^8 pfu	r-MIP-2	Acetaminophine injury, mice, IV	↑DNA synthesis, ↑survival
Phaneuf *et al*[46], 2000	1-4 × 10^{11} vp	h-HGF (CMV)	Healthy, mice, IV	↑DNA synthesis, ↓apoptosis and ALT
Shiota *et al*[39], 2000	1×10^9 pfu	r-HGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Nomi *et al*[95], 2000	1×10^9 pfu	r-HGF (CAG)	D-Gal/LPS liver failure, rats, IP	↓Apoptosis, ↑survival
Hecht *et al*[48], 2001	1×10^8 TU	h-HIL-6 (CMV)	D-Gal liver failure, mice, IP	↑Survival, ↑proliferation
Hwang *et al*[81], 2003	1×10^{11} vp	h-HGF (CMV)	TAA liver failure, mice, IV	↑Survival, ↑DNA synthesis, no hepatic necrosis
Iwaki *et al*[49], 2003	2×10^9 pfu	m-MIF antisense	BCG-LPS liver failure, mice, IV	↑Survival
Oe *et al*[45], 2004	7×10^8 pfu	h-VEGF + or r-HGF (CAG)	DMN cirrhosis 70% PH, rats, IV	↑SECs and hepatocytes proliferation
Oe *et al*[82], 2005	7×10^8 pfu	r-HGF, or h-VEGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation, ↑regeneration
Wullaert *et al*[84], 2005	2.5×10^9 pfu	m-ABIN-1 (CMV)	TNF + Gal-liver injury, mice, IV	↑Survival, ↓apoptosis,
Ichiba *et al*[94], 2005	1×10^9 pfu	r-TPO (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Khai *et al*[44], 2006	1×10^{11} vp	h-HB-EGF or h-HGF (RSV)	Fas-induced injury, mice, IV	↓Apoptosis and ↑proliferation by both
Ozawa *et al*[47], 2006	5×10^8 pfu each	r-HGF, +/or h-TGFβ2R (CAG)	DMN cirrhosis 10% PH, rats, PV	↑Proliferation, ↑survival, ↓cirrhosis
Tan *et al*[96], 2006	1×10^{11} vp	m-HNF6 (CMV)	70% PH, mice, IV	↑Proliferation
Yuasa *et al*[85], 2007	1×10^9 pfu	r-HGF, (CBA)	85% PH, rats, IV	↓Apoptosis, ↑proliferation, ↑survival
Ueno *et al*[83], 2007	5×10^8 pfu	r-HGF (CAG)	DMN cirrhosis 70% PH, rats, sPV	↑Proliferation, ↑survival, ↓cirrhosis
Atta *et al*[93], 2009	7×10^9 pfu	h-HGF, h-VEGF (CMV)	Healthy, dogs, IV	↑SEC and hepatocytes proliferation
Naked plasmid DNA				
Yang *et al*[90], 2001	10-40 μg/wk × 8	h-HGF (CMV)	Healthy, mice, IV	↑Proliferation
Xue *et al*[89], 2003	50 μg × 3	r-HGF	CCl_4 cirrhosis 70% PH, mice, IM + EP	↑Proliferation
Zhang *et al*[91], 2005	200 μg/kg per 12 h × 4	r-ALR	CCl_4 liver injury, rats, IV, IP	↓ALT and AST, ↑proliferation, ↑survival
Horiguchi *et al*[86], 2009	-	h-HGF	DMN cirrhosis, dogs, IA	↓ALT and AST, ↓fibrosis, ↑survival
HVJ Liposomes				
Ueki *et al*[88], 1999	20 or 40 mg weekly × 4	h-HGF (SRa)	DMN cirrhosis, rats, IM	↓Apoptosis, ↑survival, ↑r-HGF, ↓fibrosis
Ogushi *et al*[92], 2003	50 nmol	NF-κB decoy ODN	*P. acnes*-LPS liver injury, mice, PV	↑Survival, ↑proliferation, ↓apoptosis
Nishino *et al*[87], 2008	20 μg	h-HGF (SRα)	DMN cirrhosis 70% PH, rats, PV	↑Proliferation, ↑survival, ↓apoptosis
Takahashi *et al*[50], 2009	50 nmol	NF-κB decoy ODN	90% PH, mice, PV	↑Survival, ↓apoptosis

AAF: Acetylaminofluorene; ALT: Alanine transaminase; AST: Aspartate transaminase; ABIN-1: A20 binding inhibitor of nuclear factor κB; ALR: Augmenter of liver regeneration; BCG: Bacille Calmette-Guerin; CAG: Chicken β-actin promoter and cytomegalovirus enhancer; CBA: Chicken β-actin; D-Gal: D-galactosamine; TNF: Tumor necrosis factor; HVJ: Hemagglutinating virus of Japan; DMN: Dimethylnitrosamine; EP: Electroporation; Gal: Galactosamine; h: Human; h-HIL-6: Human hyper-interleukin-6 (IL-6) cDNA gene coding the human sIL-6R (amino acid residues 1-323) and human IL-6 (amino acid residues 29-212) fused by a synthetic DNA linker; HNF6: Hepatocyte nuclear factor 6; IA: Intra-arterial injection (hepatic artery); IM: Intramuscular injection; IP: Intraperitoneal injection; IV: Intravenous injection; M: Murine; HGF: Hepatocyte growth factor; MIF: Macrophage migration inhibitory factor; MIP-2: Macrophage inflammatory protein-2; VEGF: Vascular endothelial growth factor; CMV: Cytomegalovirus; ODN: Oligodeoxynucleotides; PH: Partial hepatectomy; LPS: Lipopolysaccharide; SECs: Sinusoidal endothelial cells; *P. acnes*: *Propionibacterium acnes*; PV: Portal vein injection; r: Rat; sPV: Selective portal vein injection; SRα: Simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat; TAA: Thioacetamide; TGFβ2R: Truncated transforming growth factor β type 2 receptor; TPO: Thrombopoietin; TU: Transducing units (1 vp = 25 TU); vp: Viral particles (1 vp = 100 pfu).

ture studies as a preparation for clinical trials. An overview of the different elements of gene therapy for liver regeneration studies are given below.

Vector type

Given the merits of adenoviruses as a powerful vector that has the highest transduction rate for liver cells, it is not surprising that two-thirds of all reviewed studies used it to prove the effect of the therapeutic gene (Table 2). It was the only viral vector used. The non-viral vectors employed in the rest of the reviewed studies are divided between naked DNA and liposomes. Despite the lower transfection rate of the non-viral vectors, their safety makes them suitable candidates for preclinical studies.

Vector dose

The administered adenoviral dose ranged between 1×10^8 pfu and 4×10^9 pfu with a dose of 1×10^9 pfu used in 80% of the studies[39,44-47,80-85,97]. The average vector dose for mice was no different from that for rats despite considerable differences in their body weights. Phaneuf *et al*[46] have examined the effect on liver regeneration of increasing doses (1×10^9 to 4×10^9 pfu) of adenoviral vector encoding for human HGF. They have found that DNA synthesis of hepatocytes and liver weight increased in a dose-dependent fashion, such that the maximal effect was seen after the infusion of 3×10^9 pfu, which resulted at day 5 in a > 130% increase in relative liver mass, with little cytopathic effect. The average single dose of naked DNA was 10-50 μg and that of liposomes was 50 nmol[50,86-91,98].

Therapeutic genes

By far the most studied therapeutic gene was HGF, which is not surprising given the fact that it is the single most important growth factor implicated in liver regeneration. It has been used in about two-thirds of studies, either alone

or in combination with other growth factors (Table 2). Other genetic materials used include growth factors, cytokines or transcription factors involved in direct liver cell proliferation, e.g. VEGF, HB-EGF, C/EBPβ, and IL-6. Two studies have reported the use of antisense ODN to NF-κB (NF-κB decoy ODN) encapsulated in hemagglutinating virus of Japan (HVJ) liposomes to prevent endotoxin- or massive hepatectomy-induced liver failure[50,92]. Antisense ODNs are not natural genes, and they are short (15-20 bases in length) synthetic oligonucleotides that are designed to hybridize to RNA through Watson-Crick base pairing. Upon binding to the target RNA, ODNs prevent expression of the encoded gene product. Although stimulation of the transcription factor NF-κB in Kupffer cells, with production of inflammatory cytokines, has been shown to be involved in liver proliferation, excessive production of cytokines is thought to be responsible for liver failure following excessive hepatectomy[50].

Route of administration

The liver is an attractive target for *in vivo* gene transfer studies because hepatocytes are readily accessible *via* the blood stream. The endothelium of hepatic sinusoids displays fenestrations that are 100 nm wide and that allow macromolecules such as viral particles to cross the endothelium and reach hepatocytes. Moreover, the hepatic blood flow represents one-fifth of the cardiac output. Thus, any particle injected into the blood circulation can quickly reach the liver[54]. For this reason, the vascular route constitutes the most commonly used in 80% of the reviewed studies. The intravenous route is the commonest among the vascular routes not only because it is the easiest route compared with intra-arterial or portal vein administration, but also due to the enhanced transduction rate following the recent modification of the hydrodynamic technique mentioned above.

Duration of transgene expression

Few of the reviewed studies have reported the duration of expression of the transduced gene or its protein[39,45,81,83,85,87,88,93-95]. Those studies that had extended observation periods have shown that the duration of transgene expression does not extend beyond 1 wk following vector administration[39,81,83,87,88,94]. These data agree with the accumulated knowledge that gene therapy using adenoviral vectors or non-viral naked DNA and liposomes confers a limited duration of gene expression. Moreover, it should be noted that the efficiency of gene transduction, which directly affects the duration of gene expression, is lower in cirrhotic liver than in normal liver due to capillarization of sinusoidal endothelial cells as a result of the decreased size or loss of the fenestrae of sinusoidal endothelial cells[99]. Nishino *et al*[87] have demonstrated that only 5%-6% of hepatocytes in cirrhotic rat livers were successfully transfected with human HGF plasmid enveloped in HVJ liposomes.

Non-hepatic gene transfection

There was a tendency towards excluding gene therapy studies for liver regeneration in which gene transduction involved organs other than the liver, e.g. skeletal muscles. Although this could be appropriate for the sake of presenting a homogeneous group of investigations, it was felt however that this would have omitted an important cluster of studies that represented an emerging direction in gene therapy for liver regeneration. In this regard, two studies used liposomes and naked plasmid to transduce skeletal muscles with HGF in animals with liver cirrhosis. They demonstrated expression of the transduced HGF gene and elevation of its plasma levels that exerted proliferative and antifibrotic effects on the liver[88,89].

FUTURE PERSPECTIVES

In 20 years of gene therapy research, there have been few studies that have aimed at enhancing liver regeneration. However, the accumulated knowledge from these studies has allowed the validation of proof of principle gene therapy investigations for promoting liver regeneration in different animal models of liver diseases. Future progress in this field is expected to tackle several points.

First, determination of the combination of gene therapy that works better for a specific disease condition. As mentioned above, enhancing liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis/cirrhosis or toxic injury requires the combined effects of genetic materials such as growth factor genes and antisense ODN. This should be based on the outcomes drawn from experimental comparative studies of different combinations of therapeutic genes for each defined disease. An example of such comparative studies is that of Ozawa *et al*[47]. In rats with liver cirrhosis, combination gene therapy of HGF, a powerful liver mitogen, and truncated type Ⅱ TGF-β receptor that specifically inhibits TGF-β signaling that is responsible for progression of liver fibrosis[100], resulted in decreased liver fibrosis and improved liver function, compared with monotherapy with either gene alone. These studies provide an opportunity to shed light on how the administered genes influence the pathogenesis of the multifactorial disease process. Also, it could identify synergistic combinations that could enhance regeneration, disease resolution and reduce the amount of transferred genetic material. An example of such studies would make use of HGF and NF-κB decoy ODN, which prevents excessive cytokine production, to prevent hepatocyte apoptosis and enhance regeneration after massive resection or liver injury[50,92].

Secondly, evaluation of the trade-off of risk against the benefits of viral *vs* non-viral gene therapy. Unlike gene therapy for liver genetic diseases that require a high rate of liver transduction to express the therapeutic protein efficiently in the systemic circulation, at a clinically relevant concentration, gene therapy for liver regeneration or resolution of fibrosis aims at locally expressing the desired proteins, which act in an autocrine or paracrine fashion[93]. Thus, despite non-viral systems having a lower transfection rate, they are safer, easy to produce in large quantities, and can be repeatedly administered, which can

aid in gauging the amount and duration of gene expression. Moreover, hydrodynamic injection in murine models and its clinically relevant retrodynamic hepatic vein gene delivery in large animals have dramatically increased transfection efficiency of non-viral systems.

Thirdly, employing the recently developed vectors that target specific liver cell types, and promoters that are capable of liver-specific sustained transgene expression in gene therapy studies to augment liver regeneration and treat associated liver injury. These new developments can be summarized as follows: (1) Cell-specific expression of therapeutic genes of interest is an extremely attractive strategy in gene therapy. Several investigators have developed selective hepatic cell delivery systems using receptors that are unique to and highly expressed by different liver cell types: (A) The asialoglycoprotein receptor (ASGPR) on the hepatocyte membrane is a specific targeting marker for gene and drug delivery. Studies have targeted the hepatocyte ASGPR using its natural ligand, asialoorosomucoid[101,102]. Chiba *et al*[103] recently have developed cationically modified biocompatible phospholipid polymer conjugated with hepatitis B surface antigen for the specific transfer of genes into human hepatocytes; (B) Quiescent HSCs lack specific receptors or motifs on their cell surface, thus, attempts to target HSCs have been a challenging task[104]. (a) The mannose 6-phosphate/insulin-like growth factor Ⅱ (M6P/IGF-Ⅱ) receptor expression is increased on activated HSCs, particularly during fibrosis. The receptor has binding sites for IGF-Ⅱ and M6P-containing ligands[105]. Beljaars *et al* have developed a carrier system that consists of human serum albumin modified with M6P, which binds to the M6P/IGF-Ⅱ receptors on HSCs[104-107]; (b) Vitamin A receptors on HSCs have been used to deliver siRNA against collagen-specific chaperone heat shock protein 47 *via* vitamin A-coupled liposomes[108]; and (c) Liposomes labeled with a cyclic RGD-peptide that recognizes the collagen type Ⅵ receptors[109,110]; (C) Sinusoidal endothelial cells (SECs) possess unique hyaluronan receptors that recognize and internalize hyaluronic acid (HA). SECs have been targeted using HA, the endogenous ligand for the HA receptor for endocytosis[111,112]; and (D) Kupffer cells possess receptors that recognize galactose and N-acetylgalactosamine. Studies have shown that galactosylation can target various DNA preparations including liposomes, low-density lipoprotein and chitosan polymer to Kupffer cells[113-115]; and (2) Liver-specific sustained transgene expression can be obtained at very high levels from optimized promoters[116]. Many experimental gene therapy vectors described in this review express transgenes under the control of non-specific promoters such as CMV, Rous sarcoma virus, simian virus 40 (SV40) and mammalian elongation factor 1α (EF1α) (Table 2). These promoters direct strong gene expression but are shut off rapidly *in vivo*[117,118]. A tissue-specific promoter is a promoter that has activity in only certain cell types. Use of a tissue-specific promoter in the expression cassette can restrict unwanted transgene expression as well as facilitate persistent transgene expression[119]. Ongoing developments are based on two liver-specific promoters, the albumin promoter and the α1 antitrypsin promoter. Wooddell *et al*[116] have demonstrated that when using a plasmid vector that contains albumin promoter combined with an α-fetoprotein (AFP) MERⅡ enhancer, 5' intron from the factor Ⅸ gene, and the 3'UTR from the albumin gene, including intron 14, the reporter gene expression levels remained high for 1 year, at levels comparable to those obtained from the CMV promoter on day 1. Ziegler *et al*[120] have shown that intravenous administration of a recombinant AAV2 vector encoding human α-galactosidase A under the transcriptional control of a liver-restricted enhancer/promoter consisted of human serum albumin promoter (nucleotides -486 to +20), to which were appended two copies of the human prothrombin enhancer (nucleotides -940 to -860). The enhancers were placed 5' of the promoter in the forward orientation. This vector mediated sustained hepatic expression of α-galactosidase A for 12 mo and was associated with a significantly reduced immune response to the expressed enzyme. Several investigators have reported encouraging long expression of transgenes using different modifications of α1 antitrypsin promoter[117,121-123]. Jacobs and his colleagues have compared 22 hepatocyte-specific expression cassettes and have found that a promoter that consists of an 890-bp human α1-antitrypsin promoter and two copies of the 160-bp α1-microglobulin enhancer results in the highest expression levels[124]. Comparisons between different liver-specific promoters have shown that α1-antitrypsin promoters induce higher levels and prolonged expression of transgenes than other liver-specific promoters such as AFP and albumin promoter[125-127]. The most recent investigations have shown the unlimited possibilities for gene therapy modifications. Li *et al*[128] have developed a small DNA fragment (347 bp) from the AAV chromosome 19 integration site that is capable of providing efficient and enhanced liver-specific transcription when used in recombinant AAV vectors. Previously described tissue-specific promoters for gene therapy are typically too big for AAV vectors. Wolff *et al*[129], in an effort to increase long-term expression of transgene products, have designed a plasmid DNA vector under the control of a tissue-specific promoter and have included microRNA target sites in the transcripts, in order to silence expression in antigen-presenting cells.

CONCLUSION

The success of several proof of principle studies of gene therapy for liver regeneration, coupled with the recent extensive search for the mechanisms of selective targeting of specific liver cells, should pave the way towards future clinical trials. As liver regeneration is usually an integral part of the therapeutic goals of many liver diseases, gene therapy to enhance liver regeneration needs to be combined with gene therapy for associated liver disease. Consequently, clinically relevant gene transfer protocols should be developed to address specific goals of such combined gene therapy trials.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4031

World J Gastroenterol 2010 August 28; 16(32): 4031-4038
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Extracellular matrices for gastrointestinal surgery: *Ex vivo* testing and current applications

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Received: April 4, 2010 Revised: May 26, 2010
Accepted: June 2, 2010
Published online: August 28, 2010

Abstract

AIM: To assess the effects of bile and pancreatic juice on structural and mechanical resistance of extracellular matrices (ECMs) *in vitro*.

METHODS: Small-intestinal submucosa (SIS), porcine dermal matrix (PDM), porcine pericardial matrix (PPM) and bovine pericardial matrix (BPM) were incubated in human bile and pancreatic juice *in vitro*. ECMs were examined by macroscopic observation, scanning electron microscopy (SEM) and testing of mechanical resistance.

RESULTS: PDM dissolved within 4 d after exposure to bile or pancreatic juice. SIS, PPM and PDM retained their integrity for > 60 d when incubated in either digestive juice. The effect of bile was found to be far more detrimental to mechanical stability than pancreatic juice in all tested materials. In SIS, the loss of mechanical stability after incubation in either of the digestive secretions was less distinct than in PPM and BPM [mFmax 4.01/14.27 N (SIS) *vs* 2.08/5.23 N (PPM) *vs* 1.48/7.89 N (BPM)]. In SIS, the extent of structural damage revealed by SEM was more evident in bile than in pancreatic juice. In PPM and BPM, structural damage was comparable in both media.

CONCLUSION: PDM is less suitable for support of gastrointestinal healing. Besides SIS, PPM and BPM should also be evaluated experimentally for gastrointestinal indications.

Key words: Extracellular matrix; Intestinal regeneration; *Ex-vivo* testing; Gastrointestinal surgery; Gastrointestinal fistula; Bioscaffolding

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Hoeppner J, Marjanovic G, Helwig P, Hopt UT, Keck T. Extracellular matrices for gastrointestinal surgery: *Ex vivo* testing and current applications. *World J Gastroenterol* 2010; 16(32): 4031-4038 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4031.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4031

INTRODUCTION

Extracellular matrices (ECMs) have been introduced for clinical therapy of gastrocutaneous, enterocutaneous and anal fistulas and for buttressing of gastrointestinal staple lines. Moreover, they have been evaluated experimentally as bioscaffolds for tissue regeneration in different gastrointestinal hollow organs[1-14] and for reinforcement of gastrointestinal anastomoses[15,16]. In particular, small-intestinal submucosa (SIS) has been tested clinically and experimentally. SIS is a biodegradable, commercially available, acellular, immunologically inert collagen matrix, which is extracted from the submucosal layer of porcine small bowel.

Several clinical studies have been performed to evaluate SIS for plug repair of gastrointestinal fistula. Prospective studies have shown high rates of success in the treatment of anal fistulas[17,18], but enterocutaneous[19,20] and gastrocutaneous[21,22] fistulas have also been treated successfully by implantation of SIS plugs. Furthermore, it has been shown that sealing of intestinal anastomoses promotes the healing of intestinal anastomoses[15,16]. For successful application in luminal gastrointestinal organs, temporary maintenance of structure and stability of ECM against gastrointestinal fluids should be guaranteed. To date, SIS has never been examined because of its resistance against gastrointestinal digestive juices. Other biological collagenous acellular scaffolds, which are promising for the support of gastrointestinal healing, are extracted from porcine dermis, porcine pericardium or bovine pericardium.

The aim of our study was to assess the effect of physiological intraluminal intestinal components human pancreatic juice and bile on 3D surface ultrastructure and mechanical resistance of different natural biological scaffolds *in vitro*.

MATERIALS AND METHODS

Materials

Single-layer SIS was prepared as previously described[23]. Sections of porcine jejunum were obtained from the local slaughterhouse and immediately placed in 0.9% saline solution. Jejunal sections were cut into 10-cm lengths and luminally cleaned with 0.9% saline solution. The mesenteric tissues were removed from the segment of the small intestine, followed by mechanical removal of the tunica serosa and tunica muscularis from its outer surface by gentle abrasion using a scalpel handle and saline-moistened gauze. The segment was inverted and the tunica mucosa was mechanically removed by similar mechanical abrasion and reverted to its original orientation. The remaining 0.1-0.2-mm thick translucent tube actually consisted of the tunica submucosa. The stratum compactum that originally was in contact with the more superficial luminal mucosa was now the luminal surface of the SIS graft. After sterilization of the SIS graft by 2 h incubation in 0.1% perchloric acid, it was rinsed with sterile normal saline and stored in refrigerated 0.05% gentamicin at 4℃. Storage time for the graft materials ranged from 3 to a maximum of 7 d until the material was used for *in vitro* testing.

Four-layer SIS was provided as Surgisis® from Cook Surgical (Lafayette, IN, USA). Porcine dermal matrix (PDM) was provided as Xenoderm® from MBP (Neustadt-Gleve, Germany). Cleansed porcine pericardial matrix (PPM) was provided from aap Biomaterials (Dieburg, Germany). Bovine pericardial matrix (BPM) was provided as Lyoplant® from Braun Dexon (Melsungen, Germany). The materials were divided under sterile conditions into pieces of 1 cm × 1 cm for scanning electron microscopy (SEM) and for measurement of degradation time. For assessment of mechanical properties, samples of 1 cm × 3 cm were used.

Incubation

Human bile had been collected during laparotomy from patients in whom cholecystectomy was performed. Microbiological assessment of bile excluded an infectious biliary syndrome. Pancreatic juice was obtained from patients with pancreatic duct drainage after pancreatic head resection for chronic pancreatitis. Patient consent to use the secretions for the study was given. Both fluids were checked for bacterial contamination in aerobic and anaerobic microbiologic cultures, and only sterile bile and pancreatic juice were used for experiments. ECM specimens of 1 cm × 1 cm and 1 cm × 3 cm were incubated at 37℃ for 1, 7, 14 or 60 d in bile and pancreatic juice. For reference, the material was also incubated in sterile phosphate buffered saline (PBS). The pH of human bile used for incubation was 8.37. Pancreatic enzyme concentrations were measured by routine diagnostic methods at the central laboratory of the University Hospital Freiburg. Initial concentration of amylase was on average 86 040 U/L, concentration of lipase was 210 630 U/L in pancreatic juice, and pH was 8.42. After 24 h of incubation, concentration of amylase was on average 68 440 U/L, and concentration of lipase was 28 670 U/L in pancreatic juice. Due to this degradation of active enzymes during incubation, both media were replaced every 24 h under sterile conditions.

Measurement of degradation time and macroscopic evaluation

All incubated samples were macroscopically inspected daily for signs of degradation for 60 d. After incubation in PBS, human bile and human pancreatic juice, the size of the specimens was measured after 1, 7, 24 and 60 d.

Mechanical testing

Assessment of mechanical resistance using a sero-hydraulic material testing machine with a 200-N force transducer (UTS 20; UTS Systeme GmbH, Germany) was performed after 24 h and 14 d. Test Expert II Software (Zwick GmbH, Ulm, Germany) was used for analysis of force/distension diagrams. All mechanical experiments were carried out in triplicate. ECM strips of 1 cm × 3 cm were removed from incubation medium, rinsed in PBS and fixed in the testing device of the material testing machine. The sample was distended longitudinally at a speed of 12 mm/min until the sample broke. The required force until failure of the SIS strip was measured and reported as Fmax in Newtons.

SEM

After 7 d incubation in PBS, bile or pancreatic juice ECM samples (1 cm × 1 cm) were rinsed in PBS and subsequently fixed with 4% buffered formaldehyde for 48 h at room temperature. The samples were dehydrated in a graded series of acetone, dried in a critical-point dryer, mounted for SEM, and coated with gold in an evaporator unit. The samples were mounted such that one of the sides and the cross-section were visible. Examination was then performed in an LEO 435 VP scanning electron microscope (LEO Electron

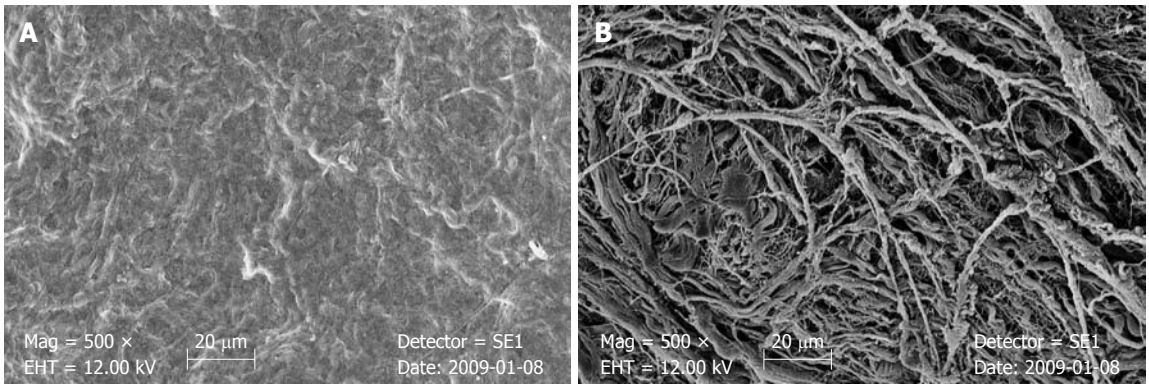

Figure 1 Scanning electron microscopy pictures of the stratum compactum surface (A) and the abluminal surface (B) of small-intestinal submucosa. Magnification × 500.

Table 1 Extracellular matrix degradation ultrastructural grading system

Characteristic	Resistance → Degradation			
	Score 0	Score 1	Score 2	Score 3
Porosity	No change	Light increase	Distinct increase	Strong increase
Surface fibrillar arrangement	No change	Light alteration	Distinct alteration	Native arrangement not recognizable
Erosion of matrix surface	No change	Light surface erosion	Distinct surface erosion	Native surface structure not recognizable

Original materials incubated for 60 min in phosphate buffered saline were used as reference.

Table 2 Degradation time with phosphate buffered saline, bile and pancreatic juice

	PBS	Bile	Pancreatic juice[1]
SIS 4-layer	> 60	> 60	> 60
SIS 1-layer	> 60	> 60	> 60
PDM	40	2	4
PPM	> 60	> 60	> 60
BPM	> 60	> 60	> 60

Degradation time is given in days. Incubation was performed at 37℃ for 60 d. [1]Enzyme concentration: amylase 86040 U/L; lipase 210630 U/L. PBS: Phosphate buffered saline; SIS: Small-intestinal submucosa; PDM: Porcine dermal matrix; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

Microscopy Ltd., Cambridge, UK). A grading system was developed for comparison of ultrastructural alterations after incubation in bile and pancreatic juice. As reference, original materials incubated for 60 min in PBS were used. Changes in porosity, surface fibrillar arrangement and erosion of the ECM surface were measured and scored (Table 1).

RESULTS

Macroscopic examination and degradation time

SIS one-layer, SIS four-layer, PPM and BPM samples were intact, without macroscopic signs of degradation after 60 d of incubation in bile, pancreatic juice and PBS. Apart from deep green-brown color after incubation in bile, there were no macroscopic differences recognizable after 7, 14 and 60 d of incubation. No shrinkage and no change in size of the patches were macroscopically detectable, regardless of the medium of incubation in SIS, PPM and BPM samples. PDM was dissolved within 40 d in PBS, within 2 d in bile, and within 4 d in pancreatic juice (Table 2).

Mechanical testing

For SIS, PPM and BPM, testing revealed the most distinctive loss of mechanical strength after incubation in bile at 24 h and 14 d. Incubation in pancreatic juice also caused a significant decrease of breaking strength at 14 d. No marked difference could be detected between saline and pancreatic juice incubation for SIS four-layer, PPM and BPM after 24 h. As a result of early structural degradation, PDM could only be tested after 24 h of incubation. No marked differences in mechanical resistance in the three different media were detected for PDM after 24 h. Numerical mean values of mechanical testing are shown in Table 3.

Surface structure of the ECMs

SEM of SIS showed a non-directed fibrous and fine surface structure of abluminal surface of the material. On the opposite side, which represents the stratum compactum of the submucosal layer of the porcine bowel wall, the surface appeared dense without a porous aspect (Figure 1). The single layers of the 4-layer SIS were distinguishable in the cross-sectional view. Arrangement of collagenous fibers in PDM appeared to be more directed. Single fibers looked chubby and showed a scaly surface (Figure 2). The collagenous fibers in PPM appeared fine and were assembled almost straight. Porosity of the material was verified by SEM, although some parts of the heterogeneous surface appeared dense (Figure 2). In BPM, SEM showed

Table 3 Mechanical resistance of the biomaterials after incubation in phosphate buffered saline, bile or pancreatic juice (mean ± SE)

	PBS		Bile		Pancreatic juice	
	24 h	14 d	24 h	14 d	24 h	14 d
SIS 4-layer	31.40 ± 4.3	26.03 ± 3.2	13.70 ± 0.9	4.01 ± 0.3	30.20 ± 1.0	14.27 ± 0.6
SIS 1-layer	4.56 ± 1.2	3.42 ± 0.4	3.60 ± 1.6	1.96 ± 0.2	2.56 ± 0.4	2.59 ± 0.4
PDM	1.05 ± 0.2	0.74 ± 0.1	0.81 ± 0.1	Dissolved	0.97 ± 0.1	Dissolved
PPM	19.03 ± 2.9	13.8 ± 4.9	10.45 ± 1.4	2.08 ± 0.6	21.95 ± 7.2	5.23 ± 1.3
BPM	27.13 ± 7.3	31.07 ± 5.2	10.34 ± 1.3	1.48 ± 0.3	30.07 ± 2.2	7.89 ± 1.8

Experiments were carried out in triplicate. PBS: Phosphate buffered saline; SIS: Small-intestinal submucosa; PDM: Porcine dermal matrix; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

Table 4 Ultrastructural alteration of extracellular matrices incubated in human bile and human pancreatic juice

	Bile				Pancreatic juice[1]			
	Porosity	Surface fibrillar arrangement	Erosion of matrix surface	Σ	Porosity	Surface fibrillar arrangement	Erosion of matrix surface	Σ
SIS 4-layer	3	2	3	8	2	1	1	4
SIS 1-layer	3	2	3	8	2	1	1	4
PPM	1	3	1	5	1	3	3	7
BPM	0	2	2	4	1	2	1	4

Incubation was performed at 37℃ for 7 d. [1]Enzyme concentration: amylase 86040 U/L; lipase 210630 U/L. SIS: Small-intestinal submucosa; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

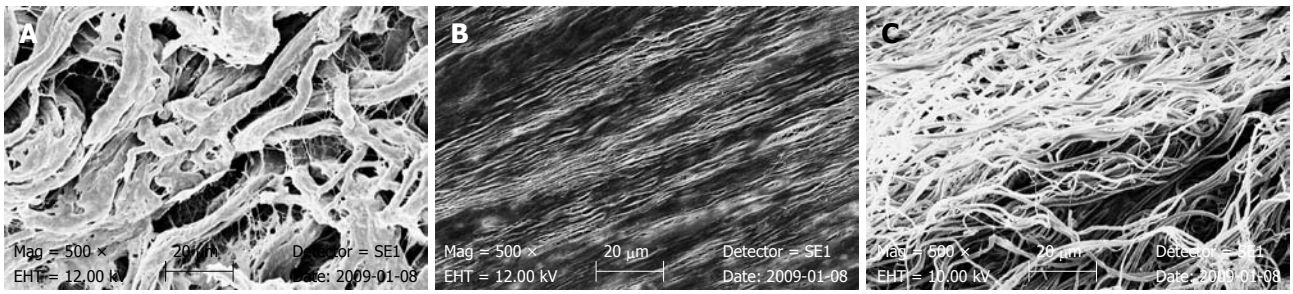

Figure 2 Scanning electron microscopy pictures of the surface structure of the samples with surface characteristics of the different original biomaterials. A: Porcine dermal matrix; B: Porcine pericardial matrix; C: Bovine pericardial matrix. Magnification × 500.

a directed but wormed arrangement of the fibers. Single collagenous fibers appeared fine-structured with a slick surface (Figure 2).

Structural changes after incubation in bile

After 7 d of incubation in bile, the stratum compactum surface of SIS appeared clearly damaged. The dense surface was scarified and a fibrous structure of the deeper parts of the material could be recognized. After biliary incubation in PPM, collagenous fibers were grouped together with a gross scaly surface. The dense surface of the single strands appeared eroded and very fine reticular structures were recognized. In BPM, SEM after 7 d exposure to bile also revealed the phenomenon of collagenous fibrous structures that appeared to be grouped together (Figure 3 and Table 4).

Structural changes after incubation in pancreatic juice

After exposure to pancreatic juice, SEM revealed structural changes in SIS. In the stratum compactum, surface porosity of SIS was increased and the fibrillar structure of the deeper parts was recognizable. Surface structure of the single fibrils and the surface texture of the SIS sheet appeared to be altered. A slight decrease in thickness of the SIS was seen after pancreatic juice incubation for both types of SIS samples in the cross-sectional view. Single layers of four-layer SIS could not be separated in the cross-sectional view. In PPM, the straight arrangement of the collagenous fibers was no longer recognizable after pancreatic juice incubation. The PPM surface appeared dense and partially eroded with a fine undirected fibrillar aspect. In BPM, the fibers of the matrix appeared grouped together with a chubby surface (Figure 3 and Table 4).

DISCUSSION

A prerequisite to the *in vivo* implantation of a biomaterial as a tissue substitute in a gastrointestinal luminal organ is its resistance against gastrointestinal contents, with maintenance of integrity until gastrointestinal healing has progressed and integrity of the host tissue is restored. Physi-

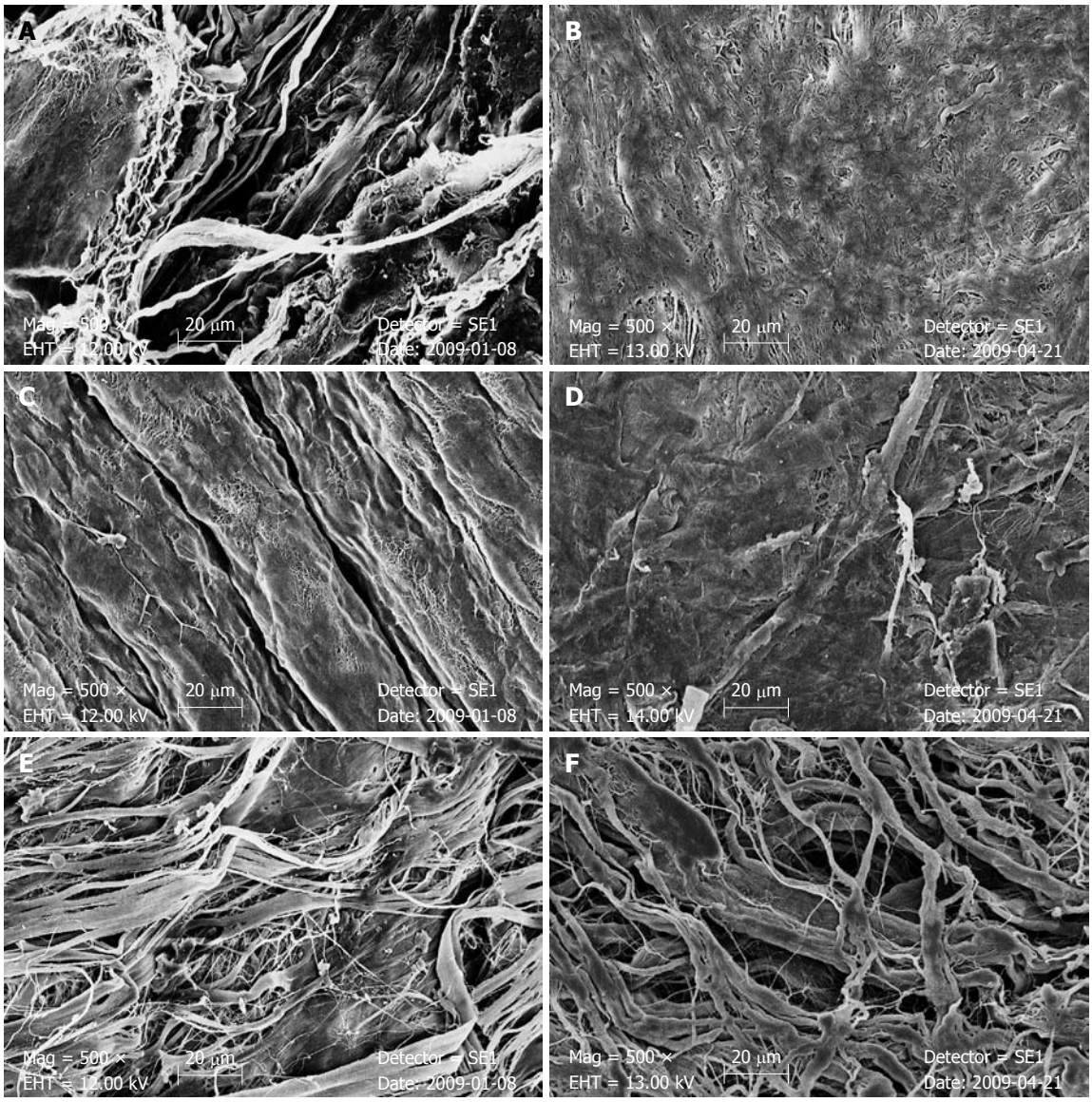

Figure 3 Scanning electron microscopy pictures of small-intestinal submucosa, porcine pericardial matrix and bovine pericardial matrix after 7 d of bile or pancreatic juice incubation. A: Small-intestinal submucosa (SIS) incubated in bile; B: SIS incubated in pancreatic juice; C: Porcine pericardial matrix (PPM) incubated in bile; D: PPM incubated in pancreatic juice; E: Bovine pericardial matrix (BPM) incubated in bile; F: BPM incubated in pancreatic juice.

ological digestive fluids are extremely aggressive substances that are able to destroy most biological tissues. At present, only one experimental study has examined the resistance of ECM against digestive juices[24]. Human placental extracts, human collagen patches, bovine elastin, and bovine collagen matrices were tested for their resistance against bile and pancreatic juice. Only human collagen patches showed sufficient maintenance of integrity, whereas human placental extracts and all bovine materials failed in *in vitro* incubation in bile and pancreatic juice. Recent bioscaffolds like SIS, which have been successfully evaluated for therapy of gastrointestinal fistula, but also have proven potential for gastrointestinal tissue substitution and regeneration at different locations and organs, have so far not been examined systematically for structural and mechanical resistance against digestive contents. This study was designed to examine the effects of biological digestive fluids on the mechanical and structural resistance of current ECM.

It has been shown that ECM, implanted *in vivo* in gastrointestinal luminal organs, is able to induce regenerative responses in the host, and that anatomical tissue structure and tissue function are restored[1-13]. Different expressions of morphological and functional regeneration have been reported in the literature (Table 5). In the past, the majority of experimental work on ECM as a bioscaffold for gastrointestinal regeneration has been performed with SIS[1-13]. Other biological ECMs have only been tested in one single study in an *in vivo* setting in rodents[14]. Nearly complete and anatomical regeneration with SIS as a bioscaffold has been shown for the esophagus and the small bowel. Complete mucosal regeneration and regeneration of muscular layers has been reported within 3-6 mo[2,4,10,11]. After implantation in the rodent stomach, mucosal and muscular

Table 5 Structural regeneration in experimental application of small-intestinal submucosa in the alimentary tract

Autor	Location	Model	Structural regeneration		
			Mucosa	Muscularis	Nerve
Badylak *et al*[4]	Esophagus	Dog	+	+	NR
Lopes *et al*[11]	Esophagus	Rat	+	+	+
de la Fuente *et al*[3]	Stomach	Rat	(+)	-	-
Ueno *et al*[6]	Stomach	Rat	+	+	+
Rosen *et al*[1]	Bile duct	Dog	+	-	NR
De Ugarte *et al*[9]	Duodenum	Rat	(+)	-	NR
Souza Filho *et al*[8]	Duodenum	Dog	(+)	-	NR
Demirbilek *et al*[12]	Jejunum	Rabbit	+	-	NR
Chen *et al*[2]	Small bowel	Dog	+	+	NR
Ansaloni *et al*[10]	Ileum (isolated loop)	Rat	+	+	+
Wang *et al*[13]	Ileum	Rat	+	(+)	NR
Ueno *et al*[5]	Ceacum	Rat	+	+	+
Hoeppner *et al*[7]	Colon	Pig	(+)	(+)	-

+: Complete regeneration; (+): Partial/marginal regeneration; -: Missing regeneration; NR: Not reported.

restoration, along with regeneration of innervation of the stomach wall were evident within 6 mo[6]. In contrast, limited mucosal regeneration and complete absence of muscular regeneration have been reported in the repair of duodenal and lower colonic defects with SIS[7-9]. These differences in SIS-induced gastrointestinal tissue regeneration could be explained by the effects of varying intraluminal chemical and bacterial environments on the integrity and intactness of the collagenous matrix and the 3D structure of the matrix. Biliary and pancreatic enzyme aggression is likely to play a major role in these processes. Toxicity of bile is explained by the detergent capacity of bile salts and alkaline pH of bile. The destructive effects of pancreatic juice on biological tissues and membranes are best explained by its content and the high concentration of enzymes like proteases, glucosidases, elastases and lipases. It is assumed that these enzymes are able to degrade the ECM components that are present in biological and artificial scaffolds[24]. Physiologically, bile and pancreatic juice are slowly secreted and diluted by other intraluminal gastrointestinal contents such as chyme. In our study, ECM was exposed to concentrated and very aggressive media *in vitro*, although they almost never encounter such demanding conditions *in vivo*.

The most pronounced effects of bile and pancreatic juice in our study were seen in PDM. It was dissolved within a few days in both digestive juices. SIS, PPM and BPM maintained their integrity for at least 60 d of incubation in pure bile or pancreatic juice. In ECM of porcine origin, SIS and PPM, the loss of mechanical stability after incubation in the two digestive media was less distinct than in BPM. Our examination revealed bile to be much more efficacious in degrading ECM than pancreatic juice in all tested materials. In SEM, ECM incubated in bile was more eroded than after incubation in pancreatic juice. Moreover, SEM showed obvious changes in the 3D surface structure and arrangement of the collagenous fibers in SIS, PPM and BPM. It is assumed that the 3D structure of the fibrillar collagens and adhesive glycoproteins in the naturally occurring biopolymer SIS are involved in tissue regeneration induced by SIS[25], therefore, it has to be assumed that those changes in 3D structure impair gastrointestinal tissue regeneration. Moreover, destruction of regulatory proteins that are present in SIS, such as fibronectin, heparin sulfate proteoglycan, fibroblast growth factor-2, transforming growth factor-β and vascular endothelial growth factor, by aggressive contents of bile and pancreatic juice could also impair tissue regeneration[25-27]. This is a possible explanation for limited mucosal and missing muscular regeneration in duodenal patch repair by SIS[8,9]. Although nearly complete regeneration of biliary epithelium was reported, no formation of a muscular layer was seen when SIS was used for defect repair of the common bile duct in a canine model[1]. Based on these *in vivo* reports, it can be assumed that in the presence of higher intraluminal concentrations of bile and pancreatic enzymes, in particular, muscular regeneration is impaired if SIS is used as a bioscaffold.

Different clinical studies have reported successful application of SIS as a plug system for the therapy of enterocutaneous fistulas. For this indication, SIS is commercially available as Surgisis AFP Anal Fistula Plug® (Cook Biotech Inc.). For plug repair of anorectal fistulas, Champagne *et al*[17] have reported an overall success rate of 83% in a prospective study in 46 patients with a follow-up of 24 mo. In another prospective examination in 60 patients, Schwandner *et al*[18] have reported effective closure in anorectal fistula systems in 62% of cases, with a follow-up of 12 mo. In both studies, no serious adverse effects like impairment of continence function were reported.

Besides the prospective trials for therapy of anorectal fistulas, only case reports have been published for other enterocutaneous fistulas. Small-bowel-derived fistulas have been reported to be closed successfully in three patients[19,20]. Recently Toussaint *et al*[21] have reported a case series of five patients with gastrocutaneous fistulas after gastric sleeve and gastric bypass with a success rate of 80%. Furthermore, effective closure of persistent gastrocutaneous fistulas after removal of a gastric feeding tube[22], as well as successful therapeutic approaches for rectovaginal and ileal pouch-vaginal fistulas have been reported in case series and reports[28].

Although only experimental work has been published, SIS is commercially available for reinforcement of linear gastrointestinal staple lines (Surgisis Biodesign Staple Line Reinforcement®, Cook Biotech Inc.). It has been proven experimentally that SIS-reinforced staple lines in the porcine small bowel have increased mechanical stability if tested for bursting pressure in small bowel *in vitro*[29] and immediately after application of staple lines *in vivo*[30]. To date, no information is available concerning the effects of SIS in buttressing circular staple lines and its consequences for the intestinal healing process in staple lines. Most experimental and clinical examinations concerning buttressing of staple lines have been carried out on bovine pericardium. Especially in the field of obesity surgery, bovine

pericardium is commercially available and widely used for reinforcement of staple lines. In a prospective randomized trial, Angrisani *et al*[31] have reported significant effects in prevention of staple line bleeding and a reduction of operating time due to dry operating fields compared to non-buttressed staple lines in laparoscopic gastric bypass. However, not only beneficial effects have been reported for bovine pericardium. Ibele *et al*[32] have recently published a retrospective analysis of 500 patients in which buttressing of the circular staple lines with bovine pericardium during laparoscopic Roux-en-Y gastric bypass was associated with an increased staple line leak rate.

Evaluation of ECM for anastomotic reinforcement in terms of sealing of colonic anastomoses by SIS has been experimentally performed in animal models. In rodents, sealing of colonic anastomoses by SIS showed microscopically and mechanically improved intestinal healing in the most vulnerable early phase of anastomotic healing[16]. In the porcine model, although no information about the effects of SIS on early anastomotic healing and long-term effects of SIS on circular stapled colonic anastomoses beyond 30 d was gained, the feasibility and safety of anastomotic sealing by SIS were demonstrated[15].

In summary, most clinical and experimental evaluation of ECM application in gastrointestinal surgery has been performed on SIS. Apart from staple line reinforcement, other biologically derived ECMs have only been rarely examined for gastrointestinal tissues. We therefore compared the mechanical and ultrastructural characteristics of SIS with ECM derived from porcine dermis, porcine pericardium and bovine pericardium in the presence of aggressive gastrointestinal fluids. Compared to SIS and PPM, mechanical resistance of BPM after 2 wk of exposure to bile or pancreatic juice is clearly weaker. These differences, however, could not be reproduced for structural degradation. Therefore, neither ECM of porcine nor bovine origin can be judged as more resistant to one or other of the human digestive juices. Early degradation of PDM in the presence of bile and pancreatic juice could be an explanation for the failure of acellular dermal matrix as a bioscaffold for intestinal regeneration placed in gastrointestinal continuity, whereas after prevention of exposure to bile and pancreatic juice by implantation in a defunctionalized blind jejunal limb, acellular dermal matrix remained sufficient and allowed mucosal regeneration[14]. These findings are important for application of ECM to gastrointestinal luminal organs, because our data suggest that deviation of bile and pancreatic juice upstream from the repair, should be applied in further experimental and clinical testing.

SIS, PPM and BPM retain their integrity in the presence of high concentrations of human bile and pancreatic juice. Ultrastructural degradation with changes in porosity, surface fibrillar arrangement and erosion of matrix surface were detectable in all three ECMs. The extent of ultrastructural alterations in SIS was more pronounced after incubation in bile than in pancreatic juice. In PPM and BPM, these differences were less distinct. Therefore, we conclude that PPM and BPM should also be evaluated as bioscaffolds for intestinal regeneration, as sealing materials for anastomotic reinforcement, and for plug repair of gastrointestinal fistulas in *in vivo* studies. As a result of early dissolution in the presence of digestive juices, PDM is less suitable for application in gastrointestinal luminal organs. To verify the results from *in vitro* testing, the bioscaffolds used should be tested *in vivo* by implantation in different gastrointestinal luminal organs, with and without deviation from bile, pancreatic juice and stool at the site of repair in upcoming experimental studies. Finally, PPM and BPM should also be evaluated experimentally for treatment of gastrointestinal fistula and reinforcement of intestinal anastomoses *in vivo*.

COMMENTS

Background

Extracellular matrices (ECMs) have been introduced for clinical therapy of gastrocutaneous, enterocutaneous and anal fistulas and for buttressing of gastrointestinal staple lines. They have been experimentally evaluated as bioscaffolds for tissue regeneration at different gastrointestinal hollow organs and for reinforcement of gastrointestinal anastomoses.

Research frontiers

ECMs have been tested for different indications and at different locations in the gastrointestinal tract. It is not known if there are any relevant structural and mechanical changes in ECM caused by exposure to digestive juices. *In vivo* models have reported varying degrees of morphological intestinal regeneration after implantation of ECM as a bioscaffold at different gastrointestinal locations. This phenomenon could be explained by the effects of digestive juices on the structural and mechanic traits of the ECM. In this study, the authors investigated the effects of bile and pancreatic juice on different ECMs.

Innovations and breakthroughs

Small-intestinal submucosa (SIS) was found to be mechanically the most resistant tested material. It was demonstrated that porcine dermal matrix (PDM) is not suitable for therapeutic purposes in intestinal tissue regeneration due to its early degradation. However, SIS, porcine pericardial matrix (PPM) and PDM retained their integrity for up to 60 d when exposed to bile and pancreatic juices. Ultrastructural alterations were found to be more important in SIS when exposed to the juices.

Applications

As a result of their proven ultrastructural and mechanical resistance, PPM and bovine pericardial matrix (BPM) should be evaluated for treatment of gastrointestinal fistulas, as bioscaffolds for intestinal regeneration, and for reinforcement of intestinal anastomoses *in vivo*.

Terminology

ECMs are biodegradable, acellular collagen matrices that are derived from biological tissues. SIS is extracted from the submucosal layer of porcine small bowel. PDM is extracted from the porcine dermis. PPM and BPM are extracted from porcine and bovine pericardia.

Peer review

In general, this is an interesting study with new perspectives on various biomatrices that can be used for intestinal regeneration. These analyses could have an impact on the development of therapeutic approaches in the field of bioengineering.

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S- Editor Wang YR **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4039

World J Gastroenterol 2010 August 28; 16(32): 4039-4046
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Curcumin suppresses gastric NF-κB activation and macromolecular leakage in *Helicobacter pylori*-infected rats

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Supported by Thailand Research fund (code RMU 4980032) and Graduate Thesis Grant, Graduate School, Chulalongkorn University, Thailand
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Received: January 7, 2010 Revised: February 10, 2010
Accepted: February 17, 2010
Published online: August 28, 2010

Abstract

AIM: To investigate whether curcumin could attenuate nuclear factor (NF)-κB p65 expression and macromolecular leakage in the gastric mucosa of *Helicobacter pylori* (*H. pylori*)-infected rats.

METHODS: Twenty-five male Sprague-Dawley rats were equally divided into five groups: control rats (Control), control rats supplemented with 600 mg/kg curcumin, *H. pylori*-infected rats (*Hp*), *H. pylori*-infected rats supplemented with 200 mg/kg curcumin (*Hp* + cur Ⅰ), and *H. pylori*-infected rats supplemented with 600 mg/kg curcumin (*Hp* + cur Ⅱ). In *H. pylori*-infected groups, rats were inoculated with *H. pylori* suspension twice a day at an interval of 4 h for 3 d. Two weeks later, 200 or 600 mg/kg curcumin was given once daily to curcumin-supplemented groups for 7 d. On the day of the experiment, macromolecular leakage in gastric mucosa was examined by intravital fluorescence microscopy. The stomach tissue was removed to examine NF-κB p65 expression in gastric epithelial cells by immunohistochemistry.

RESULTS: The expression of NF-κB p65 in gastric epithelial cells and the macromolecular leakage from gastric mucosal microcirculation significantly increased in the *Hp* group compared with the Control group. The percentages of NF-κB p65 immunoreactive cells in Control and *Hp* groups were 10.72% ± 2.10% *vs* 16.02% ± 2.98%, $P = 0.004$, respectively. The percentages of macromolecular leakage in Control and *Hp* groups were 10.69% ± 1.43% *vs* 15.41% ± 2.83%, $P = 0.001$, respectively. Curcumin supplementation in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups significantly decreased NF-κB p65 immunoreactive cells and macromolecular leakage compared with results in the *Hp* group. The percentages of NF-κB p65 immunoreactive cells in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups were 11.79% ± 2.13% ($P = 0.017$) and 11.42% ± 1.68% ($P = 0.010$), respectively. The percentages of macromolecular leakage in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups were 12.32% ± 2.13% ($P = 0.025$) and 12.14% ± 1.86% ($P = 0.018$), respectively.

CONCLUSION: *H. pylori*-induced gastric inflammation in rats is associated with increased NF-κB activation and macromolecular leakage which can be reduced by curcumin supplementation.

Key words: Curcumin; *Helicobacter pylori*; Nuclear factor-κB p65; Macromolecular leakage

Peer reviewer: Ki-Baik Hahm, MD, PhD, Professor, Gachon Graduate School of Medicine, Department of Gastroenterology, Lee Gil Ya Cancer and Diabetes Institute, Lab of Translational Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea

Sintara K, Thong-Ngam D, Patumraj S, Klaikeaw N, Chatsuwan T. Curcumin suppresses gastric NF-κB activation and macromolecular leakage in *Helicobacter pylori*-infected rats. *World J Gastroenterol* 2010; 16(32): 4039-4046 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4039.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4039

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative bacterium. The infection causes chronic gastritis and peptic ulcer diseases in patients. *H. pylori* infection is also related to mucosa-associated lymphoid tissue lymphoma and gastric cancer diseases in patients[1,2]. The pathogenesis of *H. pylori* infection is associated with the bacterial virulence factors. After *H. pylori* bacteria adhere to gastric epithelial cells, they inject their virulence factors into the host cells *via* a type Ⅳ secretory system[3]. The virulence factors can induce the activation of nuclear factor (NF)-κB in gastric epithelial cells[4].

NF-κB is an important regulator of many cellular processes including the control of the immune response and inflammation[5,6]. NF-κB is a dimeric complex composed of the five mammalian Rel proteins, p65, c-Rel, p50/NF-κB1, p52/NF-κB2, and RelB, in almost any combination. In resting cells, the inhibitors of NF-κB (IκB) form complexes with NF-κB. Upon stimulation, specific intracellular signalling pathways are activated, leading to the activation of the IκB kinase complex (IKK complex). The activated IKK complex phosphorylates the IκB at specific amino acids for the poly-ubiquitination of these NF-κB inhibitors. The ubiquitination of IκB and its subsequent degradation by a proteasome are required for NF-κB activation. NF-κB is now free to translocate into the nucleus and regulate NF-κB-dependent gene expression[7]. The target of activated NF-κB includes the genes encoding proinflammatory cytokines and chemokines that are the causes of *H. pylori*-induced gastric inflammation[4,8,9].

In *H. pylori*-associated gastric inflammation, inflammatory mediators could induce vascular damage. A previous study demonstrated that *H. pylori*-infected patients showed erythema, edema, and vasodilation as well as neutrophil infiltration in the mucosa[10]. Our previous study suggested that leukocyte adhesion in postcapillary venules was increased in *H. pylori*-infected rats. Moreover, the degree of leukocyte adhesion was correlated with the level of the proinflammatory cytokine, tumor necrosis factor (TNF)-α[11]. In addition, previous studies have demonstrated that water-soluble extracts of *H. pylori* induced leakage of macromolecules from rat gastric mucosal microcirculation[12-14].

Curcumin (diferuloylmethane) is an active ingredient of *Curcuma longa* (turmeric) and is pharmacologically safe for human and animals. Curcumin has many biological activities, including anti-inflammatory properties[15]. Most of the anti-inflammatory effects can be explained by the efficient inhibition of NF-κB mediated by this substance[16-18]. Recently, a previous study showed that curcumin can inhibit NF-κB activation in *H. pylori*-infected gastric epithelial cells[19]. Curcumin is also a potent antibacterial agent against *H. pylori* as shown in *in vitro* study[20]. In contrast, curcumin did not eradicate *H. pylori* in *H. pylori*-infected patients[21].

However, it is not clear whether curcumin has any *in vivo* effects in *H. pylori*-induced gastric inflammation. Therefore, we examined the anti-inflammatory effect of curcumin, which may reduce mucosal macromolecular leakage through the suppression of gastric epithelial NF-κB p65 expression induced by *H. pylori* infection in rats.

MATERIALS AND METHODS

Experimental design

Male Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Nakorn pathom, Thailand) weighing 200-250 g were used. All experiments and procedures carried out on the animals were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Rats were housed in a controlled temperature room at 25 ± 1℃ under standard conditions (12-h day-night rhythm). Twenty-five rats were divided into five groups (five rats each) as follows.

Control rats (Control): rats were fed normal saline (1 mL/rat) orally *via* intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

Control rats supplemented with 600 mg/kg curcumin (Cur): rats were fed normal saline (1 mL/rat) orally *via* intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 600 mg/kg curcumin (95% purified curcumin, Cayman Chemical, Ann Arbor, MI, USA) dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats (*Hp*): rats were inoculated with *H. pylori* suspension according to Thong-Ngam *et al*[22]. Briefly, *H. pylori* suspension (10^{10} CFU/mL; 1 mL/rat) was given to the rats by intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats supplemented with 200 mg/kg curcumin (*Hp* + cur Ⅰ): 2 wk after *H. pylori* inoculation, 200 mg/kg curcumin dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats supplemented with 600 mg/kg curcumin (*Hp* + cur Ⅱ): 2 wk after *H. pylori* inoculation, 600 mg/kg curcumin dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori

H. pylori strains used for all experiments were originally

obtained from peptic ulcer patients who visited the King Chulalongkorn Memorial Hospital. The bacteria were grown in Brucella broth (pH 7.0) supplemented with 10% goat serum for 24 h at 37℃ in an automatic CO_2-O_2 incubator (85% N_2, 10% CO_2, and 5% O_2).

Animal preparation

The method of preparing animals for *in vivo* fluorescent microscopy was adapted from a previous study[14]. The animal was anesthetized with thiopental (General Drug House, Thailand; 60 mg/kg, intraperitoneal). A constant level of anesthesia was maintained throughout the experiment by a supplement dose (20% of original dose) every 30-45 min[11]. A tracheotomy was performed. The arterial blood pressure was recorded in the common carotid artery using a pressure transducer (Nihon Kohden, Tokyo, Japan). The abdominal cavity was opened *via* a midline laparotomy. A 1.0 cm incision was made using an electrical microcautery device (Hyfrecator plus®, Conmed, Utica, NY, USA) at the posterior wall, being parallel to the "limiting ridge" of the exteriorized stomach[12]. Next, the stomach was gently extended and placed on a designed board. The incision in the anterior wall was opened using microclamps and covered with Saran wrap to allow visualization of the posterior mucosal surface[12]. The animals were terminated after studying intravital fluorescent videomicroscopy.

Intravital fluorescent videomicroscopy

Observations were made from the glandular portion of the posterior mucosa. Fluorescence tracer [0.3 mL of 0.5% fluorescein isothiocyanate (FITC)-labeled dextran (FITC-dx, MW = 250 000, Sigma-Aldrich, USA)] was injected into the jugular vein[23]. The posterior mucosal microcirculation was visualized under an intravital fluorescence videomicroscope (Nikon Optiphot-2, Nikon, Tokyo, Japan), and examined under × 20 objective lens (Nikon). The selected area included the characteristic honeycomb-like network of mucosal capillaries and at least one postcapillary venule (PCV; diameter 15-30 μm)[24]. Five minutes after FITC-dx administration, a recording was performed as a baseline using a video-recorder (Sony SVT-124p, Sony, Tokyo, Japan). Thirty minutes later, recording was performed again.

Measurement of macromolecular leakage in gastric mucosa

Based on the recorded video images, we measured macromolecular leakage from the PCV in the selected area. Computerized image analysis (GLOBAL LAB® image Ⅱ program, USA) was used to measure fluorescence intensity in the interstitial space and in the PCV at both time points during the experiment. The fluorescence intensities between outside and inside vessels (I_{out}/I_{in}) at baseline and 30-min time points were measured[25]. The molecular leakage in percentage was calculated using the equation: Macromolecular leakage (%) = [(I_{out}/I_{in}) at 30 min - (I_{out}/I_{in}) at baseline]/[(I_{out}/I_{in}) at baseline] × 100.

Assessment of H. pylori infection

The presence of *H. pylori* infection in individual rats was determined by urease test and histological examination by a pathologist. After studying intravital fluorescent microscopy, the rat was terminated by injection of an overdose of thiopental. Then the stomach was removed and longitudinally dissected along the greater curvature. A 2 mm^3 segment of gastric mucosa from the antrum was immediately cut and placed in the urease test tube.

Regarding histological examination, the stomach was fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 at room temperature for 24 h. The tissue was processed, embedded in paraffin, and cut at 5 μm thickness. The sections were stained with hematoxylin and eosin, and microscopically examined for the presence of *H. pylori*. The presence of *H. pylori* was also detected by Warthin-Starry staining in unclear cases. The level of bacterial colonization was recorded by using a grading system as follows, score 0: no bacteria detected; score 1: mild colonization in some gastric crypts; score 2: mild colonization in most gastric crypts; score 3: moderate colonization in all gastric crypts; and score 4: dense colonization in some gastric crypts. The results were presented as the bacterial colonization scores for each group. Moreover, gastric inflammation was classified by using the Sydney system[26]. The infiltrations of mononuclear and polymorphonuclear leukocytes in the gastric mucosa defining the inflammatory scores were recorded. Score 0 to 3 represented normal, mild, moderate, and marked histopathology changes, respectively.

Immunohistochemistry

The stomach sections were deparaffinized with xylene and gradually dehydrated in ethyl alcohol. Next, antigen retrieval was performed by immersing the sections in citric acid buffer (pH 6.0) in a microwave oven for 13 min. Endogenous peroxidase activity and nonspecific binding were blocked with 3% hydrogen peroxide (Merck, Hohenbrunn, Germany) for 5 min and 3% normal horse serum (Gibco, Carlsbad, CA, USA) for 20 min, respectively. After that, the sections were incubated with polyclonal antibody against the p65 subunit of NF-κB (sc109; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 in a humidified chamber for 1 h at room temperature. Then the sections were incubated with biotinylated anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) in the humidified chamber for 30 min. The reaction was visualized using the substrate diaminobenzidine (DAKO). The sections were then counterstained with hematoxylin.

Under light microscope, the expression of NF-κB p65 was cytoplasmic with scattered positive nuclear staining[27]. Thus, NF-κB p65 immunoreactive cells were defined as those with dark brown stain in their nuclei. To quantify, one thousand gastric epithelial cells were counted for each rat under × 40 objective lens. All counting was manually performed by an investigator who was blinded to the treatment groups. The data were shown as percentage of immunoreactive cells calculated from this equation: Percentage of immunoreactive cells (%) = (number of immunoreactive cells × 100)/1000.

Table 1 Demonstrated level of *Helicobacter pylori* colonization and gastric inflammation scores

Group	No.	Level of *H. pylori* colonization					Score of gastric inflammation			
		0	1	2	3	4	0	1	2	3
Control	5	5	-	-	-	-	5	-	-	-
Cur	5	5	-	-	-	-	5	-	-	-
Hp	5	-	5	-	-	-	-	3	2	-
Hp + cur Ⅰ	5	-	5	-	-	-	3	2	-	-
Hp + cur Ⅱ	5	-	3	2	-	-	3	2	-	-

Level of *Helicobacter pylori* (*H. pylori*) colonization: Score 0 = no *H. pylori* detected; Score 1 = mild colonization in some gastric crypts; Score 2 = mild colonization in most gastric crypts; Score 3 = moderate colonization in some gastric crypts; Score 4 = dense colonization in some gastric crypts. Score of gastric inflammation: Score 0 = normal gastric tissue; Score 1 = mild inflammation and histopathology changes; Score 2 = moderate inflammation and histopathology changes; Score 3 = marked inflammation and histopathology changes. Control: Control rats; Cur: Control rats supplemented with 600 mg/kg curcumin; *Hp*: *H. pylori*-infected rats; *Hp* + cur Ⅰ: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin; *Hp* + cur Ⅱ: *H. pylori*-infected rats supplemented with 600 mg/kg curcumin.

Statistical analysis

All data were presented as mean ± SD. The means were compared by one-way analysis of variance (one-way ANOVA) followed by LSD post hoc test. Correlation between the percentages of NF-κB p65 immunoreactive cells and macromolecular leakage was analyzed using Pearson's correlation. All the statistical tests were performed using the computer program SPSS, version 13.0, for Windows (SPSS Inc., Chicago, IL, USA). Probability value of less than 0.05 was considered to be statistically significant.

RESULTS

H. pylori colonization and histological changes

H. pylori infection in rats was judged based on a urease test and histological examination. From histological examination, *H. pylori* colonization was observed in the *Hp* group (Figure 1) as well as in the *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups, whereas colonization was not observed in the Control and Cur groups. Figure 2 demonstrates histological differences between Control and *Hp* groups. The data are shown in Table 1.

Effects of *H. pylori* infection on NF-κB p65 expression and role of curcumin

NF-κB p65 expression in gastric epithelial cells was studied using immunohistochemistry (Figure 3). *H. pylori* infection increased NF-κB p65 expression in gastric epithelial cells. The percentage of immunoreactive cells significantly increased in the *Hp* group (16.02% ± 2.98%) compared with the Control group (10.72% ± 2.1%, $P = 0.004$) (Figure 4A).

The expression of NF-κB p65 in gastric epithelial cells was diminished by curcumin supplementation in both *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups. The percentage of immunoreactive cells significantly decreased in *Hp* + cur Ⅰ (11.79% ± 2.13%, $P = 0.017$) and *Hp* + cur Ⅱ (11.42% ± 1.68%, $P = 0.010$) compared with the *Hp* group (Figure 4A). However, there was no significant difference between the number of immunoreactive cells in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ. Curcumin administration in the Cur group did not alter the baseline NF-κB p65 expression (Cur group, 9.47% ± 3.46%, $P = 0.447$) in gastric epithelial cells.

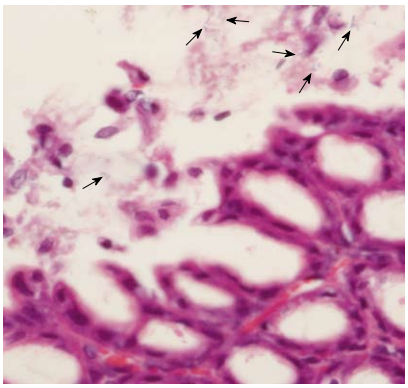

Figure 1 Histological examination (HE stain, × 1000) of *Helicobacter pylori*-infected rats. *Helicobacter pylori* (arrows) in the gastric mucosa identified by the pathologist.

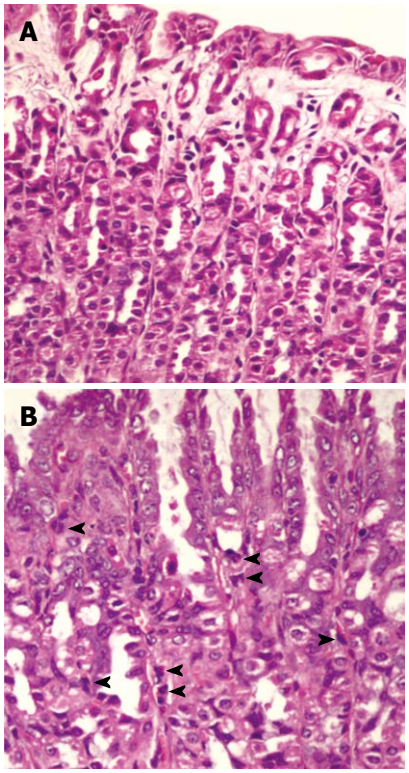

Figure 2 Pathological changes of rat gastric mucosa in control (A) and *Helicobacter pylori*-infected rats (B) (HE stain, × 400). A: Normal gastric mucosa; B: *Helicobacter pylori*-infected gastric mucosa showing inflammatory cell infiltration in the lamina propria (arrowheads).

Effects of *H. pylori* infection on macromolecular leakage and role of curcumin

The macromolecular leakage was studied by intravital fluorescent videomicroscopy. The captured images of

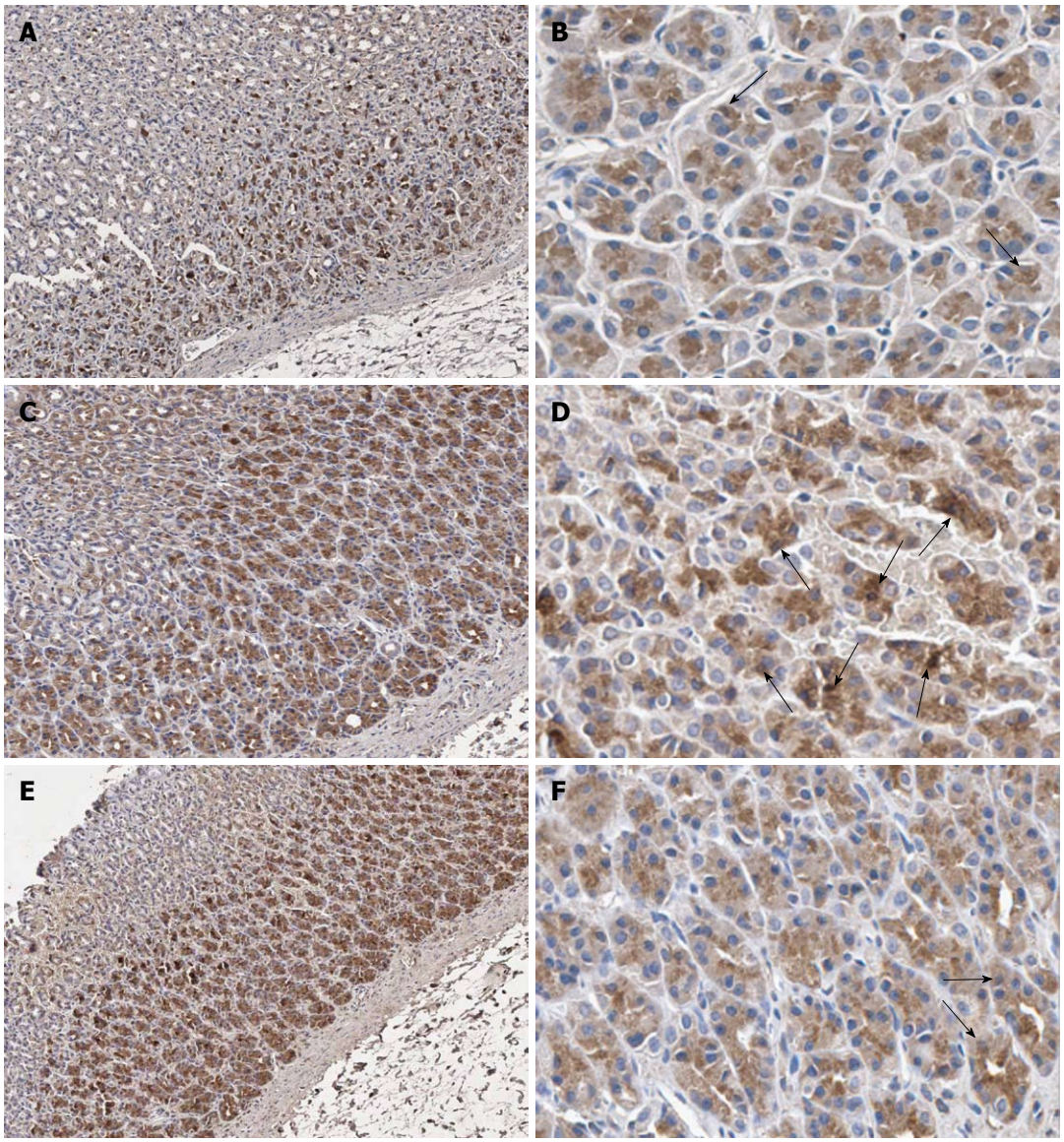

Figure 3 Immunohistochemical staining of nuclear factor-κB p65 antibody in representative tissue specimens. A, B: Control rats; C, D: *Helicobacter pylori* (*H. pylori*)-infected rats; E, F: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin. Nuclear counterstaining was performed with hematoxylin. The examples of immunoreactive cells are those with dark brown stain in their nuclei (arrows). Images were obtained at × 100 (A, C and E) and × 400 (B, D and F).

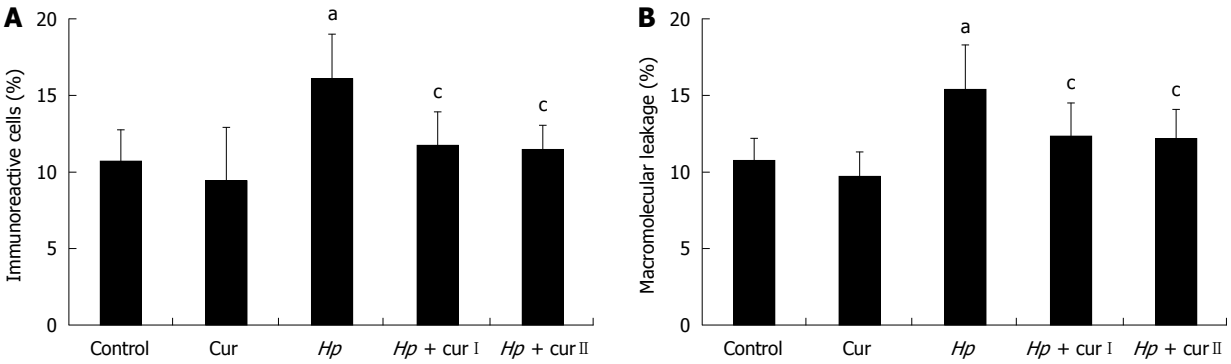

Figure 4 mean ± SD of the percentage of nuclear factor-κB p65 immunoreactive cells (A) and macromolecular leakage (B) in all experimental groups. $^{a}P < 0.05$ *vs* control rats (Control); $^{c}P < 0.05$ *vs Helicobacter pylori* (*H. pylori*)-infected rats (*Hp*). Cur: Control rats supplemented with 600 mg/kg curcumin; *Hp* + cur Ⅰ: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin; *Hp* + cur Ⅱ: *H. pylori*-infected rats supplemented with 600 mg/kg curcumin.

gastric mucosal microcirculation from Control, *Hp*, and *Hp* + cur I groups at the 30-min time point are shown in Figure 5. *H. pylori* infection led to a significant increase of macromolecular leakage in the *Hp* group (15.41% ±

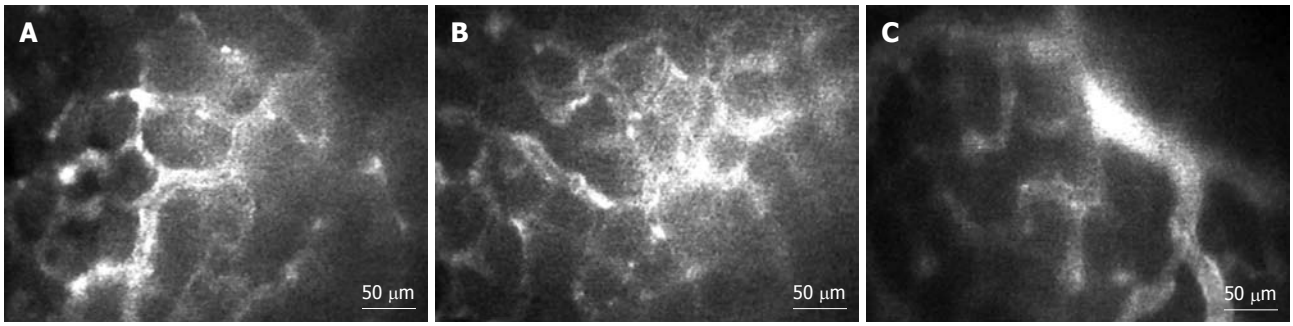

Figure 5 Intravital fluorescent microscopic images (× 200) demonstrate macromolecular leakage from vessels to the interstitial space at 30-min time points. A: Control rats; B: *Helicobacter pylori* (*H. pylori*)-infected rats; C: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin.

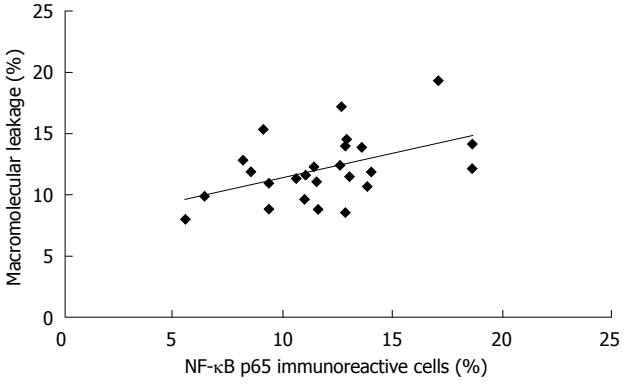

Figure 6 Correlation between the percentage of nuclear factor-κB p65 expression in gastric epithelial cells and the macromolecular leakage (r^2 = 0.2228, P = 0.017). NF-κB: Nuclear factor-κB.

2.83%) compared with the Control group (10.69% ± 1.43%, P = 0.001) (Figure 4B).

Oral treatment with curcumin over 1 wk attenuated *H. pylori* infection-induced macromolecular leakage significantly in both *Hp* + cur I (12.32% ± 2.13%, P = 0.025) and *Hp* + cur II (12.14% ± 1.86%, P = 0.018) groups (Figure 4B). However, there was no significant difference between the extent of macromolecular leakage in *Hp* + cur I and *Hp* + cur II. In addition, macromolecular leakage showed no significant differences between the Control group and the Cur group (Cur group, 9.74% ± 1.5%, P = 0.463).

Relationship between NF-κB p65 expression and macromolecular leakage

The percentages of NF-κB p65 immunoreactive cells and of macromolecular leakage from the same rat in all groups were plotted against each other (Figure 6). Interestingly, the level of NF-κB p65 expression was moderately correlated with the degree of macromolecular leakage (r^2 = 0.2228, P = 0.017).

DISCUSSION

The present study demonstrated that *H. pylori* infection activated NF-κB in gastric epithelial cells. This result corresponds to previous observations studied in both *in vitro* and *in vivo* models[4,19,28]. NF-κB is an important transcription factor which activates many genes involved in inflammatory and immune responses[5,6]. Activated NF-κB induced by *H. pylori* infection upregulates cytokine production and is associated with gastric inflammation[9]. Clinically, NF-κB was also seen to be activated in the stomach of patients with *H. pylori*-induced gastritis[29-31]. Therefore, gastric epithelial NF-κB activation may play an important role in the initiation of *H. pylori*-induced gastric inflammation.

From the results, this study can also demonstrate an increased macromolecular leakage after *H. pylori* infection. This alteration is in good agreement with previous reports[12,14]. Several mechanisms may contribute to the increased macromolecular leakage. A previous study demonstrated that the transmigration of activated neutrophils expressing a specific protein could regulate endothelial permeability, allowing macromolecules to leak[32]. Furthermore, many proinflammatory cytokines such as TNF-α and interleukin (IL)-1β could be directly modulating vascular permeability[33-35]. Neutrophil transmigration and proinflammatory cytokine production were also suggested in a NF-κB-dependent manner in gastric mucosa during *H. pylori* infection[4,8,36-38]. *H. pylori* infection may directly influence the leakage *via* the transportation of *H. pylori* toxin and activation of NF-κB in endothelial cells. Previously, *in vitro* studies indicated that endothelial cells infected with *H. pylori* have changes in protein expression and function[39,40]. Our results substantiate these findings by showing that increased NF-κB p65 expression in gastric epithelial cells is accompanied by increased macromolecular leakage during *H. pylori* infection. Thus, the increased macromolecular leakage may result from inflammatory mediator production and vascular permeability changes through *H. pylori*-induced NF-κB activation.

Our experiments show that curcumin supplementation can suppress *H. pylori*-induced gastric inflammation, as indicated by decreased NF-κB p65 expression in gastric epithelial cells and decreased macromolecular leakage in the gastric microcirculation. The activation of NF-κB is essential for transcription of many genes involved in inflammatory and immune responses influencing gastric inflammation induced by *H. pylori* infection. In the present study, NF-κB p65 expression in the nucleus indicated that curcumin may possibly suppress the translocation of activated NF-κB into transcriptional sites. *H. pylori*-

induced NF-κB activation affects recruitment of neutrophils and vascular permeability that reflect gastric inflammation. Curcumin decreased these parameters, indicating that curcumin could decrease gastric inflammation.

Inhibition of *H. pylori* growth was unlikely to be a mechanism that contributed to the effect of curcumin observed in this study, since positive results regarding *H. pylori* infection were still obtained from both urease test and histological examination after curcumin treatment in *Hp* + cur I and *Hp* + cur II animals. Recently, Di Mario *et al*[21] demonstrated that 7-d treatment with curcumin significantly improved gastric inflammation in *H. pylori*-positive patients despite *H. pylori* persistence. However, eradication by curcumin may be dependent on a high dose of curcumin, the safety of which has to be confirmed in animals and humans[41].

A previous study demonstrated that curcumin at the doses of 200 mg/kg and 600 mg/kg had an anti-inflammatory property[42]. In this study, 200 mg/kg curcumin was a sufficient dose for reducing gastric epithelial NF-κB p65 expression and mucosal macromolecular leakage. The possible mechanism cited was that curcumin inhibited *H. pylori*-induced NF-κB activation. This finding corresponded to an earlier *in vitro* study showing that *H. pylori*-induced NF-κB activation and the subsequent release of IL-8 were inhibited by curcumin[19]. In addition, the correlation between NF-κB p65 expression and macromolecular leakage found in our study suggests that *H. pylori*-induced mucosal macromolecular leakage may be mediated *via* NF-κB activation in gastric epithelial cells. Thus, the decreased macromolecular leakage may be explained by the reduction of inflammatory mediators due to epithelial NF-κB inhibition by curcumin.

In conclusion, the present study showed that *H. pylori* infection induced gastric epithelial NF-κB activation and increased mucosal macromolecular leakage. Curcumin supplementation may exert its anti-inflammatory effect by reducing macromolecular leakage through the suppression of NF-κB p65 expression in gastric epithelial cells. Hence, curcumin might be a novel therapeutic strategy against gastric inflammation induced by *H. pylori* infection.

COMMENTS

Background

The pathogenesis of *Helicobacter pylori* (*H. pylori*) infection is associated with bacterial virulence factors. The virulence factors can induce the activation of nuclear factor (NF)-κB in gastric epithelial cells, causing gastric inflammation and inducing vascular damage. Curcumin has many biological activities, including anti-inflammatory properties resulting from inhibition of NF-κB.

Research frontiers

Curcumin (diferuloylmethane) is an active ingredient of *Curcuma longa* (turmeric) that has many biological activities mediated by the efficient inhibition of NF-κB. *H. pylori* infection induces gastric epithelial NF-κB activation and increases mucosal macromolecular leakage. The hotspots of this study indicate that curcumin supplementation may exert its anti-inflammatory effect by reducing macromolecular leakage through the suppression of NF-κB p65 expression in gastric epithelial cells.

Innovations and breakthroughs

A previous study showed that curcumin is a potent antibacterial agent against *H. pylori* and can inhibit NF-κB activation in *H. pylori*-infected gastric epithelial cells *in vitro*. However, it is not clear whether curcumin has any *in vivo* effects on *H. pylori*-induced gastric inflammation. Therefore, in this study, the authors examined the anti-inflammatory effect of curcumin, which was shown to reduce mucosal macromolecular leakage through the suppression of gastric epithelial NF-κB p65 expression induced by *H. pylori* infection *in vivo* in rats.

Applications

Curcumin might be a novel therapeutic strategy against gastric inflammation induced by *H. pylori* infection.

Peer review

The merits of the manuscript are in showing the conclusion that curcumin can reduce the gastric inflammation reflected in attenuated levels of NF-κB and leakage of dextran.

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4047

World J Gastroenterol 2010 August 28; 16(32): 4047-4054
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

High expression of ErbB2 contributes to cholangiocarcinoma cell invasion and proliferation through AKT/p70S6K

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Supported by A grant from Mahidol University, Thailand (to Suthiphongchai T); and a scholarship from the Royal Golden Jubilee Ph.D. Program, the Thailand Research Fund (to Treekitkarnmongkol W)
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Received: March 7, 2010 Revised: April 21, 2010
Accepted: April 28, 2010
Published online: August 28, 2010

Abstract

AIM: To compare the impact of ErbB2 on cell invasion and proliferation in cholangiocarcinoma (CCA) cell lines.

METHODS: Level of endogenous ErbB2 expression in three CCA cell lines, namely HuCCA-1, KKU-100 and KKU-M213, was determined by real-time reverse-transcriptase polymerase chain reaction. Two ErbB2 inhibitory methods, a small molecule ErbB2 kinase inhibitor (AG825) and siRNA, were used to disrupt ErbB2 function in the cell lines. CCA cell invasion, motility and proliferation under ErbB2-disrupted conditions were detected using Transwell and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. In addition, ErbB2 downstream effectors were investigated by Western blotting analysis.

RESULTS: Suppression of ErbB2 activity, using a specific kinase inhibitor (AG825), reduced invasion, motility and proliferation of all three CCA cell lines. The ability of this drug to inhibit neoplastic properties (invasion, motility and proliferation) increased concomitantly with the level of ErbB2 expression. Similarly, knockdown of ErbB2 level by siRNA inhibited cell invasion and proliferation of KKU-M213, a high-ErbB2-expressing cell, better than those of the lower-ErbB2-expressing cells, HuCCA-1 and KKU-100. Thus, both inhibitory methods indicated that there is more ErbB2-dependency for malignancy of the high-ErbB2-expressing cell, KKU-M213, than for that of low-ErbB2-expressing ones. In addition, interrupting ErbB2 activity decreased phosphorylation of AKT and p70S6K, but not extracellular signal-regulated kinase 1/2, in the high-ErbB2-expressing CCA cell line.

CONCLUSION: Our data indicated that high ErbB2 expression enhances CCA invasion, motility and proliferation *via* the AKT/p70S6K pathway, which suggests the possibility of targeting these molecules for CCA therapy.

Key words: AKT; Cholangiocarcinoma; ErbB2; Invasion; p70S6K; Cell proliferation

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Treekitkarnmongkol W, Suthiphongchai T. High expression of ErbB2 contributes to cholangiocarcinoma cell invasion and proliferation through AKT/p70S6K. *World J Gastroenterol* 2010; 16(32): 4047-4054 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4047.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4047

INTRODUCTION

Cholangiocarcinoma (CCA) is an incurable and lethal cancer. Its incidence has been increasing worldwide during the past three decades and all cases have almost the same mortality rate[1]. Strikingly, the highest incidence of CCA

has been found in Northeastern Thailand (96 per 100 000 men), followed by China and Japan[2]. As a result of the lack of a specific tumor marker and its silent symptoms, this tumor is difficult to diagnose and the majority of patients present at the late stage (stage Ⅲ or Ⅳ) of disease progression[3]. From 70% to 80% of CCA patients are inoperable and are generally treated by chemotherapy and radiation, which unfortunately have no significant impact on long-term survival[3]. Thus, new diagnostic/prognostic markers and targeted therapies for CCA need to be investigated.

Human epidermal growth factor receptor (EGFR) 2, ErbB2 (also known as HER2/Neu), belongs to subclass I of receptor tyrosine kinases in the ErbB family. Unlike other members of the ErbB family, the conformation of the ErbB2 ectodomain is similar to the ligand-activated state of other ErbBs[4]. Therefore, activation of ErbB2 can occur without ligand binding[5]. Overexpression of ErbB2 results in receptor dimerization (either homodimerization with its own or heterodimerization with other ErbBs). Upon dimerization, cytoplasmic domains of ErbBs become autophosphorylated and act as docking sites for downstream proteins that bear an Src-homology 2 or a phosphotyrosine-binding domain, which recognizes specific phosphorylated tyrosine sites in the receptors, and leads to activation of such downstream pathways as mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT[6]. Furthermore, ErbB2 can block internalization of EGFR for degradation, which results in an elevation in the membrane steady state level[7]. Thus, aberrant ErbB2 expression can lead to an extensive activation of its downstream signals and an aberration from the normal regulation, which promotes cancer development.

ErbB2 overexpression is found in approximately 30% of human breast carcinomas and in many other types of human malignancies, such as prostate, pancreas, colon and ovarian cancers[8]. Cancer patients with high ErbB2 expression tend to have a more aggressive disease, identified by clinical outcomes such as high metastasis and low response to treatment. Typically, ErbB2-positive tumors have high proliferation rates and more extensive invasion, with frequent metastasis[9]. In addition, clinical studies using ErbB2-targeted therapy with trastuzumab (a humanized monoclonal antibody against ErbB2) have shown that the response rates of breast cancer patients with ErbB2 overexpression are higher than those with normal ErbB2 expression[10].

Several lines of evidences have implicated the role of ErbB2 in aggressive forms of CCA. High expression of ErbB2 has been found in a variety of non-cancerous biliary proliferative diseases, such as in hepatolithiasis and primary sclerosing cholangitis, both of which are risk factors associated with CCA[11,12]. Immunohistochemical data have demonstrated that 20%-30% of tumor specimens from CCA patients show moderate to strong immunostaining for ErbB2[13], and most of them confer poor clinical outcomes (high metastasis and low survival rate)[13-16]. Moreover, about 30% of transgenic mice that constitutively express wild-type Neu develop gallbladder cancer and intrahepatic CCA within 8 mo[17]. Although many reports have indicated the involvement of ErbB2 in cholangiocarcinogenesis and CCA progression, the mechanism of aberrant ErbB2 expression in promoting CCA progression remains unclear.

In this study, the role of ErbB2 in governing the malignant phenotype (invasion and proliferation) of CCA was investigated by suppressing ErbB2 function in three human CCA cell lines that expressed different levels of ErbB2. Two strategies, inhibition of its kinase function and siRNA, were used to reduce ErbB2 activity. We demonstrated that downregulation of ErbB2 expression and activity suppressed CCA cell invasion, motility and proliferation, particularly in the high-ErbB2-expressing cells. Downstream signaling pathways of ErbB2 also were investigated.

MATERIALS AND METHODS

Cell culture

HuCCA-1 cell line was a generous gift from Professor Stitaya Sirisinha (Mahidol University, Thailand)[18] and KKU-100[19] and KKU-M213 cell lines were kindly provided by Dr. Banchob Sripa, (Khon Kaen University, Thailand). These three CCA cell lines were developed from Thai patients. The cells were maintained in HAM's F-12 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2 mmol/L glutamine, 15 mmol/L HEPES and 14 mmol/L sodium bicarbonate, 100 U/mL penicillin G and 100 U/mL streptomycin. All cell cultures were incubated at 37℃, in a 5% CO_2 humidified atmosphere.

Quantitative polymerase chain reaction

Cells (80% confluent) were harvested with 0.1% trypsin/EDTA and RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Two micrograms of RNA were converted into cDNA using the Superscript™ RNase H Reverse Transcriptase kit (Invitrogen), which was amplified by quantitative polymerase chain reaction (PCR) (ABI 7500; Applied Biosystems, Foster City, CA, USA) in a 20-μL reaction volume that contained 0.5 U HotStart *Taq* polymerase (Qiagen), 1 × FastStart Universal SYBR Green Master cocktail (Roche, Germany) and 4 pmol of specific primer pairs (5'-CCAGGACCTGCTGAACTGGT-3' and 5'-TGTACGAGCCGCACATC-3' for ErbB2[20] and 5'-CTCTTCCAGCCTTCCTTCCT-3' and 5'-AGCACTGTGTTGGCGTACAG-3' for β-actin[21], used as internal control). The reactions were started with an initial heat activation step at 95℃ for 15 min and the following thermal cycling conditions: 94℃ for 30 s, 58℃ for 30 s and 72℃ for 1 min. ErbB2 mRNA levels among the test cells were determined using the $2^{-\Delta Ct}$ method[22].

Immunoblot assay

Cells transfected with siRNA (for 72 h) or treated with AG825 (for 6 h) were washed twice with PBS and lysed on ice with freshly prepared lysis buffer that contained 150 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 5 mmol/L EGTA, 5 mmol/L EDTA, 0.1% SDS, 1% so-

dium deoxycholate, 1% Nonidet P-40, 1 × protease inhibitor cocktail (Roche Diagnostics, Germany), 50 mmol/L NaF, 2 mmol/L Na_3VO_4, 40 mmol/L β-glycerophosphate, and 1 mmol/L dithiothreitol. Cells were centrifuged at 12000 × *g* for 15 min. Protein lysate (80 μg) was separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Munchen, Germany). After incubating with a blocking solution (5% skimmed milk/TBST), membranes were treated with primary antibodies specific for ErbB2, phospho-ErbB2 Y1248 (Labvision, Fremont, CA, USA), β-actin, AKT, phospho-AKT T308 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1/2, phospho-ERK1/2, p70S6K, and phospho-p70S6K T389 (Cell Signaling, Beverly, MA, USA), and then with horseradish-preoxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Signals were detected using enhanced chemiluminescence (ECL plus) (GE Healthcare, Little Chalfont, Bucks, UK) and quantified by Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

siRNA transfection

Two Silencer® validated siRNAs against ErbB2 (Ambion, Austin, TX, USA) were used to target mRNA at different exons. CCA cells were transiently transfected with siRNA using Effectene (Invitrogen) following the manufacturer's protocol. In brief, 3.25 μg of siErbB2 was mixed with Effectene and Enchancer (32.5 and 26.0 μL), incubated for 5 min, and then added to HAM's F-12 medium that contained 10% FBS. The mixture was added to 80% confluent CCA cells in 60-mm dishes that contained 10% FBS medium. After 6 h of incubation, medium was removed, cells were washed with PBS and replenished with fresh medium. Cells transfected with Silencer® Cy™-3 labeled non-targeting siRNA (Ambion) were used as a negative control. Protein expression, cell invasion and motility were determined at 72 h post-transfection and cell proliferation was analyzed during 24-96 h post-transfection.

In vitro invasion and motility assay

Cell invasiveness was determined using a Transwell chamber (6.5-mm diameter polyvinylpyrrolidone-free polycarbonate filter of 8-μm pore size) (Corning, NY, USA) precoated with 30 μg Matrigel (BD Biosciences, San Jose, CA, USA). A 200-μL aliquot of cells (10^5) transfected with siRNA or treated with various concentrations of AG825 in 0.2% FBS medium was added to the upper compartment of the Transwell, and 10% FBS medium was added to the lower chamber. After 6 h of incubation at 37℃ in a humidified CO_2 incubator, non-invaded cells in the upper compartment were removed with a cotton swab, and the invaded cells were fixed and stained with 0.5% crystal violet in 25% methanol for 30 min, followed by washing twice with tap water. Finally, the invaded cells were counted under a microscope with a 10 × objective in five random fields. Cell motility assay was performed as described for the invasion assay but using a Matrigel-free system.

Cell viability assay

Cells (3000/well) transfected with siRNA or resuspended in various concentrations of AG825 were plated onto 96-well plates and incubated for 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as follows. Fifty micrograms of MTT were added to each well and the cells were incubated for a further 4 h. The supernatant was removed and insoluble formazan dye produced from MTT by living cells was solubilized by 200 μL DMSO. Percentage cell viability was quantified by measuring absorbance at 540 nm. Cell survival during the invasion assay was determined in the same way, except that 5000 cells/well were incubated for 6 h prior to addition of MTT.

Statistical analysis

Data are presented as the mean ± SE from three independent experiments conducted in triplicate. Comparison of data between groups was analyzed by one-way analysis of variance, followed by Newman-Keuls multiple comparison test (GraphPad Software, La Jolla, CA, USA).

RESULTS

ErbB2 mRNA expression level in human CCA cell lines and inhibition of its phosphorylation by AG825

The role of ErbB2 in CCA was investigated in three cell lines that were established from tumor tissues of Thai CCA patients, namely, HuCCA-1, KKU-100 and KKU-M213. Steady-state level of ErbB2 mRNA was determined by quantitative reverse-transcriptase-PCR, normalized to β-actin mRNA. KKU-M213 cell line expressed the highest level of ErbB2 mRNA, with that of HuCCA-1 and KKU-100 being comparable at 60% (Figure 1A).

Like other tyrosine kinase receptors, ErbB2 is activated by phosphorylation. Total and phosphorylated ErbB2 levels in the three CCA cell lines as analyzed by Western blotting showed correspondence with levels of ErbB2 mRNA, but in this case, the level of phosphorylated ErbB2 in KKU-100 cells was significantly lower than that in HuCCA-1 (Figure 1B). Inhibition of ErbB2 activity by treating for 6 h with its specific kinase inhibitor, AG825 (30 μmol/L), resulted in marked reduction ErbB2 phosphorylation at Y1248 in all three cell lines, without any significant effect on total ErbB2 levels (Figure 1B).

Invasive and proliferative abilities of CCA cell lines

The abilities of the three CCA cell lines to migrate and invade were determined by Transwell *in vitro* assays. Among the three cell lines, KKU-M213 showed the highest invasive and motility abilities, which were related to its level of ErbB2 expression (Figure 2A). However, KKU-100, which contained the lowest phospho-ErbB2 level, still had a somewhat high invasive ability (about four fold higher than HuCCA-1, but still lower than KKU-M213). This suggested that other pathways might be involved in enhancing the invasive ability of KKU-100. However, the proliferative rates of the three CCA cell lines as assessed by MTT assay were comparable (Figure 2B), which suggested that ErbB2 is not the key player in regulating proliferation of these cell lines.

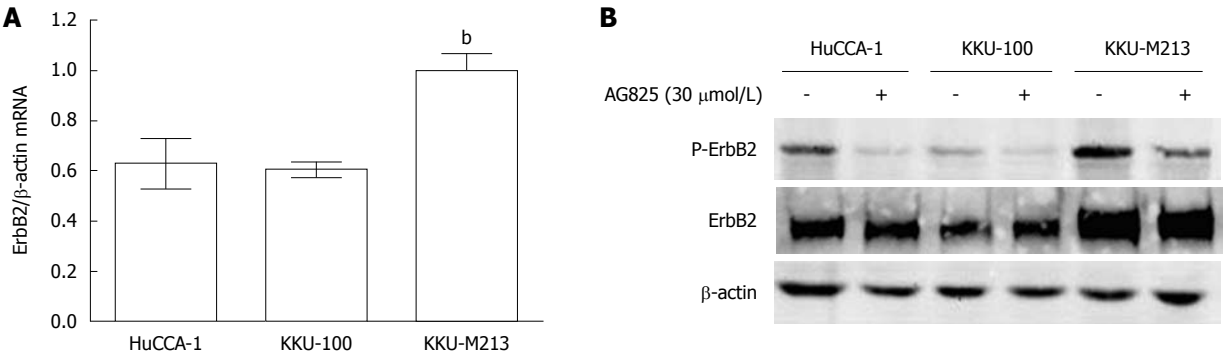

Figure 1 Levels of ErbB2 expression in three cholangiocarcinoma cell lines and inhibition of ErbB2 phosphorylation by AG825. A: Cells cultured in 10% fetal bovine serum (FBS)-containing medium were lysed and the lysates were analyzed for ErbB2 mRNA by real-time reverse-transcriptase polymerase chain reaction. Data are presented as mean ± SE of ErbB2 mRNA level normalized with β-actin mRNA obtained from three independent experiments; B: Cells were incubated without and with 30 μmol/L AG825 in 10% FBS medium for 6 h, then lysed and analyzed for ErbB2, phospho-ErbB2 (Y1248) and β-actin by Western blotting. The results shown are representative of three separate experiments. $^{b}P < 0.01$ *vs* HuCCA-1 and KKU-100.

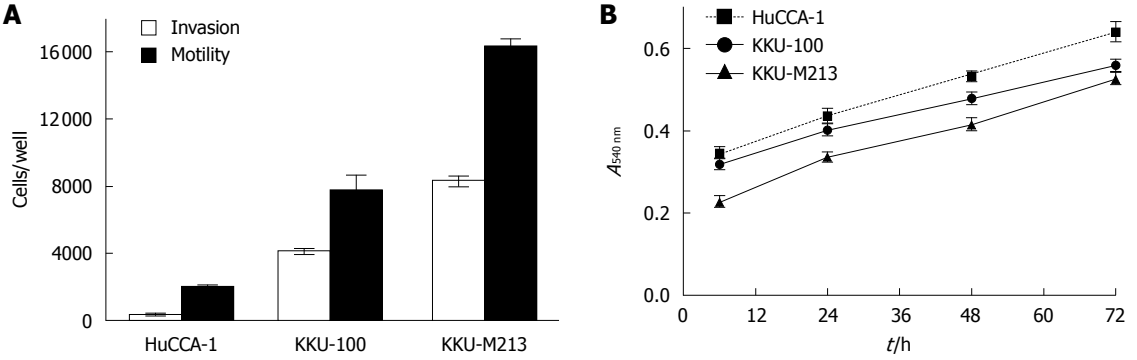

Figure 2 Invasion motility and proliferation of three cholangiocarcinoma cell lines. A: Cells resuspended in 0.2% fetal bovine serum (FBS) medium were plated in the upper compartment of a Transwell chamber coated with and without Matrigel, for invasion and motility assays. The lower compartment was filled with 10% FBS medium. After 6 h incubation, numbers of invading/motile cells were counted; B: Cells were plated on 96-well plates in 10% FBS medium. After incubation for 12-72 h, cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as mean ± SE of the percentage relative to controls obtained from three separate experiments.

Effects of ErbB2 kinase inhibitor on CCA cell invasion and proliferation

As ErbB2 has been detected in CCA and other related cancers, and its expression level has been shown to correlate with highly proliferating diseases of the bile duct and with the degree of CCA local invasion and metastasis[11,23], the role of ErbB2 on invasion and motility of the CCA cell lines was investigated by examining the effects of AG825 on these properties using a Transwell assay. Treatment of AG825 (10-100 μmol/L) for 6 h suppressed cell invasion and motility (Figure 3A-C) in all three CCA cell lines with different IC_{50} values, with KKU-M213 being the most sensitive and KKU-100 the least. During the assay period, the drug marginally affected CCA cell survival (Figure 3A-C), which indicated that inhibition of invasion and motility were not due to drug cytotoxicity. However, incubation with AG825 for 72 h resulted in inhibition of cell proliferation, with KKU-M213 showing the most sensitivity and KKU-100 the least (Figure 3D).

Effects of suppression of ErbB2 expression on CCA cell invasion, motility and proliferation

As ErbB2 kinase inhibitor experiments indicated the role of ErbB2 on malignant phenotypes (invasion, motility and proliferation) of CCA cell lines, we confirmed the specificity of this phenomenon using siRNA targeting of ErbB2 mRNA to suppress *ErbB2* gene expression. In KKU-M213, the CCA cell line that expressed the highest ErbB2 level among the three cell lines, ErbB2 level was reduced in a time-dependent manner (up to 72 h), and was partially restored at 96 h post-transfection (Figure 4A). After 72 h of siRNA transfection, ErbB2 level in all three CCA cell lines was reduced by > 70% (Figure 4B). During this post-transfection period, there was attenuation of invasion and motility of KKU-M213 and HuCCA-1 cells, but not of KKU-100 cells (Figure 5A and B). Although proliferation of KKU-M213 cells was suppressed, that of HuCCA-1 and KKU-100 cells was marginally affected (Figure 5C).

These results on suppression of *ErbB2* gene expression, together with those conducted on inhibition of ErbB2 kinase activity, indicated that a threshold level of ErbB2 is necessary to induce properties associated with malignancy (invasion, motility and proliferation) in CCA cells (viz. KKU-M213).

Involvement of ErbB2 in activation of downstream effectors, AKT, p70S6K and ERK1/2

ERK1/2 and PI3K/AKT are the two major signaling

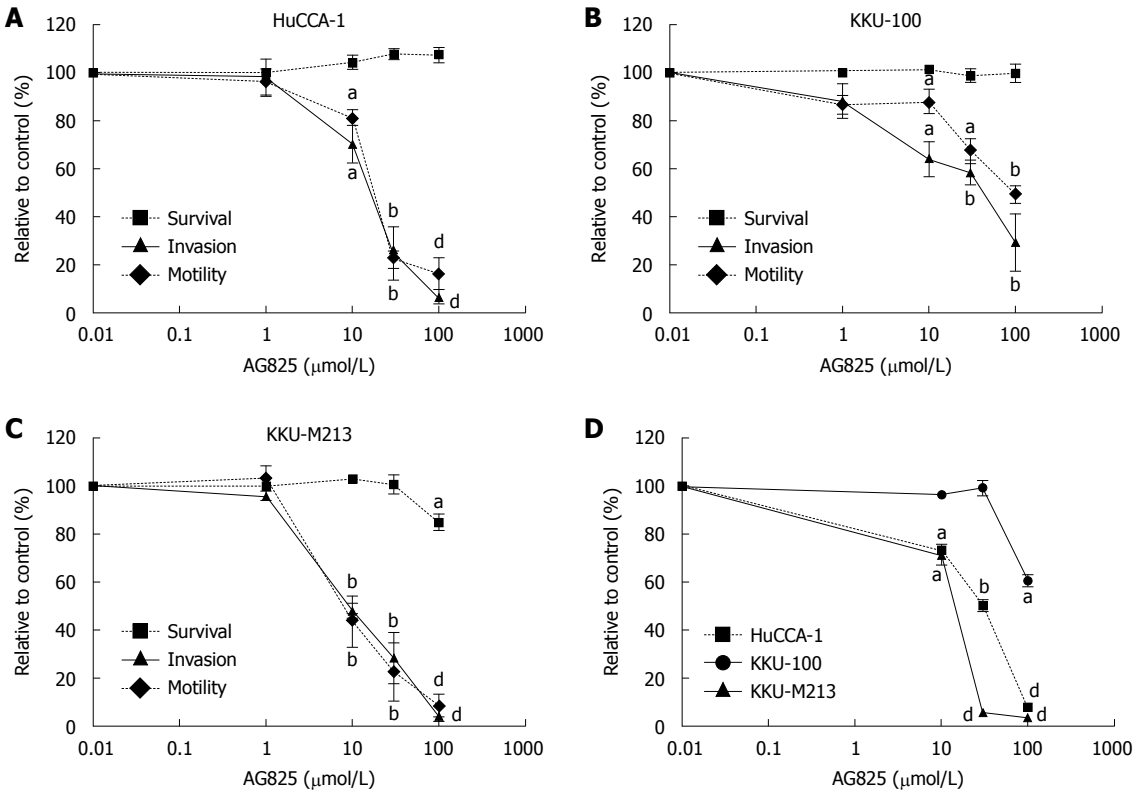

Figure 3 Effects of AG825 on cholangiocarcinoma cell invasion, motility and proliferation. A-C: Cells resuspended in medium that contained 0.2% fetal bovine serum (FBS) and indicated concentrations of AG825 were plated in the upper compartment of a Transwell chamber coated with and without Matrigel, for invasion and motility assays. After 6 h incubation, numbers of invading/motile cells were counted. Cells treated with the same vehicle were used as controls. Cell survival after 6 h of AG825 treatment was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; D: Cells were treated with various concentrations of AG825 in 10% FBS medium for 72 h, and cell survival was determined using the MTT assay. Data are presented as mean ± SE of the percentage relative to the controls obtained from three separate experiments, each done in duplicate. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control.

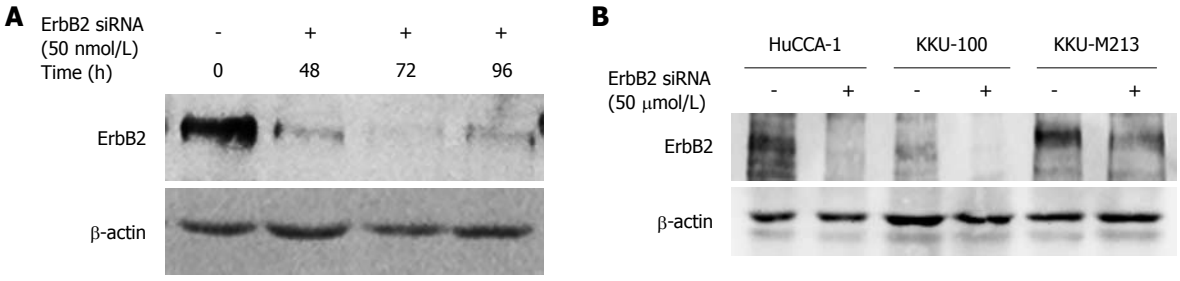

Figure 4 Inhibition of ErbB2 expression by siRNA against ErbB2. A: KKU-M213 cells were transfected with siErbB2 and the levels of ErbB2 at 0-96 h after transfection were determined by Western blotting; B: After 72 h of siRNA transfection, ErbB2 expression in HuCCA-1, KKU-100, and KKU-M213 cells was determined. Scramble siRNA (siNeg) was used as a negative control. The results shown are representatives of two (A) and three (B) separate experiments.

pathways that regulate cell proliferation, motility and invasion in response to a variety of growth factors/receptor tyrosine kinases[24]. To investigate the pathways by which ErbB2 promoted invasion and proliferation of CCA cells, we examined the effects of ErbB2 inhibition (by kinase inhibitor or siRNA) on effectors activated by ErbB2 in these two main signaling pathways. Suppression of *ErbB2* gene expression by siRNA transfection (72 h) reduced phospho-AKT level in all three CCA cell lines, but without affecting total AKT level (Figure 6A). Phosphorylation of its downstream effector, p70S6K, was similarly affected in KKU-M213 and HuCCA-1 cells, but not in KKU-100 cells (Figure 6A). On the other hand, ERK1/2 phosphorylation was marginally, if at all, affected in all three CCA cell lines (Figure 6A). Similarly, treatment of KKU-M213 cells with AG825 (30 μmol/L, 6 h) suppressed AKT and p70S6K phosphorylation but did not affect phospho-ERK1/2 level (Figure 6B). Thus, the PI3K/AKT/p70S6K and not ERK1/2 pathway is responsive to ErbB2 activity related to CCA cell invasion, motility and proliferation.

DISCUSSION

ErbB2 is overexpressed in 20%-30% of epithelial bile duct cancers[13]. Its overexpression is associated with lymph node metastasis in intrahepatic CCA[23]. Unlike

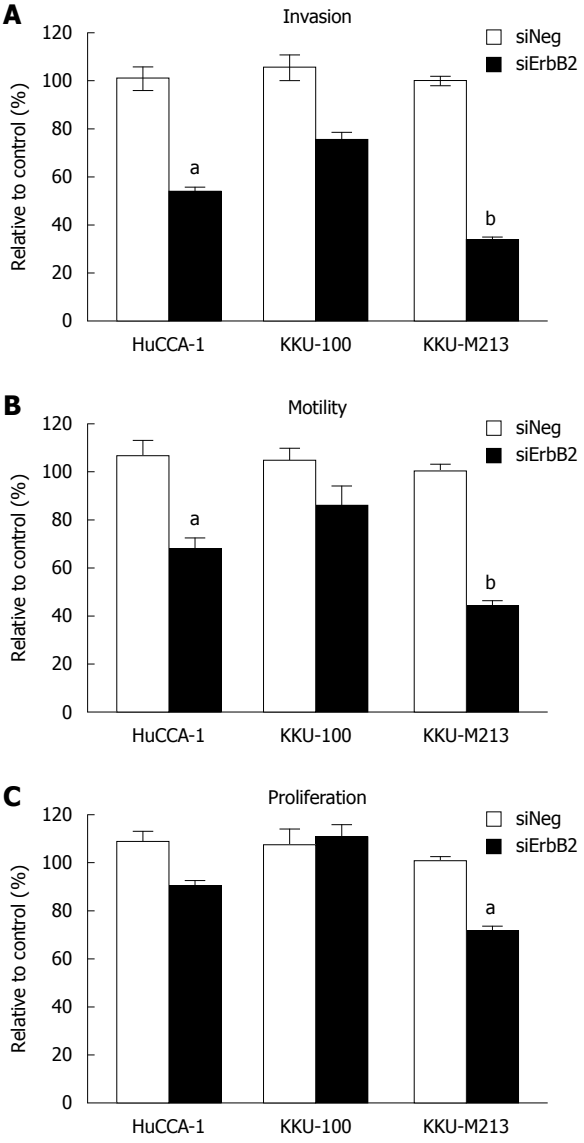

Figure 5 Effects of ErbB2 knockdown on cell invasion motility and proliferation of the three cholangiocarcinoma cell lines. Cells transfected with siRNA (negative control and ErbB2 targeted siRNA) for 72 h were analyzed for *in vitro* invasion (A) and motility (B) by using a Transwell chamber coated with and without Matrigel. After 6 h of incubation, numbers of invading/motile cells were counted. Cell proliferation (C) was analyzed by determining the number of viable cells during 24-96 h post-transfection by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as mean ± SE of the percentage relative to the controls obtained from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls.

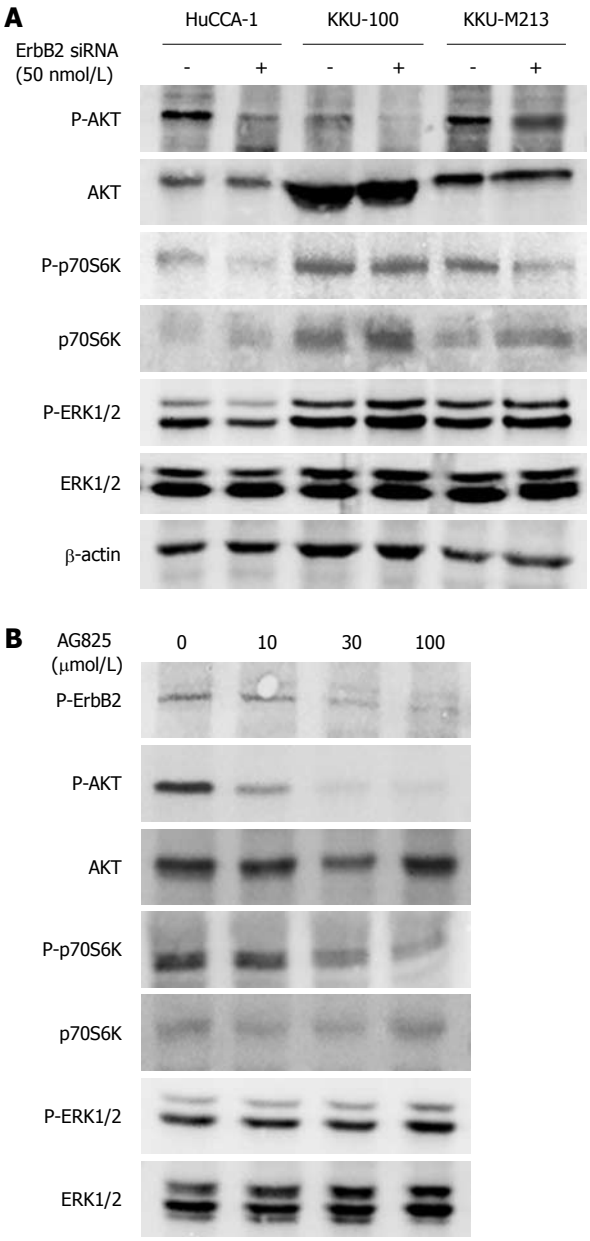

Figure 6 Effects of ErbB2 inhibition (using siRNA and specific kinase inhibitor) on ERK1/2, AKT, and p70S6K phosphorylation in the cholangiocarcinoma cell lines. A: Cholangiocarcinoma cell lines were transfected with siErbB2 and siNeg, as a control, for 72 h and the collected cell lysates were analyzed by Western blotting; B: KKU-M213 cells were treated without and with AG825 in medium that contained 10% fetal bovine serum for 6 h, then lysed and analyzed by Western blotting. The results shown are representatives of three separate experiments.

other ErbBs, ErbB2 is not expressed in normal adult liver (both hepatocytes and cholangiocytes) or in fetal liver[11,25] which makes it an interesting candidate for molecular targeting therapy for liver and bile duct cancers.

The mechanism by which ErbB2 exerts tumorigenicity in CCA is not completely understood. Here, the role of ErbB2 involved in controlling critical characteristics of CCA, namely, invasion, motility and proliferation, was investigated in three human CCA cell lines that expressed different levels of ErbB2, as shown by measurements of mRNA and phosphorylated (P-Y1248) ErbB2 levels. Data showed that ErbB2 was more important for these properties in the highest ErbB2-expressing cell line (KKU-M213), through activation of the AKT/p70S6K pathway.

Upregulation of ErbB2 is implicated in cancer aggressiveness. Studies in transgenic mice have revealed that animals bearing either an activated form of Neu (ErbB2) or overexpressing wild-type Neu frequently develop mammary tumors and lung metastases[26,27]. ErbB2 overexpression plays an important role in cell proliferation and invasion of many cancers, especially those of breast, ovary, stomach and bladder[28,29]. In breast cancer, application of antisense RNA or siRNA against ErbB2 inhibits growth

of high-ErbB2-expressing cells, but has only a little effect on lower-ErbB2-expressing cells[30,31]. In the present study, disruption of ErbB2, by both inhibition of ErbB2 kinase activity using AG825 and knock-down of ErbB2 expression using siRNA, suppressed the neoplastic phenotype of the high-ErbB2-expressing CCA cell line, KKU-M213, to a greater extent than that of the low-ErbB2-expressing cell line, KKU-100. This indicated that the neoplastic phenotype of the high-ErbB2-expressing cell line, KKU-M213, was highly dependent on ErbB2 compared to the low-ErbB2-expressing cells.

There are several possible explanations for the increase in the requirement of ErbB2 in cancer malignancy. Firstly, in cancer cells that have evolved high ErbB2 expression, this pathway has become predominant in regulating cell proliferation and invasion, which results in cells with dependency on this protein. Moreover, Kaelin[32] has suggested that an increase in the requirement of a given protein for cancer cell survival is due either to intrinsic (genetic/epigenetic) or extrinsic (microenvironment) changes. In this case, accumulation of genetic/epigenetic alteration during cancer development might cause a loss of proteins with functional redundancy to ErbB2, or a gain of those with opposing functions. However, cancer cells with ErbB2 overexpression are able to overcome the selective pressures and therefore survive and propagate, thus yielding cells with high dependency on ErbB2. Therefore, the malignant behavior of such ErbB2-dependent cells responds to inhibition of ErbB2 function, whereas those with low or no ErbB2 expression depend on other pathways for their survival, and hence are refractory to such treatment. This has provided the rationale for application of ErbB2-targeted therapy in ErbB2-overexpressing cancer. In fact, trastuzumab, a monoclonal antibody that targets ErbB2, has been used successfully for the treatment of ErbB2-positive metastatic breast cancer as well as for adjuvant therapy of early breast cancer[33]. Although ErbB2-targeted therapy has not been studied in CCA, our data on the inactivation of ErbB2 suggest that this is a crucial oncogene for maintaining the malignant phenotype of CCA, thus becoming a potential target for future therapy.

In the CCA cells, inhibition of ErbB2 by both kinase inhibitor and siRNA suppressed phosphorylation (activation) of AKT and its downstream effector, p70S6K, but not that of ERK1/2. This might be because the regulation of ERK1/2 by other signals overrides the importance of ErbB2 for its activation, or because signaling pathways downstream of ErbB2 are diverse. This diversity depends partly on dimerization partners of ErbB2. For instance, PI3K/AKT is the most important oncogenic signal downstream of ErbB2-ErbB3 heterodimerization[34]. In this case, overexpressed ErbB2 might dimerize with ErbB3, thereby activates PI3K/AKT as a major pathway. The role of AKT in the malignant characteristics of CCA has also been previously reported. Upregulation of AKT pathway has been reported to be related to low survival rate[35]. Moreover, LY294002, a specific inhibitor of PI3K/AKT pathway, attenuates CCA cell proliferation and promotes apoptosis in 10% serum-containing medium[36], and suppresses KKU-M213 and HuCCA-1 cell invasion induced by HGF[37]. In other systems such as ovarian cancer[38,39] and chick embryo fibroblasts[40], both AKT and p70S6K have been reported to regulate cell invasion, motility and proliferation. Therefore, this implies that ErbB2 regulates invasion and proliferation *via* the PI3K/AKT/p70S6K pathway.

In summary, the results presented here demonstrate that ErbB2 is particularly important for malignant properties (invasion, motility and proliferation) of human high-ErbB2-expressing CCA cells. ErbB2 acts through activation of the AKT/p70S6K pathway. These findings lend support for the therapeutic targeting of ErbB2 and/or its effector molecules in CCA with ErbB2 overexpression.

ACKNOWLEDGMENTS

The authors thank Professor Stitaya Sirisinha and Dr. Banchob Sripa for providing HuCCA-1 and KKU-100 and KKU-M213 cell lines respectively, and Prof. Prapon Wilairat for critical reading of the manuscript.

COMMENTS

Background

Cholangiocarcinoma (CCA), a malignancy of the biliary tract, is an aggressive and currently incurable cancer. The incidence is high in Thailand, China and Japan. Disease progression, especially metastasis, is correlated with ErbB2 overexpression.

Research frontiers

ErbB2 promotes many oncogenic properties, including cell growth, survival, adhesion, motility, invasion, and metastasis. Although many reports have shown the involvement of ErbB2 in CCA, the mechanism that underlies ErbB2-promoted CCA progression is not clearly understood.

Innovations and breakthroughs

Suppression of ErbB2 activity/expression reduced invasion, motility and proliferation in CCA cell lines. This is believed to be the first report to show that, in CCA cell lines with high ErbB2 expression, such neoplastic behavior is more sensitive to ErbB2 inhibition than that in cells with lower ErbB2 expression. Moreover, AKT and p70S6K, but not extracellular signal-regulated kinase 1/2, play important roles as ErbB2 downstream effectors in high-ErbB2-expressing CCA cells.

Applications

As ErbB2 is overexpressed in 20%-30% of CCA, our finding that the malignant phenotypes of CCA cells with high ErbB2 expression are highly dependent on ErbB2 supports the potential use of ErbB2-targeted therapy for the treatment of patients with ErbB2-overexpressing CCA.

Terminology

ErbB2/HER2, human epidermal growth factor receptor 2, is a member of the epidermal growth factor receptor family. Both AKT and p70S6K are downstream effectors of many receptor tyrosine kinases including ErbB2. Their activations induce proliferation and invasion of cancer cells, which leads to metastasis. This study shows that aberrant ErbB2 expression is involved in CCA cell proliferation and invasion *via* AKT/p70S6K.

Peer review

This study provides evidence that high ErbB2 expression in CCA cell lines might be an indicator that blockage of this molecule could be beneficial in cancer therapy. This was a well performed and clearly presented study.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4055

World J Gastroenterol 2010 August 28; 16(32): 4055-4060
ISSN 1007-9327 (print)

BRIEF ARTICLE

Relationship between alcohol intake and dietary pattern: Findings from NHANES Ⅲ

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Author contributions: Liangpunsakul S conducted data analyses and wrote the manuscript.
Supported by Veterans Administration Young Investigator Award/Indiana Institute for Medical Research (to Liangpunsakul S), K08 AA016570 (to Liangpunsakul S) from the NIH/NIAAA and Central Society for Clinical Research Career Development Award (to Liangpunsakul S)
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Received: March 20, 2010 Revised: April 13, 2010
Accepted: April 20, 2010
Published online: August 28, 2010

Abstract

AIM: To examine the association between macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey Ⅲ.

METHODS: A total of 9877 subjects (5144 males) constituted the study cohort. Dietary interviews were conducted with all examinees by a trained dietary interviewer in a mobile examination center (MEC). Subjects reported all foods and beverages consumed except plain drinking water for the previous 24-h time period. Physical examination and history of alcohol consumption were obtained. Pearson correlation coefficients were used to evaluate the association of the levels of alcohol consumption and the percentage of energy derived from macronutrients. Univariate and multivariate regression analyses were performed accounting for the study sampling weight to further explore the relationships between alcohol consumption and calories derived from each macronutrient.

RESULTS: Subjects who drank were younger than non-drinker controls in both genders ($P < 0.01$). Alcohol intake was inversely associated with body mass index and body weight in women. Of all macronutrients, carbohydrate intake was the first to decrease with increasing alcohol consumption. In the multivariate analyses, the level of alcohol consumption was found to be an independent predictor associated with lower intake of other macronutrients.

CONCLUSION: Our results show that there is an alteration in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat.

Key words: Alcohol; Macronutrients; National Health and Nutritional Examination Survey Ⅲ

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Liangpunsakul S. Relationship between alcohol intake and dietary pattern: Findings from NHANES Ⅲ. *World J Gastroenterol* 2010; 16(32): 4055-4060 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4055.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4055

INTRODUCTION

Aside from fat, ethanol is the macronutrient with the highest energy density. Though alcohol can serve as the energy source, how the body processes and utilizes the en-

ergy from alcohol is very complex. Because of additional energy supplementation from alcohol, we might anticipate many drinkers to be obese. In fact, data have shown that drinkers are no more obese than non-drinkers, despite higher caloric intake[1,2]. Moreover, weight loss and malnutrition are common clinical presentations among drinkers. Alcohol intake may be associated with altered patterns of food intake resulting in the replacement of alcohol for other nutrients[1]. We hypothesized that energy derived from alcoholic beverages might substitute energy from other macronutrients such as carbohydrate, protein, and fat. In this study, we examined an association between the macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey (NHANES Ⅲ).

MATERIALS AND METHODS

Study population

NHANES Ⅲ was conducted in the United States from 1988 through 1994 by the National Center for Health Statistics of the Centers for Disease Control and Prevention. The NHANES Ⅲ survey used complex, multi-stage, stratified, clustered samples of civilian, non-institutionalized populations of age 2 mo and older to collect information about the health and diet of people residing in the United States. A detailed description of the survey and its sampling procedures is available elsewhere[3]. This study was approved by the CDC Institutional Review Board and all participants provided written informed consent.

During the survey period, 18162 subjects underwent physical examination and laboratory assessment at a mobile examination center (MEC). Exclusion criteria for this study included minors (age < 20 years old), breast feeding or pregnant women, and those with missing values of specific variables (hepatitis B and C serologies, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum creatinine, and drinking history). Additionally, subjects with history of diabetes, congestive heart failure/heart disease, renal insufficiency were also excluded. History of diabetes was defined by self-report and/or taking diabetes medications and/or the subjects having been told by his/her physicians that he/she has diabetes or sugar diabetes. Subjects with history of congestive heart failure/heart disease were defined by self-report of prior myocardial infarction and/or they had been told by their physicians that they have congestive heart failure. For study purposes, we defined subjects with renal insufficiency as those with creatinine ⩾ 2.5 mg/dL. Subjects with diabetes, congestive heart failure and renal insufficiency were excluded from this study because of the possibilities of dietary restrictions due to underlying diseases. Thus, our analytic population included 5144 men and 4733 women.

NHANES Ⅲ dietary data

Dietary interviews were conducted with all examinees by a trained dietary interviewer in the MEC. Subjects reported all foods and beverages consumed except plain drinking water for the previous 24-h time period (midnight to midnight). An automated, microcomputer-based dietary interview and coding system known as the Dietary Data Collection (DDC) System was used to collect all dietary recall data. The detailed method for data collection is described elsewhere[3].

Estimation of alcohol consumption

The amount of alcohol consumed was determined based on the responses to two survey queries that questioned the number of days of drinking over the past 12 mo and the number of drinks per day on a given drinking day. To avoid any arbitrariness in the choice of a cutoff point, we further stratified the extent of alcohol consumption in subjects who reported a history of alcohol use into four groups using quartiles.

Estimation of physical activity

Physical activity assessment was part of the comprehensive interview in NHANES Ⅲ. In brief, subjects were asked to identify specified exercises in which they participated during their free time (jogging or running; riding a bicycle or exercise bicycle; swimming; aerobic dancing; other dancing; calisthenics or floor exercises; gardening or yard work; and weight lifting). They were requested to specify the number of times they participated in an identified activity during the past month. Responses were standardized as "times per week" using the conversion factors 4.3 wk/mo and 30.4 d/mo, then rounded to the nearest whole number. The frequency of performance of other reported exercises, sports or physically active hobbies was also recorded. The physical activity was specified as the sum of intensity rating multiplied by times (of each activity) per month[4].

Laboratory measurements

All venous blood samples were immediately centrifuged and shipped weekly at -20℃ to a central laboratory. The laboratory procedures followed in the NHANES Ⅲ are described in detail elsewhere[3].

Statistical analysis

Descriptive statistics such as means, SD, ranges, and percentages were used to characterize the study patients. Comparisons among groups were made using Analysis of Variance for the continuous and χ^2 test for the categorical variables. Pearson correlation coefficients were used to evaluate the association of the levels of alcohol consumption and the percentage of energy derived from macronutrients. Multivariate regression analyses were performed accounting for the study sampling weight. All statistical analyses and database management were performed using SAS-callable SUDANN software accounting for stratification, sample weight, and clustering. This analysis method also takes into account the different sample weights and the effects of the complex sample design on variance estimation.

Table 1 Clinical characteristics and dietary patterns in male cohorts (n = 5144)

	Levels of alcohol consumption (g/d)					*P* value
	Non-drinkers (n = 3557)	< 16 (n = 372)	16-35 (n = 465)	36-64 (n = 353)	> 64 (n = 397)	
Age (yr)	48.8 ± 19.5	46.4 ± 19.3	45.4 ± 17.8	42.9 ± 16.2	39.5 ± 14.1	< 0.01
Body mass index (kg/m^2)	26.3 ± 4.1	25.5 ± 3.7	25.7 ± 3.6	25.9 ± 3.7	26.0 ± 3.9	0.87
Body weight (kg)	79.3 ± 14.3	76.9 ± 12.7	78.3 ± 12.3	79.1 ± 13.6	79.4 ± 14.0	0.82
Cigarette smoking (%)	24.8	25.5	37	41.9	50.6	< 0.01
Waist to hip ratio	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.07	0.94 ± 0.07	0.94 ± 0.07	0.94
AST (U/L)	22.8 ± 12.7	22.6 ± 8.3	24.1 ± 11.9	26.4 ± 18.7	29.7 ± 26.1	< 0.01
ALT (U/L)	19.7 ± 15.2	19.1 ± 12.8	20.1 ± 13.4	21.6 ± 16.5	25.5 ± 23.2	< 0.01
Physical activity	76.7 ± 109.2	91.6 ± 108.4	91.2 ± 113.8	71.6 ± 98.1	71.5 ± 105.5	< 0.01
Total energy from food (kcal/d)	2 213 ± 838	2432 ± 850	2500 ± 848	2606 ± 857	3155 ± 1018	< 0.01
kcal from alcohol (%)	0	3.5 ± 2.1	8.0 ± 3.9	13.6 ± 6.2	24 ± 11.3	< 0.01
kcal from carbohydrate (%)	50.8 ± 10.7	47.7 ± 10.6	44.4 ± 9.8	42.2 ± 9.1	36.6 ± 8.4	< 0.01
kcal from protein (%)	16.1 ± 5.1	15.9 ± 4.6	15.5 ± 4.4	14.6 ± 4.1	12.9 ± 4.1	< 0.01
kcal from fat (%)	34.6 ± 9.0	34.1 ± 9.1	33.2 ± 9.2	30.7 ± 8.3	27.2 ± 9.2	< 0.01
kcal from monosaturated fat (%)	13.1 ± 4.0	13.0 ± 4.1	12.7 ± 4.1	11.7 ± 3.8	10.4 ± 3.8	< 0.01
kcal from polysaturated fat (%)	7.3 ± 3.4	7.0 ± 3.2	7.1 ± 3.5	6.2 ± 2.8	5.7 ± 3.3	< 0.04

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2 Multivariate linear regression analysis of total daily food energy and other macronutrients adjusting for covariates by level of alcohol intake[1]

	Levels of alcohol consumption (g/d)					*P* value
Male subjects	Non-drinkers (n = 3557)	< 16 (n = 372)	16-35 (n = 465)	36-64 (n = 353)	> 64 (n = 397)	
Total energy from food (kcal/d)	2228.5 ± 818	2419.6 ± 850	2454.5 ± 847	2559.6 ± 857	3052.1 ± 1018	< 0.05
kcal from carbohydrate (%)	50.2 ± 10.6	47.0 ± 10.6	44.1 ± 9.8	41.9 ± 9.0	36.5 ± 8.3	< 0.01
kcal from protein (%)	16.1 ± 5.1	16.0 ± 4.5	15.5 ± 4.4	14.7 ± 4.1	13.1 ± 4.1	< 0.05
kcal from fat (%)	34.8 ± 9.0	34.4 ± 9.1	33.3 ± 9.2	30.7 ± 9.3	27.1 ± 9.2	< 0.05
kcal from monosaturated fat (%)	13.1 ± 4.0	13.1 ± 4.0	12.7 ± 4.1	11.8 ± 3.9	10.4 ± 3.8	< 0.05
kcal from polysaturated fat (%)	7.2 ± 3.3	6.9 ± 3.2	7.1 ± 3.4	6.1 ± 2.7	5.6 ± 3.3	< 0.05
Female subjects	Non-drinkers (n = 4062)	< 13 (n = 161)	13-21 (n = 169)	22-38 (n = 172)	> 38 (n = 169)	
Total energy from food (kcal/d)	1683 ± 668	1794 ± 612	1881 ± 735	1838 ± 651	2256 ± 861	< 0.01
kcal from carbohydrate (%)	51.5 ± 10.8	48.0 ± 10.3	45.4 ± 10.1	43.4 ± 10.1	38.2 ± 8.4	< 0.01
kcal from protein (%)	15.8 ± 5.1	15.7 ± 4.5	15.1 ± 4.4	14.8 ± 4.3	12.4 ± 3.9	< 0.01
kcal from fat (%)	34.1 ± 9.4	33.8 ± 9.1	34.0 ± 10.1	31.5 ± 9.0	27.2 ± 9.0	< 0.01
kcal from monosaturated fat (%)	12.7 ± 4.0	12.7 ± 4.0	13.0 ± 4.5	12.1 ± 4.1	10.1 ± 3.4	< 0.05
kcal from polysaturated fat (%)	7.3 ± 3.7	7.1 ± 3.2	7.0 ± 3.6	6.8 ± 3.6	5.9 ± 3.3	< 0.05

[1]Adjusted for age, body weight, physical activity, and smoking status.

RESULTS

Relationship between dietary pattern and alcohol consumption in males

Among male participants (n = 5144), 69% reported no history of alcohol use (Table 1).

There were no differences in waist-to-hip ratio among groups. In this study cohort, only subjects who drank < 16 g of alcohol/d weighed less than non-drinker controls. The percentage of subjects who smoked increased in accordance with the level of alcohol consumption. As expected, markers of hepatic inflammation, AST and ALT, were increased with the level of alcohol consumption. The total energy consumption per day increased with the level of alcohol consumption. The increment in such energy was mainly due to the calories provided by alcohol. In the univariate analysis (Table 1), we found that the percentage of energy derived from carbohydrate, protein, and fat decreased with increasing alcohol consumption. Carbohydrate intake started to decrease at a daily consumption of ≤ 35 g/d. When the daily levels of alcohol consumption continued to increase (> 35 g/d), subjects consumed less protein and fat. In those who drank alcohol at a level of > 64 g/d, the energy intake which was derived from protein and fat was reduced by 4% and 7%, respectively. In the multivariate linear regression analyses adjusting for covariates (such as age, body weight, smoking status and physical activity), the level of alcohol consumption was found to be an independent predictor associated with lower percent calories derived from macronutrients. The adjusted calories from macronutrients stratified by alcohol consumption are shown in Table 2. In the correlation analyses, the amount of alcohol consumed per day was inversely associated with the percentage of calories derived from each macronutrient (Figure 1A-C).

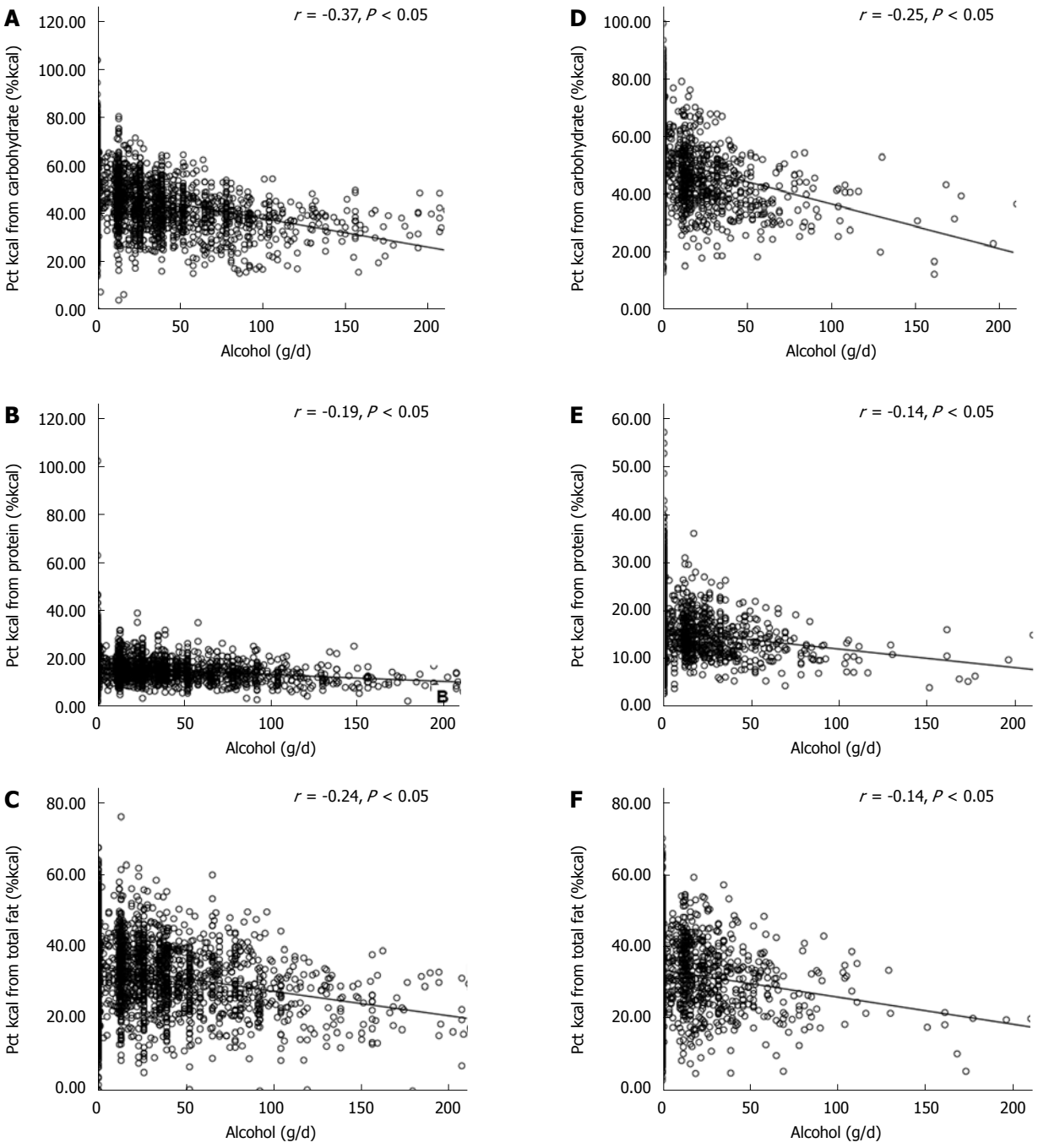

Figure 1 Relationship between the levels of alcohol consumption and the percentage of calories derived from each macronutrient in males (A-C) and females (D-F).

Relationship between dietary pattern and alcohol consumption in females

As observed with male participants, women who drank higher amounts of alcohol were younger than non-drinker controls ($P < 0.05$). Alcohol intake was inversely associated with body mass index and body weight in women. Again, no differences in waist-to-hip ratio were observed among groups (Table 3). As with male subjects, we found a higher prevalence of cigarette smokers in those who drank higher amounts of alcohol. Because of the energy provided from alcohol, the total energy consumed per day was higher in those who drank any amount of alcohol when compared to controls. We again found that female drinkers started to consume less carbohydrate even when they drank < 13 g/d. The calories derived from protein and fat were reduced once the subjects drank > 21 g of alcohol/d. In the multivariate linear regression analyses, the level of alcohol consumption was found to be an independent predictor associated with lower percent calories derived from macronutrients (Table 2). In the correlation analyses, the amount of alcohol consumption per day was inversely associated with the percentage of calories derived from each macronutrient, as observed in male subjects (Figure 1D-F).

Table 3 Clinical characteristics and dietary patterns in female cohorts (n = 4733)

	Levels of alcohol consumption (g/d)					*P* value
	Non-drinkers (n = 4062)	< 13 (n = 161)	13-21 (n = 169)	22-38 (n = 172)	> 38 (n = 169)	
Age (yr)	49.1 ± 19.5	45.6 ± 18.4	45.4 ± 17.6	41.9 ± 15.2	41.3 ± 14.3	< 0.01
Body mass index (kg/m^2)	26.3 ± 5.0	25.1 ± 4.5	24.9 ± 4.9	24.8 ± 4.5	24.8 ± 4.5	< 0.05
Body weight (kg)	67.3 ± 13.9	64.8 ± 11.8	65.7 ± 14.1	66.3 ± 11.9	66.4 ± 13.0	< 0.05
Cigarette smoking (%)	19	25	30.1	35.4	44.9	< 0.01
Waist to hip ratio	0.87 ± 0.08	0.85 ± 0.07	0.86 ± 0.08	0.86 ± 0.11	0.86 ± 0.08	0.94
AST (U/L)	19.8 ± 12.1	18.9 ± 6.7	23.4 ± 30.9	20.9 ± 11.5	20.5 ± 9.4	< 0.01
ALT (U/L)	14.6 ± 13.8	13.8 ± 8.8	15.1 ± 14.2	14.6 ± 10.5	14.6 ± 10.7	< 0.01
Physical activity	56.9 ± 90.9	78.6 ± 105.7	81.6 ± 107.3	70.5 ± 94.8	70.7 ± 104.3	< 0.01
Total energy from food (kcal/d)	1667 ± 667	1816 ± 646	1903 ± 735	1895 ± 651	2316 ± 860	< 0.01
kcal from alcohol (%)	0	3.7 ± 2.2	6.8 ± 3.4	11.6 ± 4.4	23.2 ± 11.4	< 0.01
kcal from carbohydrate (%)	52.4 ± 10.8	48.9 ± 10.5	46.1 ± 10.1	43.8 ± 10.1	38.4 ± 8.7	< 0.01
kcal from protein (%)	15.8 ± 5.0	15.6 ± 4.5	15.0 ± 4.4	14.7 ± 4.2	12.3 ± 3.9	< 0.01
kcal from fat (%)	33.4 ± 9.4	33.2 ± 9.2	33.6 ± 10.1	31.4 ± 9.0	27.3 ± 9.3	< 0.01
kcal from monosaturated fat (%)	12.3 ± 4.0	12.4 ± 4.1	12.8 ± 4.4	11.9 ± 4.1	10.1 ± 3.8	< 0.01
kcal from polysaturated fat (%)	7.3 ± 3.6	7.1 ± 3.3	7.1 ± 3.5	6.9 ± 3.6	6.0 ± 3.4	< 0.04

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

DISCUSSION

In this large population-based study, we found that (1) alcohol consumption was inversely related to body mass index and body weight, primarily in women; (2) energy derived from alcohol replaced that from macronutrients in both genders; and (3) carbohydrate was the foremost macronutrient in which the energy was replaced by that from alcohol. With increasing alcohol consumption, we found a significant reverse relationship between alcohol and all macronutrient intakes.

Although alcohol is an energy source, how the body processes and uses the energy from alcohol is more complex than can be explained by a simple calorie conversion value. Energy derived from alcohol has been consider as "empty calories" because alcohol contains no beneficial nutrients. Additionally, it can also replace the energy derived from other macronutrients. As shown in this study, particularly in female subjects, alcohol provides an average of 23% of the calories when intake is > 38 g/d (Table 2). Despite higher caloric intake from alcohol, females who drank at this level were less obese than non-drinkers. It is postulated that chronic drinking triggers the microsomal ethanol-oxidizing system (MEOS)[1,5,6], an inefficient system of alcohol metabolism. Much of the energy from MEOS-driven alcohol metabolism is lost as heat rather than used to supply the body with energy. The association between gender, body weight, and alcohol intake is debatable. Though our results are consistent with those reported by Colditz *et al*[1], there have been previous reports that men who drank weighed more than non-drinkers[7,8]. The inconsistency in these results is likely due to the study design and data collection.

We observed that the major difference in nutrient intake for both genders was a significantly lower intake of carbohydrates by drinkers (r = -0.37 and -0.25, in male and female subjects, respectively). Our findings are similar to those reported by Thompson *et al*[9], where they observed a decreased absolute intake of carbohydrate, protein, and fat with increasing alcohol intake.

Several limitations in using NHANES datasets deserve discussion. First, the cross-sectional design in NHANES does not enable us to truly address potential temporal associations between significant alcohol consumption and the variables of interest. Second, the accuracy of the alcohol consumption data, as with other retrospective study designs, is unknown. Since the extent of alcohol consumption will be derived from self-report questionnaires, it is vulnerable to a recall bias in each participant.

In summary, our results showed that there is an alteration in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat. Female drinkers were less obese than non-drinkers, suggesting that alcohol calories may be less utilized in female subjects. However, further research is needed to explore the role of gender and body weight in alcoholics.

COMMENTS

Background

Aside from fat, ethanol is the macronutrient with the highest energy density. Though alcohol can serve as the energy source, how the body processes and utilizes the energy from alcohol is very complex. Because of additional energy supplementation from alcohol, the authors might anticipate many drinkers to be obese. In fact, data have shown that drinkers are no more obese than non-drinkers, despite higher caloric intake. Moreover, weight loss and malnutrition are common clinical presentations among drinkers. Alcohol intake may be associated with altered patterns of food intake resulting in the replacement of alcohol for other nutrients. In this study, the authors examined the association between the macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey (NHANES Ⅲ).

Research frontiers

In this large population-based study, the authors found alterations in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first population-based study to address the relationship between alcohol consumption and dietary pattern. The authors found that alcohol consumption was inversely related to body mass index and body weight, primarily in women. With increasing alcohol consumption, they found a significant reverse relationship between alcohol and all macronutrient intakes.

Applications

Energy derived from alcohol has been considered as "empty calories" because alcohol contains no beneficial nutrients. In this study, the authors also found that energy derived from alcohol consumption can replace that from other macronutrients.

Peer review

Dr. Liangpunsakul describes in his manuscript the relationship between alcohol consumption and the macronutrient dietary patterns using data from the NHANES 1988-1994. He found that increasing alcohol intake is associated with an altered daily dietary pattern and that the energy of alcoholic beverages substitutes that from carbohydrates, protein and fat.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4061

World J Gastroenterol 2010 August 28; 16(32): 4061-4065
ISSN 1007-9327 (print)

BRIEF ARTICLE

Hepatocellular carcinoma in patients with chronic hepatitis C virus infection without cirrhosis

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Received: December 11, 2009 Revised: January 26, 2010
Accepted: February 3, 2010
Published online: August 28, 2010

Abstract

AIM: To investigate and characterise patients with chronic hepatitis C virus (HCV) infection presenting with hepatocellular carcinoma (HCC) in the absence of cirrhosis.

METHODS: Patients with chronic hepatitis C infection without cirrhosis presenting with HCC over a 2-year period were identified. The clinical case notes, blood test results and histological specimens were reviewed to identify whether additional risk factors for the development of HCC were present.

RESULTS: Six patients (five male, one female) with chronic hepatitis C infection without cirrhosis presented to a single centre with HCC over a 2-year period. Five patients were treated by surgical resection and one patient underwent liver transplantation. Evaluation of generous histological specimens confirmed the presence of HCC and the absence of cirrhosis in all cases. The degree of fibrosis of the background liver was staged as mild (n = 1), moderate (n = 4) or bridging fibrosis (n = 1). Review of the clinical case notes revealed that all cases had an additional risk factor for the development of HCC (four had evidence of past hepatitis B virus infection; two had a history of excessive alcohol consumption; a further patient had prolonged exposure to immune suppression).

CONCLUSION: HCC does occur in patients with non-cirrhotic HCV infection who have other risk factors for hepatocarcinogenesis.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Non-cirrhotic; Screening

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Nash KL, Woodall T, Brown ASM, Davies SE, Alexander GJM. Hepatocellular carcinoma in patients with chronic hepatitis C virus infection without cirrhosis. *World J Gastroenterol* 2010; 16(32): 4061-4065 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4061.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4061

INTRODUCTION

Hepatocellular carcinoma (HCC) is a well-recognised complication of cirrhosis regardless of aetiology; the risk of malignancy differs according to the underlying cause of liver damage. As a consequence, patients with cirrhosis undergo routine interval screening in most liver centres using a combination of serum α-foetoprotein (AFP) and liver ultrasound, although solid evidence to support this

approach post-dates adoption of the strategy[1,2]. Surveillance is restricted to those at higher risk in some centres. Patients with cirrhosis secondary to chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) are at particular risk of HCC[3]. Furthermore, there are numerous reports indicating that chronic infection with HBV is associated with HCC in the absence of cirrhosis.

In contrast, HCC in patients with chronic HCV infection without cirrhosis appears to be very rare[4-7]. We describe six such cases that presented to one tertiary referral centre in the past 2 years (Table 1).

MATERIALS AND METHODS

Patients with chronic HCV infection without cirrhosis presenting with HCC over a 2-year period were identified. The clinical cases were reviewed to identify any additional risk factors for hepatocarcinogenesis. Details obtained included ethnic origin, alcohol consumption, past or present infection with HBV, medical co-morbidity, medication history and family history of liver disease or HCC. Histological specimens were reviewed by an experienced liver pathologist to confirm the presence of HCC and to assess the degree of fibrosis of the background liver tissue. Furthermore, histological specimens were evaluated for the presence of additional hepatic pathology including the presence of iron, steatosis and α-1 antitrypsin globules.

RESULTS

Six cases with non-cirrhotic chronic HCV infection and HCC presented to one centre over a 2-year period. Table 1 summarises the clinical and histological characteristics of the cases including additional risk factors for hepatocarcinogenesis. The first case, a 53-year-old Caucasian male, was referred to the service having been found to be HCV-RNA positive (genotype 1). He had a history of previous injecting drug use and a high alcohol intake exceeding 30 U/wk. Evaluation of hepatitis B serology demonstrated that he was surface antigen (HBsAg) negative, and positive for core antibody (anti-HBc) in keeping with past infection. Screening liver ultrasound demonstrated a focal lesion. Computerised tomography (CT) confirmed a mass with arterial enhancement. Serum AFP was 239 IU/L (normal range < 10 IU/L). Histology of the resected mass revealed a 3.5-cm multi-focal HCC, with moderate to poor differentiation and vascular invasion. Histology of the background liver revealed minimal inflammation but moderate fibrosis. He is recurrence-free 24-mo post resection. HCV-RNA was undetectable at completion of 48-wk therapy with pegylated interferon-α and ribavirin.

The second case, a 57-year-old Caucasian male, presented with fever and abdominal pain. CT of the abdomen revealed a 5 cm × 3 cm, inflammatory mass in the right side of the abdomen in close proximity to bowel and in addition a 2 cm hypodense lesion within the liver. A right hemi-colectomy was performed which revealed extra-colonic fibrosis and abscess formation secondary to a caecal diverticulum. Biopsy of the liver mass demonstrated HCC; the background liver comprised moderate inflammation and mild fibrosis consistent with chronic HCV infection. He was HCV-RNA positive (genotype-3), HBsAg negative, anti-HBc positive with a history of previous injecting drug use. Liver function tests were normal. Serum AFP was 3 IU/L. After hepatic resection, histology revealed a 3.1 cm HCC with moderate differentiation, without vascular invasion; the background liver confirmed mild inflammation and mild fibrosis. He has completed therapy with pegylated interferon-α and ribavirin with sustained virological response. He is recurrence-free 22 mo following surgery.

Case three, a 67-year-old Nigerian lady with chronic HCV infection, had a 4 cm focal lesion detected on screening liver ultrasound. CT imaging confirmed the mass with arterial enhancement. Biopsy of the lesion demonstrated HCC and the background liver revealed moderate inflammation and mild fibrosis consistent with chronic HCV infection. She was HCV-RNA positive (genotype 1), HBsAg negative, anti-HBc positive. Liver function tests were unremarkable. Serum AFP was 206 IU/L. After hepatic resection, histology revealed a 4.5 cm HCC with moderate to poor differentiation and lymphovascular invasion on a background liver with moderate fibrosis. She was unable to tolerate antiviral therapy due to profound anaemia and remains HCV-RNA positive. She is recurrence-free 20 mo after resection.

The fourth case was a 46-year-old Caucasian man known to have chronic HCV (genotype 1) with a history of previous injecting drug use; he was HBsAg negative, anti-HBc positive. Liver biopsy demonstrated moderate activity and moderate fibrosis consistent with chronic HCV infection. He had failed to respond to pegylated interferon and ribavirin. Six years later investigation of a raised AFP (77 IU/L) revealed a 1.5 cm arterially enhancing lesion in the liver. Surgical resection revealed a 1.7 cm moderately - well differentiated HCC without vascular invasion on a background liver with bridging fibrosis but not cirrhosis. He is recurrence-free 16 mo after resection.

Case 5 was a 46-year-old Indian man with chronic HCV infection with a history of renal transplantation 15-year previously, treated with ciclosporin and prednisolone. Liver function tests were unremarkable. Screening ultrasound detected a 3 cm focal lesion in the liver; biopsy of the mass revealed HCC. He was HCV-RNA positive (genotype 1) without evidence of exposure to HBV. Serum AFP was 5 IU/L. Further imaging with CT, magnetic resonance imaging and angiography confirmed the mass and revealed two further smaller lesions. He was deemed to meet Mazzaferro criteria[8] and listed for liver transplantation. The explant revealed a 3 cm HCC with poor differentiation and vascular invasion and several small satellite lesions. The background liver demonstrated moderate fibrosis and moderate inflammation. He developed HCC within the grafted liver after 6 mo leading to death 8 mo following transplantation.

The final case, a 63-year-old Russian doctor, acquired HCV (genotype 1) 9 years previously following a needle stick injury from a patient. He had a long history of excess alcohol consumption (30 U/wk) but was immune

Table 1 Patient characteristics

Age (yr)	Sex	HCC	Background liver histology at time of HCC treatment	AFP (IU/L)	HCV status	HBV status	Alcohol (U/wk)
53	M	3.5 cm Moderate/poor differentiation Vascular invasion	Minimal inflammation Moderate fibrosis	239	RNA positive Genotype 1	HBsAg negative Anti-HBc positive	10-30
57	M	3.1 cm Moderate differentiation No vascular invasion	Mild inflammation Mild fibrosis	3	RNA positive Genotype 3	HBsAg negative Anti-HBc positive	0
67	F	4.5 cm Moderate/poor differentiation Lymphovascular invasion	Moderate inflammation Moderate fibrosis	206	RNA positive Genotype 1	HBsAg negative Anti-HBc positive	0
52	M	1.7 cm Moderate differentiation No vascular invasion	Moderate inflammation Bridging fibrosis	77	RNA positive Genotype 3	HBsAg negative Anti-HBc positive	20
46	M	3 cm Poorly differentiated with satellite lesions	Moderate inflammation Moderate fibrosis	5	RNA positive Genotype 1	Negative	0
62	M	6.5 cm Moderate differentiation Vascular invasion	Moderate inflammation Moderate fibrosis	10	RNA positive Genotype 1	Negative	30

HCC: Hepatocellular carcinoma; AFP: α-foetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

to HBV and was anti-HBc negative. At presentation, liver biopsy revealed mild fibrosis with moderate inflammation. Nine years later he was found to have a 6.5 cm HCC. Serum AFP was 10 IU/L. He underwent surgical resection; histology revealed a moderately differentiated HCC on a background liver with moderate fibrosis. He is well 5 mo after.

DISCUSSION

Six cases with non-cirrhotic chronic HCV infection and HCC presented to one centre over a 2-year period. All were considered at low risk of HCC and none was in a surveillance programme. One had symptoms but the identification of a liver mass in the remaining five patients was fortuitous. Cirrhosis was excluded confidently in all cases by careful histological review of generous tissue specimens revealing at worst bridging fibrosis. No patient had histological features to suggest any diagnosis other than HCV related injury. All patients had a normal body mass index and none were diabetic. However, all patients had an additional risk factor for liver injury or HCC: four had evidence of past HBV infection; two had a history of excessive alcohol consumption; a further patient had prolonged exposure to immune suppression.

The incidence of HCC is increasing across the developed world[9]. Cirrhosis of any cause is an important precursor for HCC, although liver disorders including chronic HBV or HCV infection, haemochromatosis, non-alcohol-related fatty liver disease (NAFLD) and alcohol-related liver disease carry a particular risk[3]. The risk is also much higher in men and older patients[10,11]. The current increase in the prevalence of chronic liver disease secondary to chronic HCV infection and NAFLD (a consequence of the increasing prevalence of obesity and an ageing population) are the main reasons for the increasing incidence of HCC in the developed world[12-15], although improved screening and diagnosis may also play a part. The underlying mechanisms that lead to malignant transformation of HCV-infected hepatocytes, however, remain uncertain, but as most HCV-related HCC occurs on a background of severe fibrosis or cirrhosis it is thought that the mechanism of carcinogenesis is more likely to be indirect, such that the process of tissue damage, regeneration and repair are important, rather than a direct oncogenic effect of HCV infection or the inflammatory response to the virus.

It is well recognised that chronic HBV infection can lead to HCC in the absence of cirrhosis[16]. HBV is a DNA virus that can integrate into the host cell genome; integration may be mutagenic directly by causing genomic instability, loss of tumour suppressor activity or over-expression of genes involved in regulation of cell cycle proliferation. In addition, HBV encodes HBx protein, which functions as a transcriptional trans-activator of cellular genes that are involved in cell proliferation control such as c-jun, c-fos and c-myc. This may lead to dysregulation of the cell cycle and interference with cellular DNA repair and apoptosis[17].

This series raises the possibility that HCV may be oncogenic. HCV is an RNA virus, which replicates in the cytoplasm and does not integrate into host cellular DNA. However, some HCV proteins, such as HCV core and non-structural proteins NS3, NS4B and NS5A have a regulatory effect on cellular promoters and interact with a number of cellular proteins involved in carcinogenesis under certain conditions[18-21]. In addition, hepatocytes from patients with chronic HCV infection are arrested in G1 and may undergo replicative senescence, which may predispose to malignancy[22]. Direct evidence for a carcinogenic role for HCV *in vivo* is lacking.

HCC has been described very rarely in chronic HCV infection in the absence of cirrhosis[4-7], which suggests that other aetiological factors may be more important. Analyses of case series in Japan have suggested that ageing increases the risk of developing HCC in patients with HCV who do not have cirrhosis, particularly in wom-

en[10,11]. In the series presented here, however, five of six cases were men and the median age at diagnosis of HCC was 55, suggesting that alternative factors are important. None of the cases described had histological evidence of additional injury such as steatohepatitis or iron accumulation that are recognised co-factors in the development of liver injury and HCC[23,24]. However, four of six patients had serological evidence of previous exposure to HBV. It is possible that integration of HBV genes had occurred, increasing the risk of HCC as in HBV infection without cirrhosis. Long-term immune suppression in another may have increased the risk of HCC[25]. Furthermore, it has been reported that renal transplant patients might have an increased susceptibility to HCC even without viral infection purely as a result of immune suppression[26]. In the final patient there was a long history of excess alcohol use but no evidence of alcohol related liver damage on biopsy.

A third of patients with HCV have been exposed to HBV because of common risk factors[27]. In the Cambridge series 35% of 1500 patients with chronic HCV infection were anti-HBc positive/HBsAg negative while 2% were co-infected with HBV. Past HBV infection is associated with an increased risk of progressive liver injury in some series of patients with chronic HCV infection and other liver disorders[28,29] although the presence of anti-HBc was not associated with progressive fibrosis in our own series[30]. HBV genomic material has been identified in liver from patients with HCC who were HBsAg negative but HCV RNA positive[31,32] and the presence of HBV genes in this setting has been linked to HCC[33]. In a prospective observational study, serum anti-HBc was a marker of high risk for HCC among patients with HCV related cirrhosis, but was not a significant risk factor in those without cirrhosis[34]. The presence of HBV genes in HCC tissue of HBsAg negative, HCV negative patients has also been described[35]. Thus, long-term persistence of HBV genes in liver tissue may cause HCC without inflammation, necrosis or regeneration. While abnormal alanine aminotransferase (ALT) fluctuation is associated with carcinogenesis in HCV positive patients[36], the presence of integrated HBV DNA in the liver may promote carcinogenesis independently. Patients with low levels of ALT and minimal histological change may still be at risk of HCC development if they have had previous exposure to HBV.

Surveillance for HCC has been conducted for many years but a survival benefit for screening with 6-monthly ultrasound and AFP monitoring has only been demonstrated recently[1]. However, surveillance is practiced widely and recommended in high-risk groups such as those with cirrhosis due to HBV, HCV, alcohol, or haemochromatosis[2]. In addition, because of the high risk of HCC in non-cirrhotic HBV infection, current guidelines recommend screening in high-risk groups (family history of HCC, Asian males > 40 years, Asian females > 50 years and Africans > 20 years and could be extended to those with high serum HBV DNA levels). In HCV infection with cirrhosis there is a high risk of HCC development (2%-8% per year); surveillance is recommended and cost effective[37]. At the current time it is unclear whether patients with bridging fibrosis should be offered screening and it is not recommended for patients with mild or moderate disease regardless of patient age or length of time of infection.

All HCV-infected patients with cirrhosis in our centre are offered 6-monthly screening with liver ultrasound by a small group of experienced liver radiologists or ultrasonographers; serum AFP is not sought in this particular cohort because of a lack of specificity and sensitivity, as demonstrated in this series. This experience has prompted review of our policy; for example should older men with chronic HCV infection without cirrhosis but with an additional risk factor for HCC, such as anti-HBc or excess alcohol consumption, undergo ultrasound screening? The increased workload for our service would be enormous and it is probable that the number of patients identified with HCC that could be cured by intervention would be too low to justify such an approach. Further data on the incidence of HCC in HCV-infected patients without cirrhosis are required before a change in policy can be recommended as routine practice; a national register of such cases could be helpful.

COMMENTS

Background

Cirrhosis of any cause is associated with a significant risk of developing hepatocellular carcinoma (HCC). Chronic hepatitis B virus (HBV) infection in the absence of cirrhosis is also a recognised risk factor for HCC and screening is recommended in some high risk groups. HCC occurs rarely in non-cirrhotic hepatitis C virus (HCV) infection and there are no recommendations for screening in these patients.

Research frontiers

Due to the observation of an increase in HCC in non-cirrhotic HCV patients, a detailed evaluation of these patients was undertaken.

Innovations and breakthroughs

In each patient with HCC and non-cirrhotic HCV infection, an additional risk factor for hepatocarcinogenesis was identified. These included previous infection with HBV, high alcohol intake and immunosuppression.

Applications

Patients with chronic HCV without cirrhosis may be at risk of developing HCC if there are other risk factors for liver injury and carcinogenesis present. It is possible that these patients should be considered for surveillance programmes although this would result in a dramatic increase in workload for radiological departments and may not be cost effective.

Peer review

This is a well written article.

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S- Editor Wang YR **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4066

World J Gastroenterol 2010 August 28; 16(32): 4066-4071
ISSN 1007-9327 (print)

BRIEF ARTICLE

Quality of life assessment in patients with chronic pancreatitis receiving antioxidant therapy

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Received: December 23, 2009 Revised: February 16, 2010
Accepted: February 23, 2010
Published online: August 28, 2010

Abstract

AIM: To undertake a baseline study comparing quality of life (QoL) in patients with chronic pancreatitis (CP) on Antox to those with CP, matched for disease duration, who were not on this medication.

METHODS: CP was defined according to the Zurich classification. Sixty eight consecutive patients with CP who were taking Antox (antioxidants) were compared with 69 consecutive control CP patients not on Antox. European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) were used to assess QoL. Out of a total of 137 patients 28 in each group were matched for disease duration (within 12 mo). Median disease duration was 8 (1-22) years in the Antox group and 7 (1-23) years in the Non-Antox cohort (P = NS, Mann-Whitney U-test). Other parameters (age, gender, etiology, endocrine and exocrine insufficiency) were similar between groups.

RESULTS: Median visual analogue pain score in the Antox group was 3 (0-8) compared with 6 (0-8) in the Non-Antox group ($P < 0.01$). Perceptions of cognitive, emotional, social, physical and role function were impaired in the Non-Antox group compared to Antox patients ($P < 0.0001$, $P = 0.0007$, $P = 0.0032$ and $P < 0.005$ and $P < 0.001$, respectively). Analgesics and opiate usage was significantly lower in the Antox group ($P < 0.01$). Overall physical health and global QoL was better in the Antox group ($P < 0.0001$, 95% CI: 1.5-3).

CONCLUSION: Contemporary quality of life assessments show that after correction for disease duration and cigarette smoking, patients with CP taking antox had better scores than non-antox controls.

Key words: Chronic pancreatitis; Antioxidants; Quality of life; Assessment; Management

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Shah NS, Makin AJ, Sheen AJ, Siriwardena AK. Quality of life assessment in patients with chronic pancreatitis receiving antioxidant therapy. *World J Gastroenterol* 2010; 16(32): 4066-4071 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4066.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4066

INTRODUCTION

Chronic pancreatitis (CP) is a chronic inflammatory con-

dition of the pancreas characterized histologically by loss of normal pancreatic parenchymal architecture with varying degrees of fibrosis and inflammatory infiltration[1]. Clinically, CP presents a spectrum of disease most often marked by chronic or recurrent abdominal pain together with varying features of pancreatic exocrine deficiency, most typically fat-related, resulting in steatorrhea, and manifestations of pancreatic endocrine deficiency, such as diabetes mellitus[1].

Although there are no national incidence registries, population-based data indicate a frequency of 8.6/100 000 per year[2] with similar incidence being recognized in the United States[3] and other Northern European countries[4,5]. The clinical course of CP can be characterized by variable background abdominal pain with episodic exacerbations. Although the later stages of the illness are marked by pancreatic exocrine and endocrine insufficiency, abdominal pain is the dominant symptom for many sufferers.

Until now there has been no specific therapy for CP. Rose *et al*[6] demonstrated that CP arose as a result of pathological exposure of the pancreatic acinar cells to short-lived oxygen free radicals - a process termed oxidative stress. The peripheral blood samples taken from patients with clinical CP have shown that antioxidants (the term given to inhibitors of the oxidative stress response), their precursors and co-factors in physiologic antioxidant pathways are depleted during the course of this illness[7]. In addition, there is elevation of peripheral blood markers of oxidative injury. Braganza *et al*[8] reasoned that exogenous supplementation with antioxidants or precursors for antioxidant pathways may augment these deficient pathways and help to quench ongoing acinar injury. From a series of exploratory studies they concluded that co-factors of the endogenous glutathione peroxidase pathway were key components for supplementation. Selenium, vitamin C (ascorbic acid) and methionine were proposed as key antioxidants[8-10]. A commercially-available formulation, Antox (Pharma Nord, Morpeth, UK) was developed comprising vitamin C, vitamin E, β-carotene, selenium and methionine.

There is anecdotal evidence from small, underpowered, randomized trials that oral antioxidant therapy reduces the frequency and severity of episodes of pain[11,12]. More recently, a well-conducted randomized trial from India demonstrated that oral antioxidant therapy was associated with a reduction in hospital admission and "pain days"[13].

In contemporary healthcare terms, perhaps the critical issue in the treatment of CP is assessment of the effect of intervention on quality of life (QoL). In this regard, formal, well-validated questionnaire-based QoL scoring systems are now available for assessment of patients with CP[14].

The European Organization for Research and Treatment of Cancer (EORTC) QoL study group has developed a modular approach to the development of QoL instruments designed for use in clinical trials[15]. A 30-item core cancer questionnaire; the EORTC Quality of Life Questionnaire (QLQ) Core questions 30 (C-30) was developed. It was initially developed and validated for use in patients with non small cell lung cancer[16]. The 30-item core questionnaire is intended to be supplemented by additional questionnaire modules to assess disease symptoms and treatment side-effects. The EORTC QLQ Pancreatic Modification (26 questions) (PAN-26) was developed and mainly used for pancreatic cancer[17]. During the development of the QLQ PAN-26, interest was expressed in the feasibility of using this assessment system in patients with CP[14].

Two questions have been added to PAN-26 to produce a questionnaire for use in CP, the QLQ PAN (CP)-28[17].

The aim of this study is to compare QoL, using an appropriately validated, disease-specific questionnaire-based approach in a cohort of patients with CP receiving oral antioxidant therapy to individuals with CP who are not receiving this medication.

MATERIALS AND METHODS

Study design

This is a prospective, single-centre clinical study comparing QoL as assessed using validated, disease-specific EORTC questionnaires[17] in a group of patients with a clinical diagnosis of CP receiving oral antioxidant therapy in the form of Antox (Pharma Nord, Morpeth, UK) to a cohort of patients with CP from the hepatobiliary and gastroenterology services of the same hospital who were not receiving oral antioxidant supplementation.

Patients and treatment algorithm

The terminology advocated by the Zurich International Workshop was used to define alcohol-related CP[18]. All patients had radiological evidence of CP on either computed tomography or magnetic resonance imaging; in addition some patients had supplementary evidence from endoscopic retrograde pancreatography or endoscopic ultrasonography. Routine monitoring of blood glucose was undertaken in the outpatient setting. Pancreatic exocrine function testing was not routinely employed in patients in this study. In general, antioxidant therapy was offered for the treatment of patients with a diagnosis of CP if there was no evidence of a pancreatic lesion potentially requiring surgical intervention, or if there was no marked pancreatic ductal dilatation amenable to endoscopic or surgical drainage. Thus, for the purposes of this study, patients were categorized into those taking Antox and those not taking this medication. Clinical characteristics of these 2 groups are shown in Table 1.

Administration of questionnaire, data registration and analysis

Patients completed the EORTC QLQ C-30 and QLQ PAN-28 questionnaires in the presence of an interviewer as part of a dedicated interview. Pain was assessed using a visual analogue score (VAS), where 0 is no pain and 10 is the maximum (scale 0-10). All interviews were undertaken by the same interviewer (NS). Interviews were conducted

Table 1 Profile of patients with chronic pancreatitis matched for similar disease duration

	CP patients on Antox (n = 28)	CP patients NOT on Antox (n = 28)	P-value
Age (yr), median (range)	53 (24-82)	53 (31-74)	0.30 (Mann-Whitney U-test)
Etiologies	Alcohol: 13 (46%) Idiopathic: 13 (46%) Others: 2 (8%)	Alcohol: 17 (61%) Idiopathic: 11 (39%)	
Gender (male:female)	16:12	18:10	0.79 (Fisher's exact test)
Duration of disease (yr), median (range)	8 (1-22)	7 (1-23)	0.85 (Mann-Whitney U-test)
Current cigarette smoking	8 (27%)	18 (62%)	0.01 (Fisher's exact test)
Alcohol before diagnosis of CP	118 (48-240) g/d per person	160 (28-240) g/d per person	< 0.01 (Mann-Whitney U-test)
Alcohol intake, current mean (range)	25 (0-48) g/d per person	33 (0-96) g/d per person	0.63 (Mann-Whitney U-test)

CP: Chronic pancreatitis.

with patients attending the Hepatobiliary and Gastroenterology clinics in this hospital during the study period February 2007 to February 2009. The interviewer was a clinical research fellow and not involved in the clinical care of any of the patients. Questionnaires were completed prospectively but analyzed retrospectively as a batch after completion of the study. Questionnaire results were not used to inform clinical decision-making. Questionnaire results were transcribed onto an electronic database (Microsoft Excel, Microsoft, Redmond, Washington, USA) for subsequent analysis. The interviews were conducted on a single time point basis: no patients underwent repeat interview. All patients in the Antox group had been receiving therapy for at least 6 mo. No patients had undergone surgery in the 6 mo prior to interview.

Disease duration-matched cohort

Interim analysis of the whole cohort data showed that there were significant differences in the median age and disease duration between patients in the Antox group and those in the Non-Antox cohort. In an effort to correct for at least one of these factors, disease duration matching was undertaken. The disease duration was recorded for each patient from the clinical chart. Patients in the Non-Antox group were then matched with corresponding individuals from the Antox group. A disease duration of the same time period ± 12 mo was selected for matching. No patient was included twice and data were paired by searching chronologically according to date of interview from first interviewee to the last.

Ethics committee approval

This study was approved by regional research ethics committee.

Statistical analysis

Continuous data are presented as median (range). Statistical comparisons were by non-parametric test using the Mann-Whitney U-test for 2 group comparisons and Fisher's exact test for contingency tables. The Wilcoxon signed ranks test (two-sided test) was used for comparison of paired data. Statistical significance was at the $P < 0.05$ level. The StatsDirect software program was used for statistical analyses (StatsDirect version 2.6.5, http://www.statsdirect.com).

RESULTS

Entire cohort comparison (NOT matched for disease duration)

Alcohol was the most common etiologic agent in 84 (61%) of patients. The median age of the group taking Antox was 56 (24-82) years compared to 47 (24-74) years in those not taking Antox. This difference was statistically significant. Disease duration and proportion of patients with diabetes mellitus were also greater in the Antox groups (although the difference in incidence of diabetes mellitus was not significant).

Entire cohort outcome (NOT matched for disease duration)

VAS, overall physical health scores and global QoL were significantly better in patients with CP taking Antox. These results are reflected in the significantly lower number of patients in the Antox group taking analgesics and opiates.

Disease-duration matched cohort outcome

Table 1 shows that the disease duration-matched cohort were also similar in terms of age, etiology of CP and gender ratio. There were more smokers in the Non-Antox group, and alcohol intake prior to diagnosis was also greater in this group.

The outcome data in the disease duration-matched patients show that patients taking Antox had lower pain scores and fewer were taking analgesics (including opiates). There was no difference in the proportions of patients who were diabetic or who were taking pancreatic exocrine supplements (Table 2). A significantly greater number of patients in the Non-Antox group had undergone either surgical or endoscopic intervention.

Detailed global outcome data from the disease duration-matched cohort are shown in Table 3. Answers to questions were ranked on a scale of 1 to 4 [(1) not at all affected; (2) a little affected; (3) quite a bit affected and (4) very much affected]. In addition to lesser pain scores (as above) factors which were significantly better in patients

Table 2 Quality of life, pain scores and analgesic usage in disease duration-matched patients with chronic pancreatitis

	CP patients on Antox (n = 28)	CP patients NOT on Antox (n = 28)	P-value
Median visual analogue pain scores (range 0-10)	3 (0-8)	6 (0-8)	< 0.01 (Mann-Whitney U-test)
Patients taking analgesics	16	26	< 0.01
Patients taking opiate analgesics	11	23	< 0.01
Diabetes	10 (36%)	11 (39%)	0.80
Pancreatic exocrine supplements	14 (50%)	16 (57%)	0.60

CP: Chronic pancreatitis.

Table 3 Detailed European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) results in disease duration-matched patients with chronic pancreatitis

Scales	Items	CP patients on Antox mean score (n = 28)	CP patients NOT on Antox mean score (n = 28)	P-value	95% CI
Physical Functioning	Q-1-5	1.5	1.97	0.005[1]	-0.8 to 0.1
Role Functioning	Q-6-7	1.84	2.75	0.001[1]	-1.5 to 0.5
Pain	Q-9, 19	2.1	3.1	< 0.0001[1]	-1.5 to 0.7
Fatigue	Q-10, 12, 18	2.05	2.86	0.0001[1]	-1.7 to 0.3
Nausea and vomiting	Q-14, 15	1.5	2.2	0.002[1]	-1.2 to 0.2
Cognitive functioning	Q-20, 25	1.6	2.4	< 0.0001[1]	-1 to 0.5
Emotional functioning	Q-21-24	1.8	2.6	0.0007[1]	-1.2 to 0.5
Social functioning	Q-26, 27	1.8	2.7	0.0032[1]	-1.2 to 0.2
Global quality of life	Q-29, 30	2.6	4.9	< 0.0001[1]	1.5 to 3
Pancreatic Pain	Q-31, 33, 35	1.9	2.9	< 0.0001[1]	-1.5 to -0.7
Digestive function	Q-36, 37	2.14	2.5	0.09[1]	-1 to 0
Jaundice	Q-44, 45	1.1	1.2	0.41[1]	-0.25 to 0
Altered bowel functioning	Q-46, 47	2.1	1.6	0.07[1]	-1 to 0
Body Image	Q-48, 51	1.3	2.5	0.0004[1]	-1.2 to -0.25
Alcohol related guilt	Q-49, 50	1.5	1.4	0.7[1]	-0.25 to 0.5
Satisfaction with health care	Q-55, 56	3.2	3.4	0.44[1]	-0.5 to 0.25
Sexual functioning	Q-57, 58	1.7	2.66	0.0003[1]	-1.5 to -0.5
Dyspnea/shortness of breath	Q-8	1.5	1.6	0.63[1]	-0.5 to 0
Difficulty sleeping	Q-11	1.9	2.9	0.0003[1]	-1.5 to 0.5
Loss of appetite	Q-13	1.8	2.5	0.04[1]	-1.5 to 0
Constipation	Q-16	1.7	1.8	0.86[2]	-1 to 0.5
Diarrhea	Q-17	1.5	1.5	0.03[2]	-1 to 0
Financial problems	Q-28	1.3	1.9	0.02[2]	-1 to 0
Bloated abdomen	Q-32	1.9	2.8	0.002[2]	-1.5 to 0.5
Night pain	Q-34	1.8	3.1	< 0.0001[2]	-2 to -0.5
Taste changes	Q-38	1.2	1.7	0.02[2]	-1 to 0
Indigestion	Q-39	1.7	2.4	0.018[2]	-1 to 0
Flatulence	Q-40	2.0	2.6	0.06[2]	-1 to 0
Weight loss	Q-41	1.2	2.4	< 0.0001[2]	-1.5 to -0.5
Decreased muscle strength	Q-42	1.8	2.3	0.11[2]	-1 to 0
Dry mouth	Q-43	1.6	2.2	0.02[2]	-1 to 0
Treatment side effects	Q-52	1.2	2.0	0.0002[2]	-1 to -0.5
Fear for future health	Q-53	2.4	3.0	0.03[2]	-1 to 0
Ability to plan ahead	Q-54	1.8	2.9	0.0001[2]	-1.5 to 0.5

[1]Wilcoxon signed rank test 95% CI; [2]Wilcoxon t-test. CP: Chronic pancreatitis; Q: Question No. (total C-30 + PAN 28 = 58 questions).

taking Antox were: physical functioning, role functioning and cognitive and emotional functioning. These translated into a significant improvement in global QoL. Digestive function, jaundice and bowel function were not significantly different.

DISCUSSION

This study has examined QoL in patients with CP. Contemporary criteria were used for definition of disease and the Zurich Workshop recommendations were used for assessment of alcohol-related CP[18]. QoL has been evaluated in 2 cohorts of patients: those taking oral antioxidant therapy for CP and those not taking this medication. Specific disease-validated questionnaires were used for assessment of QoL[17].

Antioxidant therapy has been available for the treatment of CP for over 20 years[19]. However, the lack of good-quality randomized trial evidence and the dearth of information about clinical outcome in patients taking antioxidants has meant that this treatment remains on the periphery of practical management.

Thus, the importance of the present study is that it is believed to be the first to utilize specific disease-validated questionnaire methodology to assess QoL in patients with well-defined CP taking oral Antox. Potential sources of bias in these data should be borne in mind when interpreting the results.

Patients were not randomly allocated to Antox or Non-Antox; those receiving Antox were older and had longer disease duration (Table 1). Although disease-duration matching may have corrected for some of these factors, other confounding factors could persist: there were more smokers in the Non-Antox group and a sequential, multiple interview strategy would have yielded a more accurate reflection of QoL. This is accepted but must be balanced against the inconvenience to patients resulting from completing the lengthy questionnaires involved in this study. Also on a practical basis all patients were interviewed at relatively stable points in their disease with no history of recent surgery or change in medication.

Accepting these likely sources of bias, measures of QoL showed a significant benefit in patients on Antox: pain scores, physical health scores and global QoL together with analgesic (including opiate) intake were significantly better in the Antox group.

However, these findings may simply reflect a more mature population in the Antox group who have had more time to adjust to their illness and in some of whom the disease may be "burnt out"[20].

It is accepted that disease duration in a long-term chronic illness such as CP can be unreliably recalled[21] but prospectively recorded duration data were taken from the patients' charts and thus any error should be similar in both groups. The process of matching for disease duration produces a cohort of 28 pairs who are also reasonably well matched in terms of age, gender and etiology (Table 3). Although alcohol consumption in the Non-Antox group was greater prior to diagnosis, there was no difference after diagnosis.

The outcome data from the disease duration-matched cohort show some striking findings. VAS were significantly lower in the Antox group with a corresponding lower use of analgesics including opiate analgesics. There were no differences in the proportion of patients with diabetes or those taking pancreatic exocrine supplements, suggesting that if Antox modified symptoms in CP, there was no effect on the underlying disease course. Examined in detail, using the full EORTC questionnaires, the study showed improvement in global QoL in patients taking Antox. There were no differences in jaundice and digestive function answers, again suggesting that antioxidant therapy may modify symptoms without affecting disease progression.

These data should be considered together with the results of a recent large randomized trial from India of oral multi-compound antioxidants in painful CP. Although the Indian study did not use QoL measurement and had soft principal end-points in the form of reduction in hospital admission and reduction in "pain days" the study showed benefit from treatment with antioxidants.

In summary, this study has used well-validated, disease-specific questionnaires to assess QoL in patients with well-defined CP and compared a cohort of patients taking Antox to a group who were not. When corrected for differences in disease duration and age, patients on Antox had significantly lower VAS, lower analgesic use and better global QoL. Caution in interpretation is required. We would state that these data support a renewal of interest in the role of antioxidant therapy in CP and favor the conduct of a formal, randomized placebo-controlled trial of Antox in painful CP.

COMMENTS

Background

Chronic pancreatitis (CP) is associated with severe, disabling, frequent abdominal pain. It often leads to endocrine (diabetes) and exocrine (diarrhea and weight loss) disorders. In general, patients with CP have very poor quality of life (QoL) and outcome. No treatment has been found to combat long-term pain and cure.

Research frontiers

There has been anecdotal evidence of upregulation of the oxidative stress response and deficiency in antioxidants levels in patients with CP. The cascade of events due to repeated exposure and non correction leads to pancreatic fibrosis. This in turn leads to severe, chronic abdominal pain and poor QoL. In this study we demonstrated improvement in QoL for patients who were on antioxidant therapy.

Innovations and breakthroughs

Antioxidant therapy for CP was suggested in the mid 1990s. There have been a few studies showing the benefit of antioxidant therapy in CP. However, due to the paucity of data, it has not been universally accepted. This is the first comparative study to report QoL assessment in patients with CP on antioxidant therapy and those NOT on antioxidant therapy.

Applications

The study has renewed interest in antioxidant therapy for CP. The data lack randomization. This report supports the progression to a formal randomized double-blind trial of antioxidant therapy assessing QoL and outcome in patients with CP.

Terminology

Antioxidants is the term given to inhibitors of the oxidative stress response. They are typically selenium, vitamin E, methionine and ascorbic acid. European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) (PAN-28) (EORTC, QoL core questionnaire and its pancreatic modification, PAN-28) are the QoL assessment tools validated and used for patients suffering from CP. Such a methodology is important and vital in measuring the outcome in chronic diseases.

Peer review

In general, this study by Shah *et al* has high originality and is interesting because they revealed the effectiveness of antioxidant treatment for CP which focused on QoL.

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S- Editor Wang YR **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4072

World J Gastroenterol 2010 August 28; 16(32): 4072-4078
ISSN 1007-9327 (print)

BRIEF ARTICLE

Diagnostic value of cancer-testis antigen mRNA in peripheral blood from hepatocellular carcinoma patients

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Supported by National Natural Science Foundation of China, No. 30200271
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Received: April 7, 2010 Revised: May 28, 2010
Accepted: June 4, 2010
Published online: August 28, 2010

Abstract

AIM: To evaluate the diagnostic value of cancer-testis antigen (CTA) mRNA in peripheral blood samples from hepatocellular carcinoma (HCC) patients.

METHODS: Peripheral blood samples were taken from 90 patients with HCC before operation. Expression of melanoma antigen-1 (MAGE-1), synovial sarcoma X breakpoint-1 (SSX-1), and cancer-testis-associated protein of 11 kDa (CTp11) mRNA in peripheral blood mononuclear cells (PBMC) was tested by nested reverse transcripts-polymerase chain reaction (RT-PCR). Serum α-fetoprotein (AFP) in these patients was also determined.

RESULTS: The positive rate of MAGE-1, SSX-1 and CTp11 transcripts was 37.7%, 34.4%, 31.1% in PBMC samples, and 74.4%, 73.3%, 62.2% in their resected tumor samples, respectively. The positive rate for at least one of the transcripts of three CTA genes was 66.7% in PBMC samples and 91.1% in their resected tumor samples. MAGE-1, SSX-1 and/or CTp11 mRNA were not detected in the PBMC of those patients from whom the resected tumor samples were MAGE-1, SSX-1 and/or CTp11 mRNA negative, nor in the PBMC samples from 20 healthy donors and 10 cirrhotic patients. Among the 90 patients, the serum AFP in 44 patients met the general diagnostic standard (AFP > 400 μg/L) for HCC, and was negative (AFP ⩽ 20 μg/L) or positive with a low concentration (20 μg/L < AFP ⩽ 400 μg/L) in the other patients. The positive rate for at least one of the transcripts of three CTA genes in PBMC samples from the AFP negative or positive patients with a low concentration was 69.2% and 45.0%, respectively. Of the 90 patients, 71 (78.9%) were diagnosed as HCC by nested RT-PCR and serum AFP. Although the positive rate for at least one of the transcripts of three CTA genes in PBMC samples from 53 patients at TNM stage Ⅲ or Ⅳ was obviously higher than that in PBMC samples from 37 patients at stage Ⅰ or Ⅱ (77.9% *vs* 51.4%, $P = 0.010$), the CTA mRNA was detected in 41.7% and 56.0% of PBMC samples from HCC patients at stages Ⅰ and Ⅱ, respectively.

CONCLUSION: Detecting MAGE-1, SSX-1 and CTp11 mRNA in PBMC improves the total diagnostic rate of HCC.

Key words: Hepatocellular carcinoma; α-fetoprotein; Cancer-testis antigen; Diagnosis; Nested reverse transcripts-polymerase chain reaction

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Zhao L, Mou DC, Peng JR, Huang L, Wu ZA, Leng XS. Diagnostic value of cancer-testis antigen mRNA in peripheral blood from hepatocellular carcinoma patients. *World J Gastroenterol*

2010; 16(32): 4072-4078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4072.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4072

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers in the world[1-3]. More than one million cases of HCC occur in the world each year[4]. Although many treatment modalities for HCC are available (including hepatic resection, liver transplantation, radiofrequency ablation, transarterial chemoembolization, *etc.*) at present, the prognosis of HCC patients remains dismal because it is detected at an advanced, non-resectable stage. Early diagnosis of HCC can improve the prognosis of HCC patients. So far, α-fetoprotein (AFP) is the generally accepted serological marker. Serum AFP alone contributes to the diagnosis of HCC if its level is markedly elevated (over 400 μg/L as a threshold value), which occurs in less than 50% of cases at the time of diagnosis[5-8]. Moreover, serum AFP level is negative or slightly elevated in 20%-40% patients, which can significantly reduce the sensitivity of an assay based on over-expression of AFP. The serum AFP level in patients with acute or chronic hepatitis or liver cirrhosis but without malignant disease is often elevated. Since detection of serum AFP level in blood samples appears to be nonspecific[7-10]. Therefore, the diagnostic sensitivity and specificity of AFP are unsatisfactory and questionable. It is thus necessary to select other specific methods for the diagnosis of HCC.

Transcripts of tumor-specific genes can be amplified and detected by reverse transcripts-polymerase chain reaction (RT-PCR), which is a reliable technique to detect circulating tumor cells (CTC). In 1991, Smith *et al*[11] first successfully adopted RT-PCR technique to assess tyrosinase messenger RNA (mRNA) as a tumor marker in detecting circulating melanoma cells. Since then, this technique has been applied to the detection of CTC in solid tumors[12-14]. Melanoma antigen-1 (MAGE-1)[15], synovial sarcoma X breakpoint-1 (SSX-1)[16] and cancer-testis-associated protein of 11 kDa (CTp11)[17] antigens have been designated as cancer-testis antigens (CTA). It has been reported that MAGE-1 and SSX-1 mRNA are expressed with a high percentage and specificity in HCC[16,18-20]. Our group has verified a relatively high and specific expression of CTp11 mRNA in HCC tissues but not in the corresponding adjacent non-HCC and cirrhosis tissues[21]. In this study, we evaluated the diagnostic significance of a highly sensitive nested RT-PCR assay for the MAGE-1, SSX-1 and CTp11mRNA in peripheral blood of HCC patients.

MATERIALS AND METHODS

Cell lines

Human HCC cell lines BEL7405 expressing MAGE-1 mRNA and LM3 expressing both SSX-1 and CTp11 mRNA, purchased from the Cell Bank, Chinese Academy of Sciences, and Liver Cancer Institute, Zhongshan Hospital, Fudan University, respectively, were grown in RPMI1640 medium with 10% fetal calf serum and served as a positive control of the assay used in this study.

Patients and tissue samples

Ninety consecutive patients (79 men and 11 women) with a mean age of 45.6 ± 2.7 years (range 18-79 years) undergoing operation for HCC, including hepatectomy (48 cases) or orthotopic liver transplantation (42 cases), at the 2nd Hospital of Peking University Health Science Centre, were enrolled in this study. Of the 83 patients with virus infection, 79 were infected with hepatitis B virus, 2 with hepatitis C virus, and 2 with both hepatitis B and C viruses. The serum AFP level was negative (≤ 20 μg/L) in 26 patients, positive with a low concentration (20 μg/L < AFP ≤ 400 μg/L) and a high concentration (> 400 μg/L) in 20 and 44 patients, respectively. According to the TNM classification of International Union Against Cancer[22], 12 cases were classified as stage Ⅰ, 25 as stage Ⅱ, 9 as stage Ⅲ, and 44 as stage Ⅳ, respectively. HCC and its adjacent non-cancerous tissue samples (the distance to the edge of HCC tissue > 2 cm) were collected during operation. Control samples collected by surgical biopsy included 20 liver tissue samples from cirrhotic patients and 20 normal liver tissue samples from patients without liver disease. Testis tissue (kindly provided by Urological Department of the 2nd Hospital of Peking University Health Science Centre) was used as a positive control. Clinical diagnosis was confirmed by pathological examination. Each sample was immediately frozen in liquid nitrogen and stored at -80℃ until extraction of total RNA. Informed consent was obtained from each patient before the study. The study protocol was approved by the Ethic Committee of Peking University.

Blood samples

Whole blood samples were taken from the 90 HCC patients on the day before operation. Control blood samples were collected form 20 healthy volunteers and 10 cirrhotic patients. Ten mL of blood from each patient was collected into a heparinzed tube and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation[15].

Extraction of total RNA and synthesis of cDNA

Total RNA was extracted from frozen tissue specimens (50-100 mg) and freshly isolated PBMC (1×10^7 cells) using TRIZOL reagent (GIBCOL BRL) according to its manufacturer's instructions. Total RNA (2.5 μg) was primed with an Oligo (dT)15 oligonucleotide (Promega, USA) and reverse-transcribed with Superscript Ⅱ (GIBCO BRL, USA) according to their manufacturers' instructions.

PCR amplification of CTAs

PCR amplification reaction (50 μL) contained 5 μL of

Table 1 Primers and conditions used in nested reverse transcripts-polymerase chain reaction

Primers	Primers from 5' to 3'	PCR conditions denaturation annealing extension
MAGE-1 outer primers	Forward primer: 5'-CGGCCGAAGGAACCTGACCCAG-3'	94℃ for 45 s
	Reverse primer: 5'-GCTGGAACCCTCACTGGGTTGCC-3'	65℃ for 45 s
		72℃ for 45 s
MAGE-1 inner primers	Forward primer: 5'-ACAGAGGAGCACCAAGGAGAAG-3'	94℃ for 45 s
	Reverse primer: 5'-AGTTGATGGTAGTGGGAAAGGC-3'	65℃ for 45 s
		72℃ for 45 s
SSX-1 outer primers	Forward primer: 5'-CTAAAGCATCAGAGAAGAGAAGC-3'	94℃ for 60 s
	Reverse primer: 5'-AGATCTCTTATTAATCTTCTCAGAAA-3'	57℃ for 60 s
		72℃ for 60 s
SSX-1 inner primers	Forward primer: 5'-TCAGAGAAGAGAAGCAAGGCCTTT-3'	94℃ for 45 s
	Reverse primer: 5'-TTCTCAGAAATATTTGCTTTTCC-3'	56℃ for 45 s
		72℃ for 45 s
CTp11 outer primers	Forward primer: 5'-CTGCCCCAGACATTGAAGAA-3'	94℃ for 45 s
	Reverse primer: 5'-TCCATGAATTCCTCCTCCTC-3'	57℃ for 60 s
		72℃ for 90 s
CTp11 inner primers	Forward primer: 5'-TGTGAATCCAACGAGGTG-3'	94℃ for 45 s
	Reverse primer: 5'-TTGATTCTGTTCTCTCGGGC-3'	60℃ for 45 s
		72℃ for 45 s

PCR: Polymerase chain reaction; MAGE-1: Melanoma antigen-1; SSX-1: Synovial sarcoma X breakpoint-1; CTp11: Cancer-testis-associated protein of 11 kDa.

cDNA, 1 μL each of 10 μmol/L outer/inner primers, 1 μL of 10 mmol/L dNTP mixture, 2.5 U *Taq* polymerase (GIBCO BRL, USA) in a buffer solution. Thirty-five cycles of PCR amplification of cDNA from liver tissue were performed with a pre-programmed UNO Ⅱ thermocycler (Biometra, German) under the following conditions: an initial denaturation at 94℃ for 5 min, a final extension at 72℃ for 8 min. The PCR products were 421 base pair (bp) (MAGE-1), 422 bp (SSX-1) and 297 bp (CTp11), respectively. Twenty-five cycles of PCR amplification of cDNA from PBMC were performed with its first round conditions identical to those of cDNA from liver tissue. For the second round of PCR, 1 μL of the first-round PCR products was used as a template in combination with 1 μL each of 10 μmol/L inner primers. After heated for 2 min at 94℃, the samples were subjected to 35 cycles of PCR amplification, followed by a final extension at 72℃ for 8 min. The PCR products were 299 bp (MAGE-1), 398 bp (SSX-1) and 188 bp (CTp11), respectively. The PCR conditions and outer/inner primers for MAGE-1[15], SSX-1[16] and CTp11[17] used in this study are shown in Table 1. To verify the integrity of cDNA[18], β2-microglobin (β2-MG) (primers: forward: 5'-CTCGCGCTACTCTCTCTTTCTGG-3' and reverse: 5'-GCTTACATGTCTCGATCCCACTTAA-3', 335 bp) was amplified for 30 cycles (at 94℃, 55℃ and 72℃ for 45 s). For analysis, 8 μL of reaction products was run in 2% agarose gel (Promega, USA), followed by ethidium bromide staining and digital camera photographing (Korda D3.5, USA).

Sensitivity of nested RT-PCR technique

The sensitivity of our nested PCR assay was evaluated by performing the procedure on healthy volunteer blood samples mixed with a certain number of hepatoma cells. Ten-fold serial dilution experiments from 10^4 hepatoma cells were carried out using human hepatoma cell lines BEL7405 expressing MAGE-1 transcript and LM3 expressing both SSX-1 and CTp11 mRNA. Hepatoma cells (1×10^3, 1×10^2, 1×10^1 and 1×10^0) were added to 5×10^6 PBMC from healthy donors, then total RNA was extracted and subjected to RT-PCR amplification with primers for β2-microglobin, MAGE-1, SSX-1 and CTp11 genes.

Sequence analysis of PCR products

PCR amplification-purified cDNA was cloned into the pGEM-T easy vector (Promega) by T4 DNA ligase and amplified in *Escherichia coli*, JM109. Four positive colonies were selected and assessed using EcoR Ⅰ digestion of mini-prepared DNA. Putative MAGE-1, SSX-1 and CTp11 cDNA samples were sequenced with T7 sequencing primers in Sangon Co., Shanghai, China.

Statistical analysis

Statistical analysis was performed by chi-square test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Sensitivity of nested RT-PCR technique

After two rounds of PCR amplification, MAGE-1, SSX-1 and CTp11 transcript genes could be detected in the PCR products, indicating that our assay is able to detect a hepatoma cell in 5×10^6 PBMC (Figure 1).

Expression of CTA genes in HCC tissue samples

Expression of MAGE-1, SSX-1 and CTp11 mRNA was detectabled in 74.4%, 73.3% and 62.2% of the 90 HCC tissue samples, respectively. No expression of these genes was detected in the corresponding adjacent non-HCC tissue samples, or in the normal and cirrhotic liver tissue samples. Eighty-two HCC tissue samples (91.1%) were

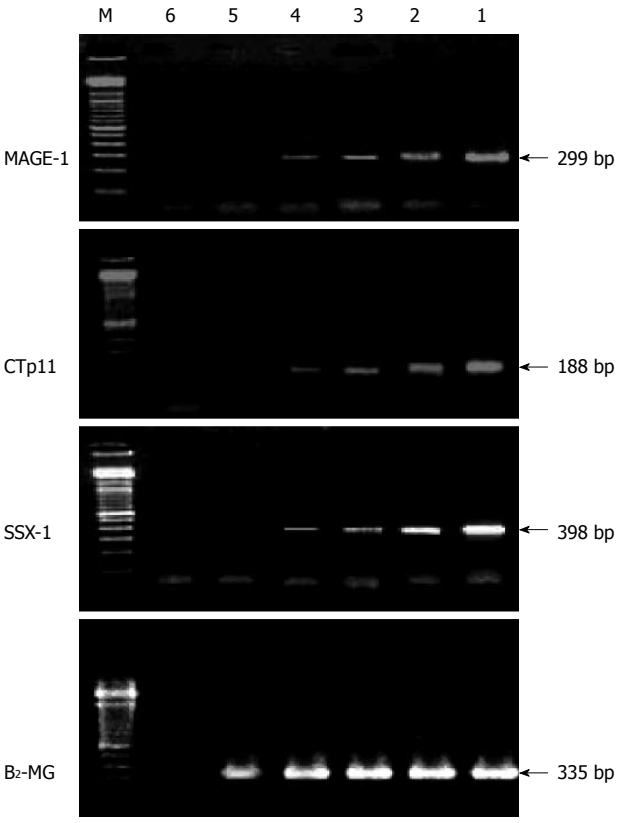

Figure 1 Sensitivity of nested polymerase chain reaction assay to tumor-specific markers in hepatocellular carcinoma cell lines. Lanes 1-4: 1 × 10^3, 1 × 10^2, 1 × 10^1 and 1 × 10^0 hepatoma cells detected by nested reverse transcripts-polymerase chain reaction using melanoma antigen-1 (MAGE-1), synovial sarcoma X breakpoint-1 (SSX-1), cancer-testis-associated protein of 11 kDa (CTp11), and β2-microglobin; Lane 5: Peripheral blood mononuclear cells (PBMC) from healthy donors only; Lane 6: negative control; Lane M: Molecular marker, 100 bp DNA ladder (Gibco).

positive for at least one of the transcripts of three CTA genes.

Nested RT-PCR results in HCC PBMC samples

After two rounds of PCR amplification, MAGE-1, SSX-1 and CTp11 were detected in 37.7%, 34.4% and 31.1% of the PBMC samples, respectively. At least one of the three genes was expressed in 66 PBMC samples (66.7%). Of the 82 MAGEE-1, SSX-1 or CTp11mRNA positive HCC tissue samples, at least one of the transcripts of three genes was detected in PBMC from 60 patients with a positive correlation rate of 73.2%. These gene transcripts could not be detected in PBMC from patients with MAGEE-1, SSX-1 or CTp11mRNA undetectable in their liver tissue samples. MAGE-1, SSX-1 or CTp11 gene was not expressed in the 30 control PBMC samples from 20 healthy volunteers and 10 cirrhotic patients. The typical electrophoresis of nested RT-PCR products amplified from cDNA in PBMC samples from HCC patients is shown in Figure 2.

Sequence analysis of PCR products

Sequence analysis of PCR products verified that the nucleotide sequences of MAGE-1, SSX-1, and CTp11 cDNA fragments were identical to those in GenBank, indicating that the RT-PCR products are MAGE-1, SSX-1, and CTp11 cDNA.

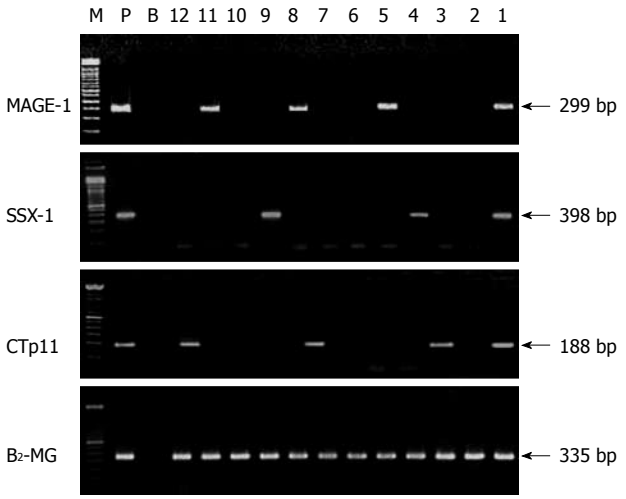

Figure 2 Electrophoresis of second round polymerase chain reaction products amplified from cDNA of peripheral blood mononuclear cells samples. Lane M: Molecular marker, 100 bp DNA ladder (Gibco); Lane P: Positive control; Lane B: Blank control; lane B2-MG (335 bp) as cDNA quality control; Lanes 1, 5, 8, 11: Positive melanoma antigen-1 (MAGE-1) transcript; Lanes 1, 4, 9: Positive synovial sarcoma X breakpoint-1 (SSX-1) transcript; Lanes 1, 3, 7, 12: Positive cancer-testis-associated protein of 11 kDa (CTp11) transcript; Lanes 2, 6 and 10: Negative transcript of all three CTA genes.

CTA transcripts in PBMC and serum AFP level

The overall positive rate of AFP with a high concentration in serum and MAGE-1, SSX-1 and/or CTp11 mRNA in PBMC samples was 48.9% and 66.7%, respectively. No correlation was observed between positive AFP rate and CTA transcripts. However, the serum AFP was negative (≤ 20 μg/L) in 26 HCC patients and positive with a low concentration (20 μg/L < AFP ≤ 400 μg/L) in 20 HCC patients. Of these 46 patients, 27 (18 cases with negative AFP and 9 cases with low concentration AFP) had MAGE-1, SSX-1 and/or CTp11 mRNA transcripts detected in their PBMC samples. By contrast, of the 30 patients with negative CTA transcripts in PBMC samples, 21 had the serum AFP level higher than 400 μg/L. Totally, 78.9% of HCC patients had either the AFP level higher than 400 μg/L in serum or positive CTA transcripts in PBMC. The parameters of AFP in serum and CTA transcripts in PBMC in combination with the results of imaging studies, would enable to make a clear diagnosis of 78.9% of HCC patients, which is much higher than the test with single AFP ($\chi^2 = 17.555$, $P < 0.01$)

CTA transcripts in PBMC from early HCC patients

The frequency of positive MAGE-1, SSX-1 and/or CTp11 transcripts detected in PBMC from patients with HCC was 41.7% at stage Ⅰ, 56.0% at stage Ⅱ, 66.7% at stage Ⅲ, and 79.5%% at stage Ⅳ, respectively. Of note, MAGE-1, SSX-1 and/or CTp11 mRNA was detected in PBMC from 51.4% of HCC patients at stages Ⅰ and Ⅱ and from 77.9% of HCC patients at stages Ⅲ and

Ⅳ, showing that advanced stages of HCC are correlated with the higher expression frequency of MAGE-1, SSX-1 and/or CTp11 gene mRNA (χ^2 = 6.632, P = 0.010). As many as 59.5% of the HCC patients at stages Ⅰ and Ⅱ could be diagnosed when CTA transcripts in PBMC and serum AFP level were combined with imaging findings. However, the serum AFP level was higher than 400 μg/L in 35.1% of the HCC patients. The diagnosis rate made by combined CTAs and AFP was significantly higher than that based on single AFP (χ^2 = 4.391, P = 0.036).

DISCUSSION

The integration of molecular and immunological techniques has led to the identification of a new category of tumor-specific antigens, also known as cancer-testis antigens, such as melanoma antigen (MAGE), synovial sarcoma X breakpoint (SSX), B melanoma antigen (BAGE), G melanoma antigen (GAGE), synaptonemal complex protein-1 (SCP-1), New York esophagus-1 (NY-ESO-1), and CTp11[23]. The CTAs are a distinct and unique class of differentiation antigens. Attributing genes to this gene group is based on their characteristics, including mRNA expression in normal tissues of testis, fetal ovary, and placenta, and mRNA expression in different cancers. Until now, at least 70 families of cancer-testis gene with 140 members have been attributed to this group and their expression has been studied in different types of tumors[23-26]. MAGE-1, SSX-1 and CTp11 belong to the CTA family members. The growing knowledge about CTAs indicates that the expression of CTA often shows a marked specificity for tumor cells[23,27-30]. These markers can be used to target tumor cells for early detection.

In the present study, CTAs (MAGE-1, SSX-1 and CTp11) were expressed with a high percentage and specificity in HCC. The positive rate for at least one of the transcripts of three CTA genes in HCC tissue samples was as high as 91.2%. Conversely, no expression was detected in the adjacent normal and cirrhotic liver tissue samples, or in the PBMC samples from healthy donors and cirrhotic patients. Based on the prevalent invasion of HCC cells to hepatic vessels, it is reasonable to consider that MAGE-1, SSX-1 and CTp11 are the appropriate tumor-specific markers for the detection of circulating HCC cells, which may play a complementary role in HCC diagnosis.

In this study, a sensitive and specific technique was developed, which is capable of detecting circulating HCC cells using MAGE-1, SSX-1 and CTp11 transcripts as tumor-specific markers by nested RT-PCR. Through the ten-fold serial dilution experiments with positive control cell lines, our results verified that exponential amplification of target cDNA converted from mRNA could allow to detect a single malignant cell within millions of normal blood cells and hence, to sensitively detect the metastatic tumor cells in peripheral blood. The sensitivity of this assay is within the range of other published reports[31-34], which is much more sensitive than antibody-based serology[35,36]. The positive rate of nested RT-PCR was as high as 66.7% for at least one of the transcripts of three CTA genes in the PBMC samples from HCC patients. In addition, detecting any of the transcripts of three CTA genes in PBMC samples from HCC patients would directly represent the presence of tumor cells in peripheral blood, suggesting that this method has a higher specificity than serum AFP and is thus able to improve the diagnosis of HCC. However, no expression of CTAs was detected in 8.9% of HCC tissue samples, showing that it is necessary to screen other CTAs or tumor specific antigens in these patients. If we can filter out 1-2 other markers, the diagnostic sensitivity of this method would be further improved.

Both albumin[37,38] and AFP[39-41] mRNA have been widely used as tumor markers for detecting HCC cells in circulation. However, the reliability is challenged, because albumin is abundantly expressed in normal liver cells[37] and AFP is expressed in liver cells infected with hepatitis virus or in cirrhotic liver[34]. In recent years, although an increasing number of genetic markers, such as telomerase reverse transcriptase[42], Des-g-carboxyprothrombin[43], squamous cell carcinoma antigen-immunoglobulin M complexes[44] and human cervical cancer oncogene[45], have been used in the diagnosis of early HCC, they have significant diagnostic limitations in their specific nature. In this study, MAGE-1, SSX-1 or CTp11 was not detected in PBMC from patients with their HCC tissue samples negative for these three CTA genes mRNA or in PBMC from 20 healthy donors and 10 cirrhotic patients, indicating that detecting CTA transcripts in PBMC from HCC patients has a high specificity for HCC.

So far, no methods or biomarkers demonstrate absolute superiority for early detection of HCC. It is difficult to simultaneously solve their sensitivity and specificity. Our assay by nested RT-PCR using MAGE-1, SSX-1 and CTp11 mRNA as tumor-specific makers showed a high sensitivity and specificity, indicating that it can establish the diagnosis of HCC.

Molecular biology technology contributes to the early diagnosis of HCC. However, its disadvantages are also obvious, including its cost and availability. PCR assay, a commonly used molecular biology technology, is more expensive and troublesome than serological tests, and is thus not the first choice in early detection of HCC. However, it plays a supplementary role in the diagnosis of HCC. Hopefully in the not so distant future, this technology will become increasingly popular and automatic with its cost decreased.

At present, the serum AFP level is still the gold standard for diagnosis of liver cancer. The AFP level is normal in 20%-40% of HCC patients at the time of diagnosis and usually remains low even in patients with advanced HCC[7-10]. AFP > 400 μg/L is considered diagnostic for HCC, although fewer than 50% of HCC patients may meet this standard[5-8]. With values of that magnitude, the specificity of AFP is close to 100% at the cost of its sensitivity fallen to less than 45%[6,7]. In this study, 51.1% of HCC patients were negative or positive for AFP with

a low concentration. MAGE-1, SSX-1 and/or CTp11 gene was expressed in 69.2% and 45.0% of HCC patients in the two groups, respectively, suggesting that mRNA, a tumor-specific marker, can be used as an adjuvant diagnostic tool. Detecting the transcripts of three CTA genes combined with serum AFP test in PBMC from HCC patients, can improve the total diagnostic rate of HCC.

In this study, the expression of CTAs in PBMC was significantly correlated with the clinical TNM classification of HCC. Although the positive frequency of CTA mRNA in PBMC was significantly higher in HCC patients at stages Ⅲ and Ⅳ than in those at stages Ⅰ and Ⅱ, MAGE-1, SSX-1 and/or CTp11 mRNA transcripts were detected in 41.7% of the HCC patients at stage Ⅰ and in 56.0% of the HCC patients at stage Ⅱ, showing that these three CTAs are reliable markers for screening hematogenous spread of early HCC cells. The combination of CTA transcripts in PBMC and serum AFP level improves early diagnosis of HCC. The classic TNM staging method[22] does not accurately reflect the actual process of HCC patients. The TNM classification criteria for HCC include tumor size, presence of portal vein invasion, and extrahepatic metastasis, *etc*. According to the TNM criteria, HCC at stage Ⅰ (T1N0M0) or stage Ⅱ (T2N0M0) should have no metastasis of tumor cells except for intra-hepatic metastasis. Assay by nested RT-PCR to detect MAGE-1, SSX-1 and/or CTp11 transcripts, the tumor-specific markers, revealed that 51.4% patients with HCC in early stages (stage Ⅰ and Ⅱ) have already had micrometastasis to the peripheral blood, indicating that blood dissemination of tumor cells has already occurred in the early stage of HCC when distant metastasis cannot be confirmed. Furthermore, it may be the reason why some early HCC patients still suffer from recurrence even after complete removal of the tumor. Detecting CTA transcripts in PBMC of early HCC patients can demonstrate hematogeneous dissemination of tumor cells more specifically than conventional methods, thus playing a supplementary role in the diagnosis of HCC. The traditional TNM staging criteria ignoring the presence of circulating HCC cells need to be perfected.

COMMENTS

Background

The prognosis of hepatocellular carcinoma (HCC) is poor because it is detection at an advanced, non-resectable stage. So far, α-fetoprotein (AFP) is a generally accepted serological marker. Its diagnostic accuracy is unsatisfactory and questionable. Serum AFP alone is helpful when its level is markedly elevated, occuring in less than 50% of cases at the time of diagnosis. Therefore, more sensitive and specific biomarkers are needed.

Research frontiers

The limitations of conventional AFP as a marker has led to a search for more sensitive and specific markers. In recent years, although an increasing number of genetic markers have been used in diagnosis of early HCC, they have significant diagnostic limitations in the specific nature. Cancer-testis antigens (CTAs) are frequently expressed in different types of cancer and have received considerable attention as ideal biomarkers of tumor cells.

Innovations and breakthroughs

The sensitivity and specificity of nested reverse transcripts-polymerase chain reaction assay using melanoma antigen-1, synovial sarcoma X breakpoint-1 and cancer-testis-associated protein of 11 kDa mRNA as tumor-specific multiple-makers are high and can thus be as an adjuvant diagnostic tool. This assay combined with serum AFP level may improve the diagnosis of HCC.

Applications

Detecting transcripts of CTA genes in peripheral blood mononuclear cells from HCC patients, combined with serum AFP test, improves the total diagnostic rate of HCC.

Peer review

The authors investigated the expression of some CTA genes in peripheral blood of HCC patients and showed that the positive rate for at least one of the three CTA genes was 67% in blood samples and 90% in resected tumor samples. They also showed a high specificity and sensitivity of their method for HCC. The study is very interesting and important for early diagnosis of HCC.

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S- Editor Wang JL **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4079

World J Gastroenterol 2010 August 28; 16(32): 4079-4083
ISSN 1007-9327 (print)

BRIEF ARTICLE

Effects of moxibustion on dynorphin and endomorphin in rats with chronic visceral hyperalgesia

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Supported by The National Basic Research Program of China, 973 Program, No. 2009CB522900; Scientific Research Grants of Shanghai Health Bureau, No. 2009209; Shanghai Leading Academic Discipline Project, No. S30304

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Received: April 28, 2010 Revised: June 19, 2010
Accepted: June 26, 2010
Published online: August 28, 2010

Abstract

AIM: To observe the analgesic effects of moxibustion in rats with chronic visceral hyperalgesia and its influence on the concentration of dynorphin (Dyn) and endomorphin (EM) in spinal cord.

METHODS: The rat model of chronic visceral hyperalgesia was established by colorectal distention (CRD). In moxibustion (MX) group, moxibustion was applied once daily for 7 d; in sham moxibustion (SM) group, moxibustion was given to the same acupoints but with the non-smoldered end of the moxa stick. Model control (MC) group and normal control group were also studied. The scoring system of abdominal withdrawal reflex was used to evaluate visceral pain for behavioral assessment. Enzyme linked immunosorbent assay was performed to determine the concentrations of Dyn and EM in spinal cord.

RESULTS: Moxibustion significantly decreased visceral pain to CRD in this rat model, and no significant difference was detected between the SM group and the MC group. In MX group, moxibustion also increased the concentrations of Dyn and EM in spinal cord, and no significant difference was found between the SM group and the MC group.

CONCLUSION: Moxibustion therapy can significantly enhance the pain threshold of rats with chronic visceral hyperalgesia, and the effect may be closely related to the increased concentration of Dyn and EM in spinal cord.

Key words: Moxibustion; Analgesia; Hypersensitivity; Dynorphins; Endomorphin

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Liu HR, Qi L, Wu LY, Ma XP, Qin XD, Huang WY, Dong M, Wu HG. Effects of moxibustion on dynorphin and endomorphin in rats with chronic visceral hyperalgesia. *World J Gastroenterol* 2010; 16(32): 4079-4083 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4079.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4079

INTRODUCTION

Acupuncture-Moxibustion is an ancient therapy with a history of 3000 years in China, and it has spread to more than 160 countries for its good effects in management

of pain, nausea induced by radiotherapy/chemotherapy, vomiting, *etc.*[1]. Many studies have proved the analgesic effect of acupuncture from the view point of neurophysiology, neurochemistry, molecular biology, and brain functional imaging[2-6]. As a twin therapy of acupuncture, moxibustion has shown its effects in treatment of irritable bowel syndrome (IBS)[7], ulcerative colitis[8], Crohn's disease[9] and chronic/acute gastritis[10], especially in alleviating visceral pain. Some studies believed that acupuncture could relieve pain by increasing the concentration or expression of dynorphin (Dyn) and endomorphin (EM) in spinal cord[11,12]. It has been reported that visceral sensory nerves are closely associated with the spinal cord fragments[13,14], so that moxibustion might achieve its analgesic effect in treating visceral pain by modulating the concentrations of Dyn and EM in the spinal cord fragments.

Our previous studies have revealed the analgesic effects of moxibustion in reducing abdominal pain in IBS patients[15] and IBS rat models[16,17]. However, the analgesic mechanism of moxibustion has not been clearly elucidated. In this study, a rat model of chronic visceral hyperalgesia was established by colorectal distention (CRD), and abdominal withdrawal reflex (AWR) scoring system was adopted for behavioral assessment in the evaluation of visceral pain after moxibustion intervention. The analgesic effect of moxibustion and increase of the concentration of Dyn and EM in spinal cord were demonstrated, which partially explained the mechanism of the analgesic effect of moxibustion in management of visceral pain.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (5 d old) were obtained from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (TCM). They were maintained in a plastic cage containing corn chip bedding with controlled temperature (22 ± 2℃), 60% ± 5% humidity and light-dark cycle (12:12 h) with a maximum of five rats per cage. Studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the Council for International Organizations of Medical Sciences and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

Study design

Neonatal rats were given daily mechanical colon distention beginning 8-21 d after their birth. After the distention was finished, the rats were kept until they reached adulthood (at least 6 wk old), and then experiments were conducted using behavioral test for visceral pain by acute CRD stimulus. Moxibustion (MX) group (n = 10): moxibustion was given to the acupoints of bilateral Tianshu (ST 25) and Shangjuxu (ST 37) using fine moxibustion stick with the smoldered end 2 cm away from the acupoints, once daily, 10 min each time, 7 times in total (Figure 1A). Sham moxibustion (SM) group (n = 10): intervention was given to bilateral Tianshu (ST 25) and Shangjuxu (ST 37) points

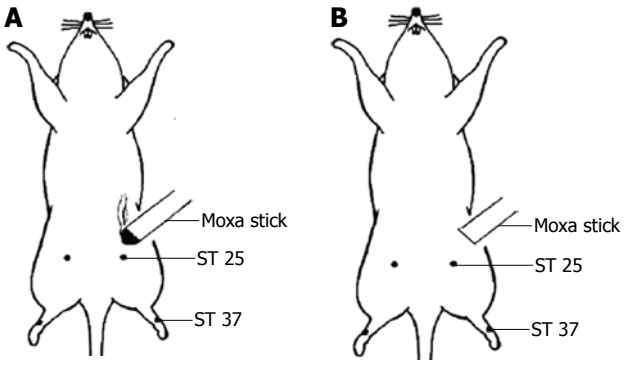

Figure 1 Moxibustion (A) and sham moxibustion (B).

Table 1 Abdominal withdrawal reflex scoring criteria

Score	
0	No behavioral response to colorectal distention
1	Immobile during colorectal distention and occasionally clicked the head at the onset of the stimulus
2	A mild contraction of abdominal muscles, but not lifting the abdomen off the platform
3	A strong contraction of the abdominal muscles and lifting the abdomen off the platform, not lifting the pelvic structure off the platform
4	Arching body and lifting the pelvic structure and scrotum

using fine moxibustion stick with the non-smoldered end 2 cm away from the acupoints, once daily, 10 min each time, 7 times in total (Figure 1B). Normal control (NC) group (n = 10) and model control (MC) group (n = 10): received no treatment except for constraining. After seven treatments, AWR was performed within 90 min, and a segment of spinal cord (L_4-S_1) was harvested and Dyn/EM concentration in spinal cord tissue was detected by enzyme linked immunosorbent assay (ELISA) (Figure 2).

Neonatal CRD irritation

Neonatal rats received CRD daily (the procedure was modified from previous reports[18,19]. Mainly, balloon (constructed from a condom; length: 20.0 mm; diameter: 3.0 mm) was inserted rectally into the descending colon. The balloon was distended with 0.5 mL air for 1 min and then deflated and withdrawn. The distention was repeated twice daily at a 30-min interval.

AWR scores

The AWR was assessed within 90 min after intervention using CRD based on semi-quantitative analysis. Prior to CRD, the rats were gently touched around anus for activating defecation. When the balloon was inserted into the descending colon, CRD was produced by rapidly inflating the balloon at strengths of 20, 40, 60, and 80 mmHg for a period of 20 s. Each score was tested three times, and each rat was tested by two people who were not involved in this research. There was a 3-min intervals between the two tests to allow the rats to adapt. The scoring criteria of AWR were referred to the method of Al-Chaer *et al*[18] (Table 1).

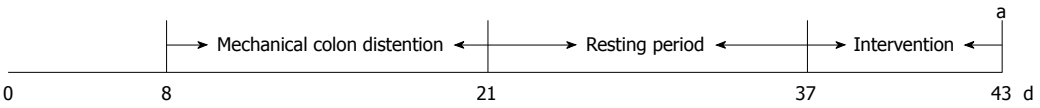

Figure 2 Experimental protocol of the study. [a]Abdominal withdrawal reflex sores after seven treatments.

ELISA for Dyn and EM

The dissected spinal cord tissue (L_4-S_1) was homogenized and weighed (10%), centrifuged for 30 min at 4℃, 4000 r/min. The supernatant was separated for assessment, and 100 μL standards and 100 μL dilution were mixed with 100 μL biotin, respectively. After incubation for 20 min at 20-25℃, 100 μL horse radish peroxidase was added. Followed by another 20 min of incubation at 20-25℃, 100 μL 3,3',5,5'-Tetramethylbenzidine substrate was added. Then 100 μL stop solution was put in after 20 min of incubation at 20-25℃. Calibration curve was drawn with OD value as the Y-coordinate and sample concentration as the X-coordinate. The concentration could be read according to the corresponding OD value. Rat Dyn and EM ELISA kits (THERMO MULTISKAN-MK3) were obtained from Finland.

Dyn in spinal cord (ng/L) = concentration × dilution times of the sample.

EM in spinal cord (ng/L) = concentration × dilution times of the sample.

Statistical analysis

The statistical analysis was done using SPSS 10.0 (SPSS Inc., USA). All data were expressed as mean ± SE for normally distributed continuous variables and as median (QL-QU) for abnormal variables. The differences in the mean values of the AWR score among the four groups (groups NC, MC, MX and SM) at each pressure of CRD were compared using the one-way analysis of variance (ANOVA, $P < 0.05$ as significant in differences). The differences in the median values of the concentration of Dyn and EM among the four groups were compared using the Kruskal-Wallis one-way analysis of variance on ranks. If the Kruskal-Wallis test result was significant ($P < 0.05$), we performed pairwise comparisons using a Wilcoxon rank sum test with a Bonferroni correction at 0.05/4 to correct for multiple comparisons. P value of < 0.05/4 was considered significant in differences.

RESULTS

Analgesic effects of moxibustion on chronic visceral hyperalgesia

At different levels of CRD stimuli (20, 40, 60 and 80 mmHg), the AWR scores in the MC group were significantly higher than in the NC group ($P < 0.01$); the AWR scores of MX group were significantly lower than that of the MC group ($P < 0.01$). There was no significant difference in the AWR scores between the MC group and SM group. This indicated that moxibustion treatment had a beneficial effect in chronic visceral hyperalgesia (Figure 3).

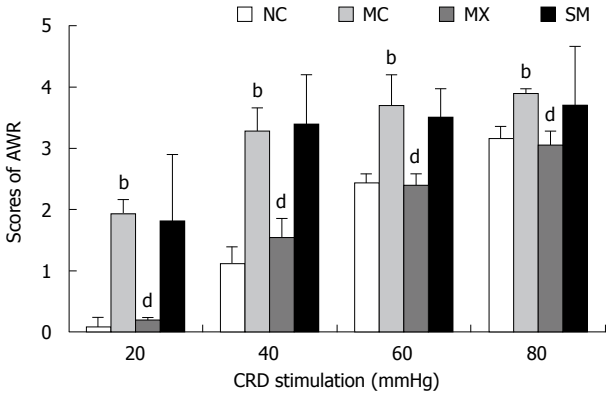

Figure 3 Analgesic effects of moxibustion on chronic visceral hyperalgesia. [b]$P < 0.01$ *vs* normal control (NC); [d]$P < 0.01$ *vs* model control (MC). AWR: Abdominal withdrawal reflex; MX: Moxibustion; SM: Sham moxibustion; CRD: Colorectal distention.

Influence of moxibustion in the Dyn concentration in spinal cord

The statistical analysis for the concentration of Dyn in spinal cord demonstrated a significant difference among the four groups, $F = 25.172$, $P = 0.000$. The concentration of MC group was significantly lower than that of NC group ($P < 0.01$). Compared with the MC group, the concentration of Dyn was significantly higher in the MX group ($P < 0.01$). No significant difference was detected in Dyn concentration between MC group and SM group (Figure 4A).

Influence of moxibustion on the EM concentration in spinal cord

Statistical analysis for the concentration of EM in spinal cord demonstrated a significant difference among the four groups, $F = 43.370$, $P = 0.000$. The concentration of MC group was significantly lower than that of NC group ($P < 0.01$). Compared with the MC group, the concentration of EM was significantly higher in the MX group ($P < 0.01$). No significant difference was detected in the EM concentration between the MC group and SM group (Figure 4B).

DISCUSSION

Visceral pain is commonly encountered by patients with functional intestinal disorders, leading to a miserable life and financial burden of the patients. Mertz *et al*[20] hold that the alterations of rectal sensitivity could be a biological indicator of IBS as IBS is featured by chronic abdominal pain. Alleviating abdominal pain is considered to be the main target in the management of IBS.

Moxibustion has been adopted as an analgesic method

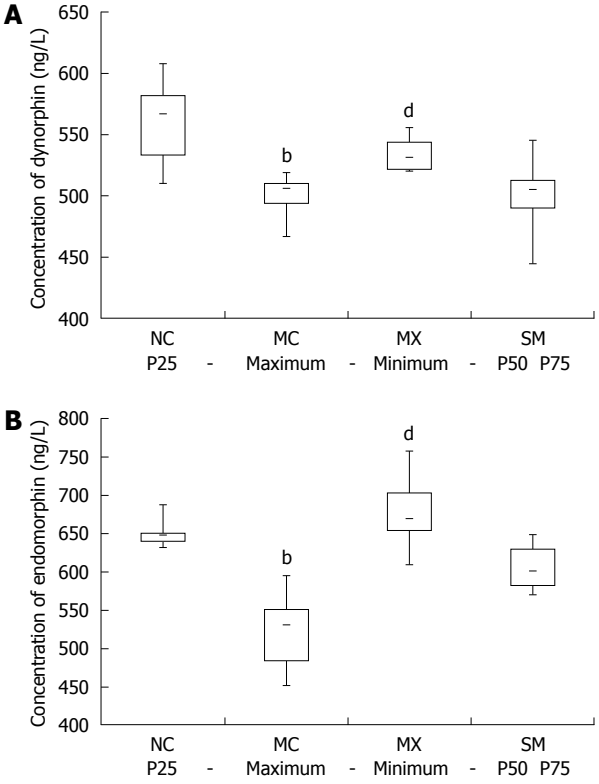

Figure 4 Influence of moxibustion on the concentration of dynorphin (A) and endomorphin (B) in spinal cord. $^{b}P < 0.01$ *vs* normal control (NC); $^{d}P < 0.01$ *vs* model control (MC). MX: Moxibustion; SM: Sham moxibustion.

for thousands of years in China, and is still frequently used in the present clinical practice. Many researches have shown its analgesic effect in treatment of primary dysmenorrhea[21], knee osteoarthritis[22], rheumatoid arthritis[23] and cancer pain[24]. Our previous studies also revealed that moxibustion could alleviate abdominal pain induced by IBS[16,17]. Although moxibustion has been practiced for thousand years, it is still difficult to establish its biological basis.

In the present study, CRD was adopted to establish a rat model of visceral hyperalgesia, and AWR was used for the behavioral assessment. The results showed that the AWR scores in the MC group were significantly higher than in the NC group at various CRD pressure levels (20, 40, 60 and 80 mmHg). Compared with the MC group, a marked reduction in AWR score was detected in the MX group ($P < 0.01$), and no significant difference was found in comparison with the SM group. It indicates that moxibustion has analgesic effects in management of visceral hyperalgesia, which is consistent with the results of our previous studies[16,17]. According to the previous studies adopting the same visceral pain model, herb-partitioned moxibustion could significantly inhibit the increase of AWR score and pain threshold induced by CRD. It has been also found that moxibustion could lower the expression of 5-HT in colon and modulate the expression of 5-HT in spinal cord, indicating a possible relationship between analgesic effect of moxibustion and central nervous system. Rats could keep quiet during the intervention of moxibustion, suggesting that modulating 5-HT was not the only way to reduce visceral hypersensitivity, some endogenous analgesic substances could also play a role in the process.

The analgesic effect of acupuncture has been widely accepted, especially in the study on chronic pain[25]. The endogenous opioid peptides (EOP) have been considered as important fundamental substances in acupuncture analgesia. According to Han JS[6,11,12,26], electro-acupuncture could activate the generation of EOP in spinal cord, such as orphanin, enkephalin, endomorphin, endorphin, and dynorphin.

The present study showed that the concentrations of Dyn and EM in spinal cord of the MC group were significantly lower than that of the NC group ($P < 0.01$). Compared with the MC group, the concentrations were significantly higher in the MX group ($P < 0.01$), and no significant difference was found from the SM group. It suggests that moxibustion could enhance the concentrations of Dyn and EM in spinal cord. Moxibustion may achieve its analgesic effect through multiple pathways and levels. Spinal cord may be the primary integrating center of moxibustion signal, increasing the concentrations of Dyn and EM in spinal cord, inducing a fragmental inhibition (including post-synaptic inhibition and pre-synaptic inhibition), and then blocking the further transmission of pain signal.

It has been shown that midbrain periaqueductal gray descending inhibitory system includes at least three transmitters: EOP, 5-HT and NA. Our findings indicate that moxibustion stimulation accelerates the synthesis and release of central EOP endorphin (dynorphin and endomorphin) and other neurotransmitters (5-HT) in the spinal dorsal horn neurons or nociceptive primary afferents, exerting analgesic effects.

In a word, moxibustion can significantly reduce AWR score and enhance the pain threshold of rats with chronic visceral hyperalgesia, and the analgesic effect may be closely related to the increased concentrations of Dyn and EM in spinal cord.

COMMENTS

Background

Previous studies into the mechanism of acupuncture analgesia have focused on the dynorphin (Dyn) and endomorphin (EM) in spinal cord. Whether analgesic effect of moxibustion is related to Dyn and EM in spinal cord remains unknown. In the previous studies, the authors have demonstrated the analgesic effect of moxibustion in reducing abdominal pain in irritable bowel syndrome (IBS) rats. However, the analgesic mechanism of moxibustion has not been clearly elucidated.

Research frontiers

More and more data have shown that the analgesic effect of moxibustion is closely related to the spinal cord fragments, which has become a hot spot of study.

Innovations and breakthroughs

Moxibustion is found effective against visceral pain. Moxibustion therapy exerts its effect on IBS by increasing the concentration of Dyn and EM in spinal cord

Applications

The experimental data can be used in further studies on moxibustion therapy for visceral pain.

Peer review

This is a good experimental investigation in which authors evaluate the effect of moxibustion, a Traditional Chinese Medicine, and possible involvement of endogenous dynorphin and endomorphin in the spinal cord in rats.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4084

World J Gastroenterol 2010 August 28; 16(32): 4084-4088
ISSN 1007-9327 (print)

BRIEF ARTICLE

Serological diagnostic factors for liver metastasis in patients with colorectal cancer

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Received: February 14, 2010 Revised: May 31, 2010
Accepted: June 7, 2010
Published online: August 28, 2010

Abstract

AIM: To investigate the serological diagnostic factors for liver metastasis in patients with colorectal cancer.

METHODS: One hundred and six adult in-patients with colorectal cancer were studied and divided into patients with liver metastasis (n = 56) and patients without liver metastasis (n = 50). Serum levels of tumor and biochemical markers for liver were measured at the time of diagnosis.

RESULTS: The mean survival time was 55.9 mo, 36.8 mo and 68.3 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively. Lactate dehydrogenase (LDH) level was significantly correlated with the survival time of colorectal cancer patients. The levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase (GGT), LDH and carcinoembryonic antigen (CEA) were significantly higher in patients with liver metastasis than in those without liver metastasis. Patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis. The cut points of LDH, GGT and CEA for screening liver metastasis were 180 U/L, 30 U/L and 5.0 μg/L, respectively. The sensitivity was 64.3%, 69.6% and 70.4%, and the specificity was 64.0%, 60.0% and 52.4%, respectively. The sensitivity of parallel test was 85.2% for LDH and CEA, and 92.6% for GGT and CEA, respectively. The specificity of serial test was 85.7% for LDH (or GGT) and CEA.

CONCLUSION: Early diagnosis of liver metastasis is of great significance. The sensitivity and specificity of combined tumor and biochemical markers are rather good in screening colorectal liver metastasis.

Key words: Liver metastasis; Colorectal cancer; Lactate dehydrogenase; γ-glutamyltransferase; Carcinoembryonic antigen

Peer reviewer: Omar Vergara-Fernandez, MD, Department of Surgery, National Institute for Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Col. Seccion XVI. Deleg. Tlalpan, CP 14000, Mexico

Wu XZ, Ma F, Wang XL. Serological diagnostic factors for liver metastasis in patients with colorectal cancer. *World J Gastroenterol* 2010; 16(32): 4084-4088 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4084.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4084

INTRODUCTION

Colorectal cancer is the 3rd most common malignancy worldwide and the second most lethal cancer type in the developed world[1]. Most patients with colorectal cancer succumb to the effects of distant metastatic lesions, especially liver metastasis rather than the primary colorectal cancer itself[2]. The liver is a primary target organ of metastatic lesions and the main cause of death. About 25%

of patients with colorectal cancer have liver metastases at the time of diagnosis and another 25%-30% of them will present with liver metastases in the following 2-3 years[3]. Without treatment, the life expectancy for patients with colorectal metastases is poor and ranges from 5 to 9 mo[2,4]. Thus early diagnosis of liver metastases of colorectal caner leads to timely treatment, which favors a better prognosis.

Laparoscopy has not been advocated as a screening test for colorectal liver metastases due to its invasiveness. Fine needle aspiration cytology also has not been advocated as a screening test, because of its high risk of complications[5]. It has been shown that the incidence of needle tract metastases is 0.4%-5.1% after fine needle aspiration and use of the procedure in abdominal tumors is fatal in 0.006%-0.031% of cases[6,7]. Most deaths are due to hemorrhage of liver tumors[3]. Imaging modalities, such as contrast enhanced computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography CT (PET-CT), may establish the diagnosis of liver metastasis of colorectal cancer[8]. However, it is more difficult to make the clinical diagnosis of early liver metastases of colorectal cancer due to the absence of typical symptoms or signs. Serological examination including tumor and biochemical markers for liver function evaluation is routinely performed, though its accuracy is not high[9]. The level of carcinoembryonic antigen (CEA) is elevated in 63% of patients, while the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is increased in about 30% of patients with liver metastases of colorectal cancer[10]. To reduce metastases-related mortality, the development of new methods for diagnosis of liver metastases of colorectal cancer is of great significance.

The purpose of the present study was to determine whether CEA and biochemical hepatic tests can be used in assessing liver metastasis in patients with colorectal cancer.

MATERIALS AND METHODS

Patients

One hundred and six in-patients with colorectal cancer admitted to Cancer Institute and Hospital, Tianjin Medical University, from December 1996 to January 2004, were included in this study. Pathological test was performed to confirm their colorectal cancer and contrast enhanced CT, MRI or PET-CT was performed to confirm their liver metastasis. Moreover, liver metastasis was confirmed by operation, biopsy or progression of the disease. The patients who had a history of liver disease and did not undergo contrast enhanced CT and MRI were excluded from this study.

Investigation indexes

Blind tests were performed for total bilirubin (TB), direct bilirubin (DB), ALT, AST, serum total protein (TP), globulin (GLOB), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and CEA. Liver biochemical test was performed within 1 wk after liver metastasis was diagnosed by contrast enhanced CT and MRI in our hospital. The methods to determine biomarkers and upper or lower limits of normality used in our laboratory are shown in Table 1.

Table 1 Methods to determine biomarkers and upper or lower limits of normality used in our laboratory

Diagnostic factors	Methods	Lower and upper limits
Total bilirubin (μmol/L)	Jendrassik-Grof	2-20
Direct bilirubin (μmol/L)	Jendrassik-Grof	0-10
Alanine aminotransferase (U/L)	Rate	0-40
Aspartate aminotransferase (U/L)	Rate	0-42
Serum total protein (g/L)	Biuret	60-80
Globulin (g/L)		27-35
γ-glutamyltransferase (U/L)	Nitrophenol rate	0-50
Alkaline phosphatase (U/L)	P-nitrophenol phosphate rate	45-132
Lactate dehydrogenase (U/L)	Rate	80-240
Carcinoembryonic antigen (μg/L)	Elisa	0-5

Statistical analysis

One-sample Kolmogorov-Smirnov test was used to determine the distribution of ALP, TP, ALB, GLOB, GGT, ALT, AST, TBIL, DBIL, LDH and CEA. Data with the skewed distribution were presented as median (Quartile interval). Two-independent-sample test and χ^2 test were respectively used to determine whether there is any significant difference between patients with and without liver metastasis. Cox regression analysis was performed for GGT, ALP, LDH, TB, DB, ALT, AST, TP, GLB, CEA, lymph node metastasis in order to find the characteristic factors for survival time. Screening test for LDH, GGT and CEA, parallel test and serial test for GGT and LDH, CEA and LDH, CEA and GGT were used to determine the diagnostic factors for liver metastasis in patients with colorectal cancer. Statistical analysis was performed by SPSS (Version: 16.0, Chicago, USA).

The screening tests were evaluated by calculating their sensitivity (SE), specificity (SP), diagnostic index (DI), false positive rate (α), false negative rate (β), crude accuracy (CA), positive predictive value (PV+), negative predictive value (PV-).

SE was defined as the proportion of patients with LM testing positive (A/A + C) where C is the number of false negative cases. SP was defined as the proportion of patients without LM testing negative (D/B + D) where B is the number of false positive cases. DI was defined as the (SE + SP)-1. α was defined as the proportion of negative cases that were erroneously reported as positive while β was defined as the proportion of positive cases that were erroneously reported as negative. CA was defined as the proportion of cases correctly diagnosed by the test (A + D/A + B + C + D) where B + C is the number of cases erroneously diagnosed by the test. PV+ was defined as the proportion of patients testing positive with LM confirmed by pathology (A/A + B) while PV- was defined as the proportion of patients testing negative proved to be free of LM by pathology (D/C + D).

Serial test was defined as positive only if all the re-

Table 2 Characteristics of patients enrolled in our study

	With liver metastasis	Without liver metastasis
Age (yr)	55.69 ± 11.773	55.21 ± 13.225
Sex		
Male	32	36
Female	18	20
Primary tumor (UICC stage)		
T1	1	0
T2	4	0
T3	12	10
T4	22	23
Lymph node metastasis (UICC stage)		
N0	29	12
N1	8	13
N2	2	6

UICC: International Union Against Cancer.

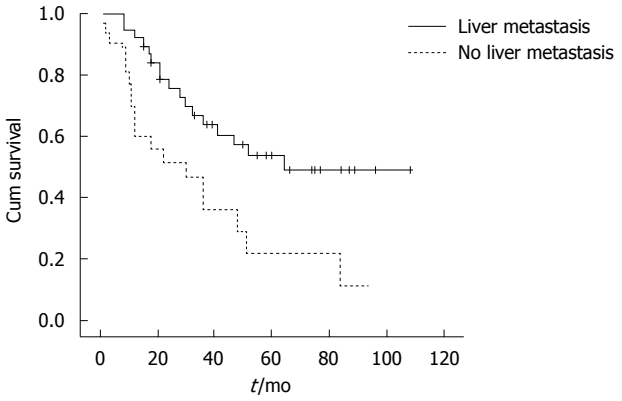

Figure 1 Survival curves for patients with and without liver metastasis of colorectal cancer.

sults were positive when we considered two or more tests together in the single patient. Parallel test was defined as positive if one of the results was positive when two or more tests were considered in the single patient.

RESULTS

Characteristics of patients

The age of our patients ranged 26-80 years with a median of 56 years. Males constituted 68 of patients with a male to female ratio of 1.788 to 1. No significant difference was found in age and sex of the patients with and without liver metastasis. The characteristics of patients with primary tumor and lymph node metastasis are shown in Table 2.

Survival analysis and cox regression

The median survival time was 47, 30 and 64 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively. Their mean survival time was 55.9 ± 5.5 mo, 36.8 ± 6.5 mo and 68.3 ± 7.0 mo, respectively. The survival curves for patients with and without liver metastasis were significantly different (P = 0.005) (Figure 1). Cox regression analysis showed that LDH was significantly correlated with the survival time of colorectal cancer patients with an increased risk of liver metastasis (P = 0.005) (Table 3).

Table 3 Cox regression analysis of patients with colorectal cancer

	χ^2	P
Serum total protein (g/L)	0.093	0.761
Globulin (g/L)	< 0.000	0.994
Alanine aminotransferase (U/L)	1.943	0.163
Aspartate aminotransferase (U/L)	0.143	0.705
Total bilirubin (μmol/L)	0.122	0.726
Direct bilirubin (μmol/L)	0.063	0.801
γ-glutamyltransferase (U/L)	1.126	0.289
Alkaline phosphatase (U/L)	1.006	0.316
Lactate dehydrogenase (U/L)	11.254	0.001
Carcinoembryonic antigen (μg/L)	0.159	0.690
Lymph node metastasis	1.601	0.206

Table 4 Levels of γ-glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and carcinoembryonic antigen in patients with and without liver metastasis of colorectal cancer

	With liver metastasis	Without liver metastasis	P
GGT (U/L)	43.00 (64.75)	24.00 (35)	0.001
ALT (U/L)	22.00 (22.75)	13.00 (12.50)	< 0.001
AST (U/L)	22.00 (17.50)	16.00 (10.00)	< 0.001
LDH (U/L)	201.5 (169.50)	164.50 (70.75)	0.003
CEA (μg/L)	13.70 (93.8)	4.87 (12.82)	0.039

GGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; CEA: Carcinoembryonic antigen.

Test of normality and two-independent-sample test

One-sample Kolmogorov-Smirnov test showed that the distribution of ALP, TP, ALB, GLOB and ALB/GLOB was normal, while that of GGT, ALT, AST, TBIL, DBIL, LDH and CEA was skewed. The levels of GGT, ALT, AST, LDH and CEA were significantly higher in patients with liver metastasis than in those without liver metastasis (P < 0.05) (Table 4). Patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis (χ^2 = 9.046, P = 0.003). No significant difference was found in ALP, TP, ALB, GLOB, ALB/GLOB, TBIL, and DBIL levels between patients with and without liver metastasis (data not shown).

Screening test

Because the diagnostic indices of LDH, GGT and CEA at 180, 30 and 5.0 for screening liver metastasis were the greatest, the cut off points were selected at 180, 30 and 5.0, respectively (Figure 2). The area under the curves of LDH, GGT and CEA was 0.671, 0. 687 and 0.675, respectively (P = 0.05). The κ of parallel test and serial test for CEA and LDH, CEA and GGT was 0.293, 0.326, and 0.357, 0.284, respectively (P = 0.05). The SE, SP, DI, false positive rate (α), false negative rate (β), CA, adjusted agreement, PV+ and PV- are shown in Table 5.

Table 5 Screening test for liver metastasis in patients with colorectal cancer

	Sen (%)	Spe (%)	DI	CA	AA	PV+	PV-	*P*	α	β
LDH	64.3	64.0	1.283	0.642	0.641	0.667	0.615	0.003	0.360	0.356
GGT	69.6	60.0	1.296	0.651	0.649	0.661	0.638	0.001	0.400	0.304
CEA	70.4	52.4	1.228	0.625	0.623	0.655	0.579	0.039	0.476	0.296
LDH and CEA (serial test)	51.9	85.7	1.376	0.667	0.695	0.823	0.581	0.007	0.143	0.481
LDH and CEA (parallel test)	85.2	42.9	1.281	0.667	0.657	0.657	0.692	0.030	0.571	0.148
GGT and CEA (serial test)	44.4	85.7	1.301	0.625	0.662	0.800	0.545	0.025	0.143	0.556
GGT and CEA (parallel test)	92.6	38.1	1.307	0.687	0.691	0.658	0.800	0.009	0.619	0.074

LDH: Lactate dehydrogenase; GGT: γ-glutamyltransferase; CEA: Carcinoembryonic antigen; Sen: Sensitivity; Spe: Specificity; DI: Diagnostic index; α: False positive rate; β: False negative rate; CA: Crude accuracy; PV+: Positive predictive value; PV-: Negative predictive value.

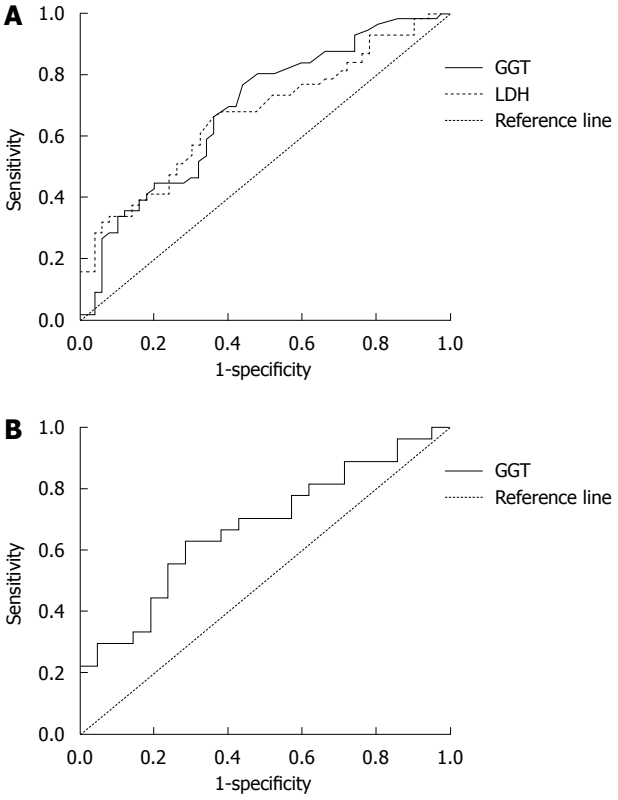

Figure 2 Receiver operator characteristic curves for lactate dehydrogenase and γ-glutamyltransferase (A) and carcinoembryonic antigen (B). LDH: Lactate dehydrogenase; GGT: γ-glutamyltransferase.

DISCUSSION

Colorectal cancer metastasis occurs in various organs, most frequent in lymph nodes and liver[1]. In this study, the patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis, indicating that regular imaging modalities, such as contrast enhanced CT and MRI, may be performed every 3 or 6 mo after surgery for patients with colorectal cancer to establish an early diagnosis of liver metastasis.

The overall life expectancy of patients with colorectal cancer is mainly determined by the progression of liver metastasis rather than by the primary carcinoma itself[3]. The median survival time was 47, 30 and 64 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively, with a mean survival time of 55.9, 36.8 and 68.3 mo, respectively. A significant difference was observed in survival curves for patients with and without liver metastasis. The levels of GGT, ALT, AST, LDH and CEA were significantly higher in patients with liver metastasis than in those without liver metastasis. Cox regression analysis showed that LDH was significantly correlated with the survival time of colorectal cancer patients, indicating that LDH may be used to predict the life expectancy of patients with liver metastasis of colorectal cancer.

CEA was demonstrated in fetal gut tissue and gastrointestinal tract tumor four decades ago, and subsequently detected in the circulation of patients and recognized as a serum marker for colorectal cancer. Expression of carbohydrate antigen (CA) 19-9 has been described in colorectal cancer, but its sensitivity is lower than CEA[11]. Lack of sensitivity and specificity precludes the use of any available serum markers, such as CEA, CA 19-9, CA 242, CA 72-4, tissue polypeptide antigen or tissue polypeptide-specific antigen, for the early detection of colorectal cancer[12]. However, a preoperative CEA serum level can predict the prognosis of recurrence and survival time of colorectal cancer patients[11,13]. Moreover, circulating levels of LDH, ALP, and GGT in malignant tissues can directly contribute to liver replacement[14,15]. In patients with metastatic colorectal cancer, CEA, ALP and LDH have been reported as prognostic factors[16-19].

A screening test was performed to show whether LDH, GGT and CEA can be used to screen liver metastasis in patients with colorectal cancer. Because the diagnostic indices of LDH, GGT and CEA at 180 U/L, 30 U/L and 5.0 μg/L for screening liver metastasis were the greatest, the cut off points were selected at 180 U/L, at 30 U/L, and at 5.0 μg/L, respectively. The sensitivity were 64.3%, 69.6% and 70.4%, respectively. The sensitivity were 64.0%, 60.0% and 52.4%, respectively. As a tumor marker, CEA test had a moderate sensitivity and a low specificity for liver metastasis in patients with colorectal cancer. Thus, tumor markers in combination with biochemical markers for liver function may improve the sensitivity and specificity for screening liver metastases in patients with colorectal cancer.

Couples of tests, usually CEA and another, would demonstrate a better accuracy than a single test[20,21], which

is consistent with the findings in our study. In the present study, the sensitivity of parallel test for LDH and CEA, GGT and CEA was 85.2% and 92.6%, respectively. The specificity of serial test for LDH and CEA was 85.7% and the specificity of serial test for GGT and CEA was 85.7% too, indicating that its sensitivity and specificity of tumor marker (CEA) in combination with biochemical markers including LDH and GGT are rather good in patients with colorectal cancer, if LDH > 180 U/L and CEA > 5.0 μg/L, or GGT > 30 U/L and CEA > 5.0 μg/L. Contrast enhanced CT, MRI or PET-CT may be performed immediately to confirm liver metastasis and timely treatment may improve the survival of patients with liver metastasis of colorectal cancer. Thus metastatic liver disease may be diagnosed before symptoms occur and liver metastases of colorectal cancer can be diagnosed more rapidly and accurately.

COMMENTS

Background

The overall life expectancy of patients with colorectal cancer is mainly determined by the progression of liver metastasis rather than by the primary carcinoma. Improved early screening modalities are still needed and molecular beacons may be sufficiently sensitive, specific, and cost-effective for screening of colorectal liver metastases.

Research frontiers

Although various diagnostic modalities, such as ultrasonography, computed tomography scan and magnetic resonance imaging have been used in demonstrating metastases, but their accuracy is low, particularly when the lesions are small. The present study demonstrated the value of carcinoembryonic antigen (CEA) and some biochemical hepatic tests in detection of hepatic metastases in patients with primary colorectal cancer.

Innovations and breakthroughs

Laboratory tests have limits in detecting LM but they can rapidly and accurately evaluate liver metastasis in patients with primary colorectal cancer. This study showed that measurements of plasma biomarkers increase the sensitivity and selectivity of liver metastasis diagnosis.

Applications

The results of this study can improve early screening modalities. Furthermore, combination of markers and even modalities with imaging or endoscopic ultrasound will be needed to achieve a sufficient reliability.

Peer review

It is a very interesting paper describing the diagnosis of liver metastases of colorectal cancer. The authors studied 106 patients with colorectal cancer, showing that the alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, lactate dehydrogenase and CEA levels are increased in patients with liver metastasis of colorectal cancer.

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S- Editor Wang YR **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4089

World J Gastroenterol 2010 August 28; 16(32): 4089-4094
ISSN 1007-9327 (print)

BRIEF ARTICLE

Altered expression of MUC2 and MUC5AC in progression of colorectal carcinoma

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Received: April 7, 2010 Revised: May 27, 2010
Accepted: June 4, 2010
Published online: August 28, 2010

Abstract

AIM: To study the expression profiles of MUC2 and MUC5AC in tumorigenesis of colorectal carcinoma and in its different pathologic types.

METHODS: Formalin-fixed, paraffin-embedded human colorectal tissue specimens were immunostained with antibodies against MUC2 and MUC5AC. Six samples of normal mucosa (NM), 12 samples of hyperplastic polyp (HP), 15 samples of tubular adenoma with low-grade dysplasia (LGD), 14 samples of tubular adenoma with high-grade dysplasia (HGD), 26 samples of conventional colorectal adenocarcinoma (CCA), 15 samples of mucinous carcinoma (MC), and 8 samples of signet-ring cell carcinoma (SRCC) were collected.

RESULTS: MUC2 was the most widely expressed protein in each study group, although the number of MUC2-positive cases was less in CCA group than in other groups ($P < 0.05$). The staining score for MUC2 was significantly decreased in the HP-LGD-HGD-CCA sequence ($r = -0.73436$, $P < 0.0001$). Among the neoplasms, MC and SRCC were more frequently associated with the high expression of MUC2 ($P < 0.05$) than with that of CCA. MUC5AC expression was detected in all groups but not in NM group. Furthermore, the staining score for MUC5AC was higher in HP, LGD, HGD, MC and SRCC groups than in NM and CCA groups ($P < 0.05$). The frequency of simultaneous expression of MUC proteins was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$).

CONCLUSION: Alterations in MUC expression occur during colorectal tumorigenesis. The transformation process in MC and SRCC may be different from that in the traditional adenoma-carcinoma sequence.

Key words: Colorectum; Tumorigenesis; MUC2; MUC5AC; Immunohistochemistry

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Bu XD, Li N, Tian XQ, Li L, Wang JS, Yu XJ, Huang PL. Altered expression of MUC2 and MUC5AC in progression of colorectal carcinoma. *World J Gastroenterol* 2010; 16(32): 4089-4094 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4089.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4089

INTRODUCTION

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with a large number of O-linked oligo-

saccharides and a few N-glycan chains, linked to a protein backbone[1]. Mucins are known to play a central role in the protection, lubrication and hydration of the external surface of human epithelial tissue layers lining the intricate network of ducts and passageways. Mucins have also been implicated in the pathogenesis of benign and malignant diseases of secretory epithelial cells. The identification of novel transmembrane mucin MUC21[2], means that a total of 20 human mucins have now been recognized. According to their structure and function, mucins can be divided into secreted mucins and transmembrane mucins. Secreted mucins can be gel-forming or non-gel-forming, and include MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9 and MUC19. Transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21, and possess a transmembrane domain for anchoring themselves to the plasma membrane of various cells. These mucin proteins are encoded by various *MUC* genes[1].

The genes for gel-forming mucins MUC2 and MUC5AC are found in a cluster on chromosome 11p15.5, and synthesis of the proteins is regulated by biologically active molecules, including cytokines, bacterial products, and growth factors[3-5]. The *MUC2* gene codes for a typical secretory mucin, which is predominantly found in colorectal goblet cells. The *MUC5AC* gene is mainly expressed in gastric and tracheo-bronchial mucosa. Changes in the expression levels and/or distribution profiles of MUC2 and MUC5AC occur in cancers of the lung, gastrointestinal tract, pancreas, hepatobiliary system and reproductive system. For example, it has been found that MUC5AC is down-regulated in peritumoral epithelium and squamous metaplasia of non-small cell lung carcinoma (NSCLC), and MUC5AC expression is reduced in NSCLC, irrespective of their histologic subtype[6]. The expression of sLe^x is related to MUC5AC protein in NSCLC, and patients with tumors co-expressing both MUC5AC and sLe^x antigen have the poorest survival[7]. MUC2 appears to act as a protective protein and has been shown to be associated with tumors of mucinous type, both in biliary system and in pancreatic system, which carry a more favorable prognosis[8]. MUC5AC expression in intrahepatic cholangiocarcinoma is associated with a higher incidence of lymph node metastasis and has been identified as an independent prognostic factor by multivariate survival analysis[9].

Interestingly, altered expression of MUC in colorectal cancer may be significantly correlated with histologic type, sensitivity to chemotherapeutic drugs, and prognosis of colorectal cancer[10-13]. Colorectal cancer constitutes a suitable model for studying the mechanisms of carcinogenesis and tumor progression in the well-established adenoma-carcinoma sequence. It is possible to observe a dynamic progression from benign adenomatous polyp to adenoma with varying degree of dysplasia, to intramucosal and invasive carcinoma[14]. Moreover, a pathway involving a hyperplastic polyp-adenoma-carcinoma sequence has also been introduced[15]. Alterations in the expression of mucin proteins and genes have been observed in colorectal adenoma and carcinoma, although their significance in neoplastic transformation of the colorectal epithelium is yet to be determined. The present study therefore aimed to study the expression profiles of MUC2 and MUC5AC during tumorigenesis and in different pathologic types of colorectal carcinoma, using immunohistochemical staining.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded human colorectal tissue specimens were obtained from Department of Pathology, Nanjing First Hospital of Nanjing Medical University. Six samples of normal mucosa (NM), 12 samples of hyperplastic polyps (HP), 15 samples of tubular adenoma with low-grade dysplasia (LGD), 14 samples of tubular adenoma with high-grade dysplasia (HGD), 26 samples of conventional colorectal adenocarcinoma (CCA), 15 samples of mucinous carcinoma (MC), and 8 samples of signet-ring cell carcinoma (SRCC) were analyzed in this study. HP was diagnosed when a serrated polyp with no overt cytological atypia showed narrowed crypt bases, predominantly lined with immature cells. Adenoma was further classified as low or high grade based on the degree of glandular intraepithelial neoplasia (dysplasia), according to the World Health Organization classification. Colorectal cancer was defined as mucinous carcinoma if more than 50% of the lesion contained a mucin lake. Cancer where more than 50% of the tumor cells were signet-ring cells was defined as SRCC. Histologically normal mucosa from margins of the specimens served as control tissue. All tissue samples were diagnosed and classified by two pathologists.

Immunohistochemistry

Paraffin-embedded blocks of different tissues were cut into 4-μm thick sections. Slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase was blocked with a 3% H_2O_2-methanol solution, and the slides were incubated in 10% normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were then incubated overnight at 4℃ with primary antibody (MUC2 or MUC5AC, 1:100; Santa Cruz, CA). The standard biotin-streptavidin-peroxidase method was then used, and the sections were lightly counterstained with hematoxylin. Histologically normal colon mucosa and gastric biopsies were used as positive controls for MUC2 and MUC5AC, respectively. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH 7.4) instead of primary antibody were used as negative controls.

Analysis of immunohistochemical data

Both goblet and non-goblet columnar cells of normal colon and hyperplastic polyps were evaluated. MUC staining was only scored in neoplastic cells of tissues containing either dysplastic epithelium or carcinoma. The range of cytoplasmic staining (0: 0%-5%; 1: 6%-30%; 2: 31%-60%; and 3: 61%-100%) and the intensity of staining (0: no stain; 1: weak staining; 2: intermediate staining; and 3:

strong staining) were assessed in at least 8 high-power fields by two observers, and averages of the grades were taken. The final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining. Staining was designated as negative if the staining score was 0 or 1, intermediate for 2, 3, or 4, and high for 6 or 9. All specimens were scored blindly.

Statistical analysis

Statistical comparison of immunohistochemical staining was performed using SAS software version 9.0 (SAS Institute, Cary, NC). The rank-sum test and Spearman's rank correlation analysis were used to determine differences between the groups and to evaluate correlations, respectively. The T approximation test in Wilcoxon's rank-sum test and Fisher's exact test were used to compare differences between CCA and other groups. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical localization of MUC2 and MUC5AC

The expression of MUC2 and MUC5AC proteins differed among the study groups, which was prominently characterized by perinuclear and diffuse cytoplasmic staining. The MUC2 was expressed in perinuclear cytoplasm of partial goblet cells in NM group (Figure 1A). The MUC2 labeling was generally increased in cytoplasm of columnar cells and goblet cells in HP group (Figure 1B), and the positive signals were also observed in apical cytoplasm of columnar cells, especially in LGD and HGD groups (Figure 1C and D). MUC2 expression was positive in the cytoplasm of cancerous cells, while the extracellular mucin remained unstained (Figure 1E-G). The staining pattern for MUC5AC was largely similar to that for MUC2 in all groups but not to that in NM group, and positive signals were found in extracellular mucin (Figure 2).

Immunohistochemical analysis of MUC2 and MUC5AC

The frequency of MUC protein expression in different groups was examined with immunohistochemical staining, and the results are summarized in Table 1. MUC2 was the most widely expressed antigen in all groups, but the number of MUC2-positive cases (46.15%) was less in CCA group than in other groups. Both the expression frequency and staining intensity of MUC2 were significantly decreased in the HP-LGD-HGD-CCA sequence ($r = -0.73436$, $P < 0.0001$). The frequency of MUC2 expression was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$). The MUC5AC expression was detected in all groups but not in NM group. Furthermore, the frequency of MUC5AC expression was dramatically lower in CCA group (30.77%) than in other groups with the exception in NM group. The proportion of high staining scores was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$), which was similar to that of MUC2 expression. Concordance between MUC2 and MUC5AC expression was also noted in individual specimens from different groups (Table 2). Concordance was defined as positive (intermediate or high) or negative MUC2 and MUC5AC expression. The frequency of simultaneous expression of MUC proteins was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$).

DISCUSSION

MUC expression has been studied in colorectal carcinoma, but few reports are available on the expression in relation to the hyperplastic polyp-adenoma-carcinoma sequence, or in different pathologic types of colorectal cancer. This study focused on the altered and *de novo* expression profiles of MUC2 and MUC5AC in the tumorigenic sequence, and in different pathologic types of colorectal cancer.

MUC2 is characteristically expressed in goblet cells of native intestinal epithelium and intestinal metastasis of gastric mucosa, but not in normal gastric epithelium. The results of the present study, with immunohistochemical staining of paraffin-embedded human tissue samples, are consistent with those of previous studies showing reduced MUC2 expression in colorectal adenocarcinoma[16-18]. Decreased MUC2 expression in nonmucinous colon cancer can result from methylation of the MUC2 promoter[1]. Gratchev *et al*[19] demonstrated that MUC2 promoter methylation is lower in normal goblet cells than in columnar cells and in specimens of mucinous colorectal carcinoma than in those of nonmucinous adenocarcinoma. Loss of functional p53 is also related to the down-regulation of MUC2 expression in colorectal carcinoma. It has been shown that MUC2 expression is transcriptionally regulated by p53 protein in several cell lines[20]. There are two potential p53-binding sites in the MUC2 promoter, each of which contributes to stimulation of promoter activity. It was reported that MUC2 immunoreactivity is inversely correlated with p53 alteration in mucinous carcinoma, i.e. the level of p53 alteration is lower in regions with a high MUC2 expression level[21]. Decreased *in vivo* expression of MUC2 is related to colon carcinogenesis accompanying increased proliferation, decreased apoptosis, and increased migration of intestinal epithelial cells[22].

MUC5AC is not expressed in normal colonic epithelium, but *de novo* expression occurs in adenoma and colorectal cancer. The number of immunoreactive cells and the intensity of MUC5AC staining are greatest in larger adenoma with moderately villous histology and dysplasia, while immunostaining is lower in highly villous polyps with severe dysplasia[23]. However, in the current study, the score for MUC5AC staining was higher in HGD group than in LGD group, in contrast to the expression of MUC2 in the two groups. This discrepancy in MUC5AC expression in the two studies might be due to the use of a different histologic type of adenoma. Although MUC5AC expression correlates with neural invasion and advanced stage of intrahepatic cholangiocarcinoma[24], the relation between MUC5AC expression and progression of colon cancers may be different. Kocer *et al*[25] found that the expression of MUC5AC in colon cancer is associated with a better

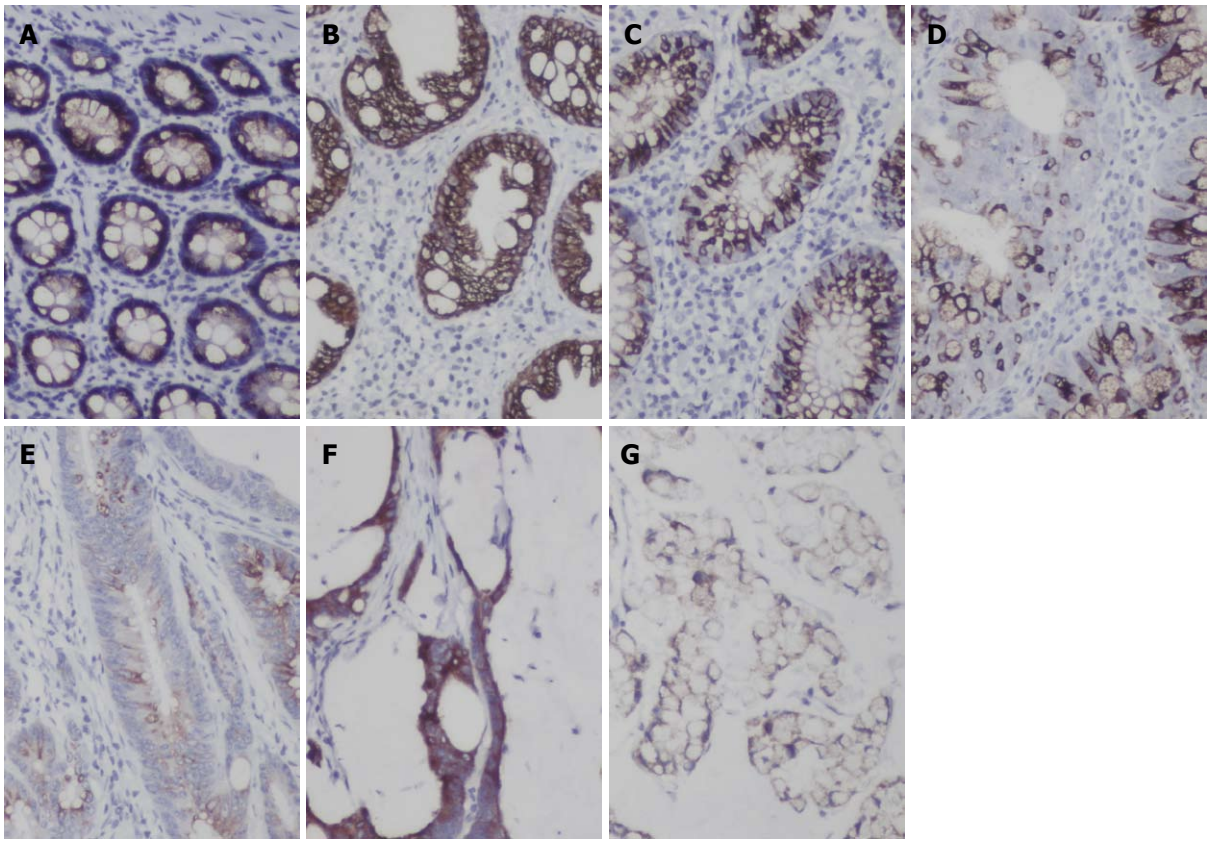

Figure 1 Expression of MUC2 (SP method, × 200). A: Normal mucosa; B: Hyperplastic polyp; C: Tubular adenoma with low-grade dysplasia; D: Tubular adenoma with high-grade dysplasia; E: Conventional colorectal adenocarcinoma; F: Mucinous carcinoma; G: Signet-ring cell carcinoma.

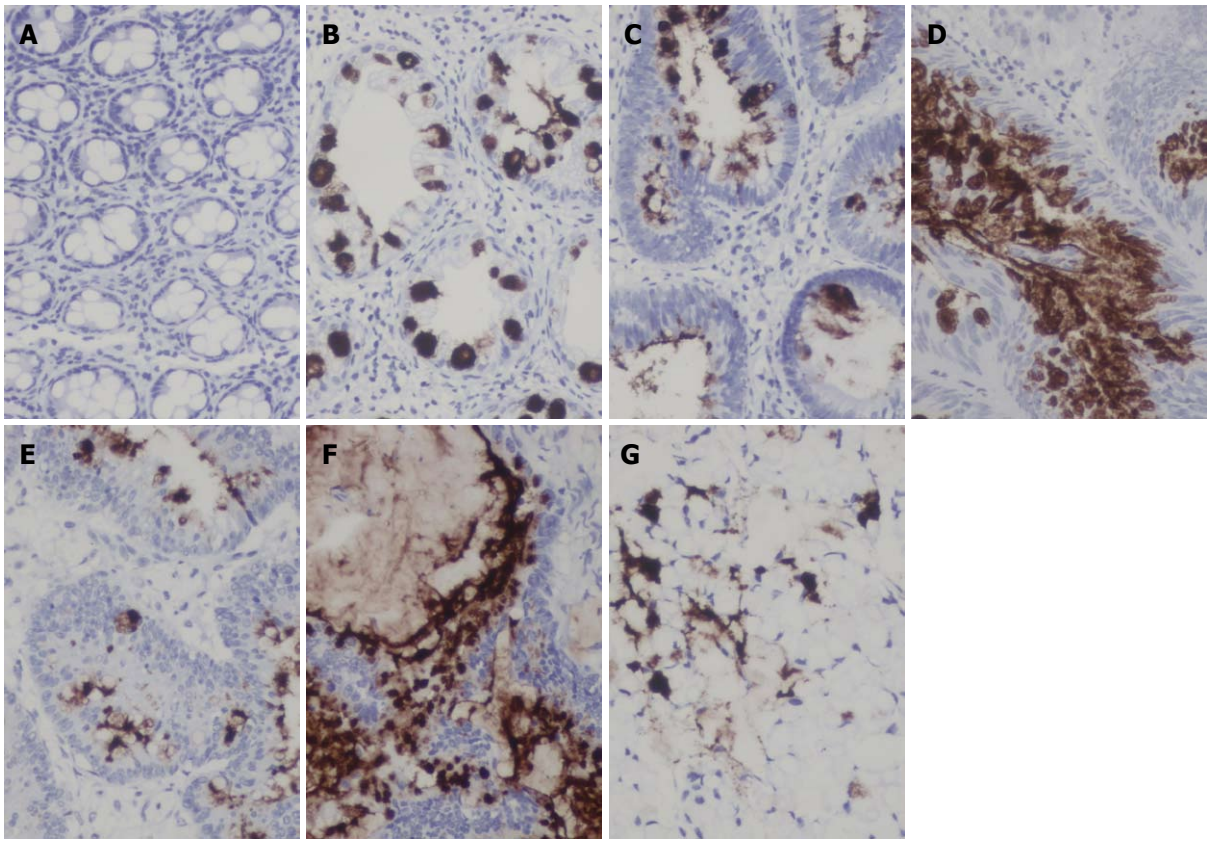

Figure 2 Expression of MUC5AC (SP method, × 200). A: Normal mucosa; B: Hyperplastic polyp; C: Tubular adenoma with low-grade dysplasia; D: Tubular adenoma with high-grade dysplasia; E: Conventional colorectal adenocarcinoma; F: Mucinous carcinoma; G: Signet-ring cell carcinoma.

Table 1 Frequency of MUC2 and MUC5AC protein expression in colorectal tissues *n* (%)

MUC staining score	NM	HP	LGD	HGD	CCA	MC	SRCC
MUC2[1]							
High	6 (100.00)	12 (100.00)	9 (60.00)	5 (35.71)	3 (11.54)	9 (60.00)	4 (50.00)
Intermediate	0 (0.00)	0 (0.00)	6 (40.00)	9 (64.29)	9 (34.62)	6 (40.00)	3 (37.50)
Negative	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	14 (53.85)	0 (0.00)	1 (12.50)
P[2]	0.0013	< 0.0001	0.0003	0.0023	-	0.003	0.0198
MUC5AC							
High	0 (0.00)	5 (41.67)	3 (20.00)	6 (42.86)	3 (11.54)	7 (46.67)	2 (25.00)
Intermediate	0 (0.00)	7 (58.33)	12 (80.00)	8 (57.14)	5 (19.23)	8 (53.33)	6 (75.00)
Negative	6 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	18 (69.23)	0 (0.00)	0 (0.00)
P[3]	0.1444	0.0007	0.0007	0.0003	-	0.0002	0.0047

[1]MUC2 protein expression is significantly decreased in HP-LGD-HGD-CCA sequence (r = -0.73436, P < 0.0001); [2]MUC2 protein expression in CCA *vs* other groups; [3]MUC5AC protein expression in CCA *vs* other groups. NM: Normal mucosa; HP: Hyperplastic polyp; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; CCA: Conventional colorectal adenocarcinoma; MC: Mucinous carcinoma; SRCC: Signet-ring cell carcinoma.

Table 2 Concordance of MUC2 and MUC5AC protein expression in colorectal tissues *n* (%)

Tissue	*n*	Positive cases		Concordance MUC2 *vs* MUC5AC	*P*[1]
		MUC2	MUC5AC		
NM	6	6 (100.00)	0 (0.00)	0 (0.00)	0.5662
HP	12	12 (100.00)	12 (100.00)	12 (100.00)	< 0.0001
LGD	15	15 (100.00)	15 (100.00)	15 (100.00)	< 0.0001
HGD	14	14 (100.00)	14 (100.00)	14 (100.00)	< 0.0001
CCA	26	12 (46.15)	8 (30.77)	4 (15.38)	-
MC	15	15 (100.00)	15 (100.00)	15 (100.00)	< 0.0001
SRCC	8	7 (87.50)	8 (100.00)	7 (87.50)	< 0.0001

[1]Concordance of MUC protein expression in CCA *vs* other groups. NM: Normal mucosa; HP: Hyperplastic polyp; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; CCA: Conventional colorectal adenocarcinoma; MC: Mucinous carcinoma; SRCC: Signet-ring cell carcinoma.

prognosis of its patients. Patients with MUC5AC-negative tumors have a poorer prognosis and a lower survival rate than those with MUC5AC-positive tumors, suggesting that the absence of MUC5AC expression in tumors is a prognostic factor for highly aggressive colorectal carcinoma. The *de novo* expression of MUC5AC in mucinous adenocarcinoma and SRCC was inconsistent in our study. The variability of MUC polypeptide expression in individual adenocarcinomas may reflect tumor cell heterogeneity and aberrant differentiation in invasive tumors, compared with their dysplastic precursors.

SRCC and MC have similar biological behaviors, but their molecular compositions may differ. Signet-ring cells rarely express adhesion molecules, implying disruption of cell-cell adhesion[18], which can explain their aggressive behavior in terms of the invasion and metastasis of tumor cells. Mucinous colorectal carcinoma is associated with microsatellite instability (MSI). It has been hypothesized that MSI may directly influence mucus production in both sporadic and hereditary colon cancer, by alternating the genes involved in mucin synthesis or degradation[26]. Colorectal cancer with a high MSI usually has a higher MUC2- and MUC5AC-positive rate than microsatellite-stable cancer[27]. Boland *et al*[28] recently suggested that colorectal tumor with MSI has more distinctive features, including a poor differentiation, mucinous or signet-ring appearance, as well as a different response to chemotherapeutics, than colorectal tumor without MSI. It is interesting that a similar phenotype (MUC2+/MUC5AC+) has been identified in adenoma and hyperplastic polyp. The classic adenoma-carcinoma pathway usually involves the loss of tumor suppressor genes, although a minority of colorectal cancers may develop in an other pathway associated with mutations in mismatch repair genes or hypermethylation of the hMLH1 gene (in sporadic cancers)[29-31].

In conclusion, alterations in MUC expression occur during colorectal tumorigenesis. The transformation process in MC and SRCC may be different from that in the traditional adenoma-carcinoma sequence. *De novo* expression of MUC5AC can occur in both mucinous and non-mucinous colorectal carcinomas, but its expression is stronger in mucinous colorectal carcinoma than in non-mucinous colorectal carcinoma. Further investigations using molecular biological techniques based on a larger clinical sample size are needed to confirm our findings.

COMMENTS

Background

Although alterations in mucins have been observed in colorectal cancer, little is known about their expression during the development and progression of colorectal tumor. Mucinous and signet-ring cell carcinomas are the two types of colorectal cancer characterized by abundant mucin secretion. However, whether the mechanism underlying the tumorigenesis of mucinous and signet-ring cell carcinomas differs from that of other colorectal cancer remains controversial.

Research frontiers

Mucinous components, such as MUC2 and MUC5AC, are associated with the distinct clinical pathologic features of colorectal cancer and the survival rate of such patients.

Innovations and breakthroughs

The abnormal expression of MUC2 and MUC5AC in mucinous and signet-ring cell carcinomas suggests that a different process may be involved in the tumorigenesis of these types of colorectal cancer.

Terminology

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with a large number of O-linked oligosaccharides and a few N-glycan chains, linked to a protein backbone. Mucins are known to play a central role in the protection, lubrication and hydration of the external surface of human epithelial tissue layers lining the intricate network of ducts and passageways. Mucins have also been implicated in the pathogenesis of benign and malignant diseases of secretory epithelial cells.

Applications

The clinical treatment of mucin-secreting tumor may differ from that of other types of colorectal cancer.

Peer review

This is a good paper that provides new data regarding MUC expression profiles in specific types of colorectal carcinoma.

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S- Editor Wang JL **L- Editor** Wang XL **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4095

World J Gastroenterol 2010 August 28; 16(32): 4095-4099
ISSN 1007-9327 (print)

BRIEF ARTICLE

Efficacy of telbivudine in HBeAg-positive chronic hepatitis B patients with high baseline ALT levels

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Supported by The China National S&T Major Project (to Yang YD), Grant No. R20090018; and the Department of Science and Technology of Zhejiang Province, China (to Zheng L), Grant No. 2009C33009
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Received: April 14, 2010 Revised: May 27, 2010
Accepted: June 3, 2010
Published online: August 28, 2010

Abstract

AIM: To evaluate the efficacy and safety of telbivudine (LDT) in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) patients who have high baseline alanine aminotransferase (ALT) levels between 10 and 20 times the upper limit of normal.

METHODS: Forty HBeAg-positive CHB patients with high baseline ALT levels between 10 and 20 times the upper limit of normal were enrolled and received LDT monotherapy for 52 wk. Another forty patients with baseline ALT levels between 2 and 10 times the upper limit of normal were included as controls. We compared the virological, biochemical, serological and side effect profiles between the two groups at 52 wk.

RESULTS: By week 52, the mean decrease in hepatitis B virus (HBV) DNA level compared with baseline was 7.03 $\log_{10}$ copies/mL in the high baseline ALT group and 6.17 $\log_{10}$ copies/mL in the control group, respectively ($P < 0.05$). The proportion of patients in whom serum HBV DNA levels were undetectable by polymerase chain reaction assay was 72.5% in the high baseline ALT group and 60% in the control group, respectively ($P < 0.05$). In addition, 45.0% of patients in the high baseline ALT group and 27.5% of controls became HBeAg-negative, and 37.5% of those in the high baseline group and 22.5% of controls, respectively, had HBeAg seroconversion ($P < 0.05$) at week 52. Moreover, in the high baseline group, 4 out of 40 patients (10%) became hepatitis B surface antigen (HBsAg)-negative and 3 (7.5%) of them seroconverted (became HBsAg-positive). Only 1 patient in the control group became HBsAg-negative, but had no seroconversion. The ALT normalization rate, viral breakthrough, genotypic resistance to LDT, and elevations in creatine kinase levels were similar in the two groups over the 52 wk.

CONCLUSION: High baseline ALT level is a strong predictor for optimal results during LDT treatment.

Key words: Chronic hepatitis B; Hepatitis B e antigen; Serum alanine aminotransferase level; Telbivudine

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Lv GC, Ma WJ, Ying LJ, Jin X, Zheng L, Yang YD. Efficacy of telbivudine in HBeAg-positive chronic hepatitis B patients with high baseline ALT levels. *World J Gastroenterol* 2010; 16(32): 4095-4099 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4095.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4095

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) affects

approximately 350 million people worldwide and is usually associated with continuing inflammatory activity and progression of liver diseases, which in turn lead to an increased risk of cirrhosis, decompensated liver disease, and hepatocellular carcinoma (HCC)[1,2]. Recently, several prospective follow-up studies of large cohorts of chronic hepatitis B (CHB) patients from Asia found that the presence of hepatitis B e antigen (HBeAg) and high levels of HBV DNA were independent risk factors for the subsequent development of advanced liver diseases[3]. Therefore, suppression of HBV replication is the main therapeutic goal in the treatment of CHB patients.

Up till now, seven drugs have been available for the treatment of CHB: they include conventional interferon α, pegylated interferon α, and nucleoside/nucleotide analogues (NUCs). NUCs for HBV therapy belong to three classes: L-nucleosides [lamivudine, telbivudine (LDT), emtricitabine], deoxyguanosine analogues (entecavir) and acyclic nucleoside phosphonates (adefovir and tenofovir). Lamivudine, adefovir, LDT, entecavir and interferon α have been approved in China for HBV treatment. LDT is an orally bioavailable L-nucleoside with potent and specific anti-HBV activity. It has been proved that LDT has a potent effect and a relatively high seroconversion rate for patients with CHB[4,5].

According to national and international guidelines, the antiviral treatment of patients with CHB is initiated when HBV DNA levels are above 2000 IU/mL and/or the serum alanine aminotransferase (ALT) levels are over 2 times the upper limit of normal (ULN), and liver biopsy shows moderate to severe active necroinflammation and/or fibrosis (e.g. at least A2F2 by METAVIR scoring)[6-8]. Many clinical trials have shown positive results of the antiviral treatments in hepatitis B patients with ALT levels between 2 and 10 times the ULN range. Nevertheless, a proportion of patients have serum ALT level over 10 times the ULN. There are few reports on the issue of whether to treat these patients right away or wait until a decline of ALT level. This paper summarizes the efficacy of LDT treatment in 40 hepatitis B patients with serum ALT level over 10 times the ULN range. We found that these patients obtained a better therapeutic effect when they received LDT treatment immediately.

MATERIALS AND METHODS

Patients and study design

This study was approved by the Ethics Review Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. All patients provided written informed consent before antiviral therapy was given. The diagnosis of CHB was made according to the diagnostic standard from the National Program for Prevention and Treatment of Viral Hepatitis[9]. All patients were diagnosed as CHB based on hepatitis B surface antigen (HBsAg) positivity for more than 6 mo. Forty HBeAg-positive CHB patients were enrolled in this study. All of them had ALT levels between 10 and 20 times the upper normal level. Another 40 HBeAg-positive CHB patients whose ALT level was between 2 and 10 times the ULN were recruited as controls. All 80 CHB patients had serum HBV DNA level > 10^5 copies/mL and had never received anti-HBV therapy before. Patients were given LDT 600 mg daily as initial antiviral treatment for at least 52 wk. Patients were excluded from this study if they were coinfected with human immunodeficiency virus, hepatitis C virus, hepatitis D virus, had liver cirrhosis or hepatic decompensation, pancreatitis, hepatocellular carcinoma, fatty liver or alcoholic hepatitis.

The present study focused on main therapeutic endpoints at 52 wk for CHB patients with high baseline ALT levels, including proportions of patients with non-detectable serum HBV DNA, serum ALT normalization, HBeAg and HBsAg seroconversion and LDT resistance. Resistance was defined as emergence of treatment-associated resistance mutations, identified by direct sequencing of the amplified HBV DNA at baseline and from sera of all patients with serum HBV DNA > 3 $\log_{10}$ copies/mL at week 52. Viral breakthrough was defined as persistent (two consecutive determinations) on-treatment increase of serum HBV DNA > 1 $\log_{10}$ copies/mL above nadir level[10].

Serum assay

Analyses of liver function, renal function and creatine kinase level were performed at baseline and at week 2, 4, 8, 12, 16, 24, 32, 36, 48 and 52 of LDT therapy using the Automatic Biochemistry analyzer (Hitachi 7600). HBsAg, HBeAg, anti-HBc, anti-HBe and anti-HBs were quantified using radioimmunoassay (Abbott Laboratories Ltd.). HBV DNA was measured using the Amplicor HBV Test (Roche Diagnostics, Basel, Switzerland) with a detection limit of 300 copies/mL. LTD-associated mutations were assessed by direct sequencing.

Statistical analysis

Quantitative data were presented as mean ± SD, categorical data were presented as counts and percentages, and HBV DNA levels were presented as log transformation. Data were analyzed using the SPSS software package version 13.0 (SPSS Inc., Chicago, IL, USA). Pearson chi-square or Fisher exact tests were used for categorical variables. In all cases, *P* values less than 0.05 were considered statistically significant.

RESULTS

Patients

Baseline characteristics for all 80 HBeAg-positive CHB patients are presented in Table 1. In the high baseline ALT CHB patient group, patients consisted of 29 males and 11 females, with ages ranging from 21 to 38 years (28.12 ± 3.71 years). Baseline data are as follows: the median level of serum HBV DNA was 7.78×10^7 copies/mL (range: 4.67×10^5-8.58×10^9 copies/mL), the median ALT level was 658.0 IU/L (range: 513.0-978.0 IU/L).

Virological response

By week 52, the mean decrease in HBV DNA level compared with baseline was 7.03 $\log_{10}$ copies/mL in the high

Table 1 Patient baseline characteristics

Variables	High baseline ALT group	Controls
Patients (*n*)	40	40
Male, *n* (%)	29 (72.5)	28 (70)
Age (yr, mean ± SD)	28.12 ± 3.71	31.12 ± 5.43
ALT (IU/L)	885.6 ± 7.89	128.4 ± 5.33
TBiL (μmol/L)	45.43 ± 6.67	29.12 ± 2.56
HBV DNA (copies/mL)		
Median	7.78×10^8	7.56×10^8
Range	4.67×10^5-8.58×10^9	5.89×10^5-7.34×10^9

ALT: Alanine aminotransferase; TBiL: Total bilirubin; HBV: Hepatitis B virus.

Table 2 Efficacy and safety at week 52 *n* (%)

Variables	High baseline ALT group	Controls	*P* value
Decrease in HBV DNA level (log_{10} copies/mL)	7.03	6.17	< 0.05
HBV DNA negative rate	29/40 (72.5)	24/40 (60)	< 0.05
ALT normalization rate	30/40 (75.0)	31/40 (77.5)	> 0.05
HBeAg negative rate	18/40 (45.0)	11/40 (27.5)	< 0.05
HBeAg seroconversion rate	15/40 (37.5)	9/40 (22.5)	< 0.05
HBsAg negative rate	4/40 (10.0)	1/40 (2.5)	< 0.05
HBsAg seroconversion rate	3/40 (7.5)	0	< 0.05
Viral breakthrough	2/40 (5.7)	3/40 (7.5)	> 0.05
Viral resistance	1/40 (2.9)	2/40 (5)	> 0.05
Increased blood creatine kinase	5/40 (12.5)	4/40 (10)	> 0.05

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

baseline ALT group and 6.17 log_{10} copies/mL in the control group, respectively ($P < 0.05$). The proportion of patients in whom serum HBV DNA levels were undetectable by polymerase chain reaction assay was greater in the high baseline ALT group than in the control group (72.5% *vs* 60%, $P < 0.05$) as indicated in Table 2.

Serological response

At week 52, 45.0% of HBeAg-positive CHB patients in the high baseline ALT group and 27.5% ($P < 0.05$) of controls became HBeAg-negative, and 37.5% of those in the high baseline group and 22.5% of those in the control group had HBeAg seroconversion ($P < 0.05$). Moreover, in the high baseline group, 4 out of 40 patients (10%) became HBsAg-negative and 3 (7.5%) of them seroconverted (became HBsAb-positive). Only 1 patient in the control group became HBsAg-negative, but had no seroconversion (Table 2).

Biochemical response

At week 52, ALT normalization was achieved for 30 of the 40 patients (75.0%) in the high baseline ALT group and 31 of 40 patients (77.5%) in the control group ($P > 0.05$).

Resistance and side effects

As indicated in Table 2, viral breakthrough and genotypic resistance to LDT were similar between patients with high baseline ALT levels and controls. Resistance developed in 2.9% of patients with high baseline ALT levels and in 5% (2/40) of control patients. Consistent with previous reports, M204I was the only mutation associated with LDT resistance in this study. After the emergence of resistance, adefovir dipivoxil was added to treatment. Resistance patients are considered treatment failures in this study.

The frequencies of adverse events through week 52 were similar in both groups treated with LDT. Elevations in creatine kinase level through 52 wk were observed in 12.5% (5/40) of patients in the high baseline ALT group and in 10% (4/10) of controls, respectively. Grade 3 or 4 elevations in creatine kinase level (at least seven times the ULN) were found only in 1 patient in the high baseline ALT group and in 1 patient in the control group, respectively; levels decreased spontaneously during LDT treatment to normal within the next two visits (6 mo). No patients in either group stopped LDT treatment because of creatine kinase elevations in this study (Table 2).

DISCUSSION

The goal of therapy for hepatitis B is to improve quality of life and survival by preventing progression of the disease to cirrhosis, decompensated cirrhosis, end-stage liver disease, HCC and death. This goal can be achieved if HBV replication can be suppressed in a sustained manner, the accompanying reduction in histologic activity of chronic hepatitis lessening the risk of cirrhosis and decreasing the risk of HCC[11]. To date, two types of antiviral drugs can be used in the treatment of CHB: interferon and nucleoside/nucleotide analogs. In China, four types of nucleoside/nucleotide analogs (lamivudine, adefovir dipivoxil, entecavir and LDT) are available. Among them, LDT is potent and induces a relatively high seroconversion rate[12]. LDT has become widely used in anti-HBV therapy in China.

Besides serum HBV DNA levels and histological grade and stage of the liver disease, baseline ALT level of CHB patients is one of the determinants for the initiation of antiviral therapy. The antiviral effect of LDT is associated with the baseline ALT level, as in interferon and lamivudine therapy[13,14]. Taking HBeAg seroconversion as an example, 32% of patients with pretreatment ALT levels between 2 and 5 × ULN and 46% of those with ALT > 5 × ULN achieved HBeAg seroconversion after 2 years of treatment with LDT[5]. Our study focused, we believe for the first time, on the antiviral effect of LDT on HBeAg-positive patients whose baseline ALT level was 10-20 × ULN, showing the HBeAg seroconversion rate was 37.5% at 52 wk, which is the same as reported for peg-interferon therapy at 48 wk[15]. More encouragingly, our results also showed 7.5% (3/40) patients had HBsAg seroconversion at 52 wk after LDT treatment.

The main mechanism of ALT elevation in CHB patients is the activated immune response to eliminate HBV, which theoretically shows the positive association between ALT level and the degree of inmune activation. High baseline ALT level has been shown to be independently associ-

ated with an increased rate of HBeAg response after either interferon or NUC treatment[16,17]. In the present study, our results clearly showed that increased serum baseline ALT levels predict a higher HBeAg seroconversion when patients are treated with LDT.

HBeAg has been recognized as a successful serologic marker in the treatment of HBeAg-positive CHB[18]. Compared with other NUCs, LDT has a relatively high seroconversion rate. Whether this is related to its immune regulation ability needs further exploration. Evans *et al*[19] reported the relatively low expression of programmed death-1 receptor on CD8+ T cells in HBeAg-positive CHB patients who received LDT therapy and had HBeAg seroconversion, compared with those counterparts who did not achieve HBeAg seroconversion.

Entecavir and tenofovir are potent HBV inhibitors and they have a high barrier to resistance. They are widely used as first-line monotherapies in developed countries. However, in China tenofovir is not available yet, and entecavir is expensive for most patients. LDT and lamivudine are still widely used. In order to reduce the incidence of resistance to these drugs, optimal treatment has been used in clinical practice. For example, pretreatment serum HBV DNA $< 10^9$ $\log_{10}$ copies/mL and ALT levels $\geqslant 2 \times$ ULN for HBeAg-positive patients were shown to be associated with a high rate of non-detectable HBV DNA, a high rate of HBeAg seroconversion and lower resistance to LDT treatment after 2 years[5]. Our study also proved that if we select the right patients to treat with LDT, there will be optimal conditions to achieve the desired results. Taken together, if baseline serum HBV DNA $< 10^9$ $\log_{10}$ copies/mL and ALT levels $\geqslant$ 2-20 $\times$ ULN for HBeAg-positive patients, we can consider the administration of LDT treatment in daily clinical practice.

In conclusion, our results indicate relatively higher HBeAg and HBsAg seroconversion in HBeAg-positive CHB patients whose baseline ALT levels were 10-20 $\times$ ULN and who received LDT monotherapy immediately. In addition, there were no significant differences in safety between these patients and their counterparts with lower ALT levels. We suggest that this treatment strategy deserves clinical application.

COMMENTS

Background

There is a proportion of chronic hepatitis B (CHB) patients with serum alanine aminotransferase (ALT) levels over 10 times the upper limit of normal. There are few reports regarding the issue of treatment for these patients, whether to treat them right away or whether to wait until the decline of ALT level.

Research frontiers

In China tenofovir is not available yet, and entecavir is expensive for most patients. Telbivudine (LDT) and lamivudine are still widely used. In order to reduce the incidence of resistance to these drugs, optimal treatment has been used in clinical practice. However, how to select optimally for LDT has not been unequivocally addressed. In this study, the authors demonstrate relatively high hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) seroconversion in HBeAg-positive CHB patients whose baseline ALT levels were 10-20 times the upper limit of normal (× ULN) and who received LDT monotherapy immediately.

Innovations and breakthroughs

Recent reports have highlighted the importance of baseline characteristics, such as serum hepatitis B virus (HBV) DNA level, ALT level and histological grade and stage, in antiviral therapy. This is the first study to report the antiviral effect of LDT on HBeAg-positive patients whose baseline ALT level was 10-20 × ULN, showing the HBeAg seroconversion rate was 37.5% at 52 wk. More encouragingly, our results also showed 7.5% (3/40) patients had HBsAg seroconversion at 52 wk after LDT treatment.

Applications

By understanding that the antiviral results are related to the baseline ALT levels in addition to HBV DNA titer, this study may represent a future strategy for therapeutic intervention in CHB patients with high baseline ALT level.

Terminology

ALT is a common indicator of liver damage and is one of the key predictors of initiation of antiviral therapy. This study suggests that high baseline ALT level is a strong predictor for optimal results during LDT treatment.

Peer review

This study shows favorable results in patients with high baseline ALT values. The authors conclude that in patients with high baseline ALT levels antiviral treatment with LDT should be started immediately.

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S- Editor Wang YR **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4100

World J Gastroenterol 2010 August 28; 16(32): 4100-4106
ISSN 1007-9327 (print)

BRIEF ARTICLE

Specific shRNA targeting of *FAK* influenced collagen metabolism in rat hepatic stellate cells

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Author contributions: Dun ZN performed the majority of the experiments, analyzed the data and drafted the manuscript; Zhang XL designed the research and wrote the manuscript; An JY and Zheng LB assisted with various experiments and helped edit the manuscript; Barrett R revised the manuscript; Xie SR contributed to the cell culture experiments.

Supported by Grants from the National Natural Science Foundation of China, No. 30872513; Hebei Provincial Natural Science Foundation of China, No. C2008001133 and No. C2010000565

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Received: February 27, 2010 Revised: June 14, 2010
Accepted: June 21, 2010
Published online: August 28, 2010

Abstract

AIM: To investigate the effects and mechanism of disruption of focal adhesion kinase (*FAK*) expression on collagen metabolism in rat hepatic stellate cells (HSC).

METHODS: The plasmids expressing *FAK* short hairpin RNA (shRNA) were transfected into HSC-T6 cells, and the level of *FAK* expression was determined by both real-time quantitative polymerase chain reaction (Q-PCR) and Western blotting analysis. The production of type Ⅰ collagen and type Ⅲ collagen in *FAK*-disrupted cells was analyzed by real-time Q-PCR. The level of collagen metabolism proteins, including matrix metalloproteinases-13 (MMP-13) and tissue inhibitors of metalloproteinases-1 (TIMP-1) was also determined by both real-time Q-PCR and Western blotting analysis.

RESULTS: The transfection of *FAK* shRNA plasmids into HSC resulted in disrupted *FAK* expression. Compared with the HK group, the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased (0.69 ± 0.03 *vs* 1.96 ± 0.15, $P = 0.000$; 0.59 ± 0.07 *vs* 1.62 ± 0.12, $P = 0.020$). The production of TIMP-1 in this cell type was also significantly reduced at both mRNA and protein levels (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$). However, the expression of MMP-13 mRNA could be significantly up-regulated by the transfection of *FAK* shRNA plasmids into HSC (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$).

CONCLUSION: These data support the hypothesis that shRNA-mediated disruption of *FAK* expression could attenuate extracellular matrix (ECM) synthesis and promote ECM degradation, making *FAK* a potential target for novel anti-fibrosis therapies.

Key words: Focal adhesion kinase; Hepatic stellate cells; Matrix metalloproteinases; RNA interference; Type Ⅰ collagen; Type Ⅲ collagen; Tissue inhibitors of metalloproteinases

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Dun ZN, Zhang XL, An JY, Zheng LB, Barrett R, Xie SR. Specific shRNA targeting of *FAK* influenced collagen metabolism in rat hepatic stellate cells. *World J Gastroenterol* 2010;

16(32): 4100-4106 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4100.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4100

INTRODUCTION

Liver fibrosis results from excessive deposition of extracellular matrix (ECM) components[1]. These components, which are mainly composed of type I collagen and type Ⅲ collagen, are produced by hepatic stellate cells (HSC). The activation, proliferation and migration of HSC play a central role in liver fibrogenesis[2,3]. Activated HSCs are the main producers of collagens and matrix metalloproteinases (MMPs) in the fibrotic liver. The MMP which is able to degrade type I collagen and type Ⅲ collagen is MMP-13. However, this can be specifically inhibited by tissue inhibitors of metalloproteinases-1 (TIMP-1), and its level is found to be high in the fibrotic liver of rats.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase, whose phosphorylation can promote the proliferation and collagen synthesis of HSC[4-8]. It had previously been shown that when FAK related non-kinase (FRNK) plasmids were transfected into fibronectin (FN)-stimulated HSC using liposome transfection, the over-expression of FRNK significantly decreased the collagen synthesis of HSC *in vitro*[9,10]. This led us to speculate that suppression of FAK expression may provide a new target in the treatment of liver fibrosis.

To date, RNA interference has been the most effective gene silencing technology. It can specifically inhibit the transcription of target genes, and in turn reduce the expression and function of the corresponding proteins[11]. We aim to inhibit FAK expression by transfecting FAK short hairpin RNA (shRNA) plasmids into HSC. To our knowledge, this is the first report that FAK expression is specifically inhibited in HSC cells. This allows us to further analyze the role of FAK in collagen synthesis and degradation in this cell type, and find out how FAK regulates the expression of MMP-13 and TIMP-1.

MATERIALS AND METHODS

Reagents

The shRNA-expressing plasmids, pEGFP-*FAK* shRNA, were purchased from Wuhan Genesil Biotechnology Co. Ltd. (Wuhan, China). One additional plasmid, p-EGFP-HK, was used to express nonsense shRNA and served as the control. Sofast™ Transfection Reagent was purchased from Xiamen Sunma Biological Engineering Co. Ltd. (Xiamen, China).

Cell line and cell culture

The cell line HSC-T6, which is the phenotypically activated HSC, was donated by Professor Xu LM, from Hepatopathy Institute of Shanghai University of Traditional Chinese Medicine. HSCs were cultured in HG-DMEM medium supplemented with 8% FBS, 100 IU/mL penicillin, 100 g/mL streptomycin, 4 mmol/L glutamine and 1 mol/L HEPES. Cells were cultured in a 5% CO_2 humidified incubator at 37℃. All experiments were conducted when cells were at an exponential stage of growth. Cells were seeded into a 25 cm^2 plastic culture flask with a total of 2-3 × 10^5 cells or were seeded in 96-well plates to a density of 3 × 10^4/mL × 200 μL/well. When cells were approximately 70%-80% confluent, shRNA plasmid was transfected into FN-stimulated HSC using a cationic polymer. The cells were divided into five groups: (1) blank control group (control); (2) FN stimulation group (FN); (3) transfection reagent group (Sofast); (4) pEGFP-HK shRNA group (HK); and (5) pEGFP-*FAK* shRNA group (*FAK* shRNA). FN was added to groups 2-5 at a concentration of 10 mg/L.

Efficiency of transfection

At 48 h after transfection, the cells were analyzed by fluorescence microscopy and flow cytometry (FCM) to obtain the efficiency of transfection.

Semiquantitative real-time quantitative polymerase chain reaction

The expressions of the gene *FAK*, *type I collagen* and *type Ⅲ collagen*, *MMP-13* and *TIMP-1* were characterized by semiquantitative real-time quantitative polymerase chain reaction (Q-PCR). Briefly, total RNA was extracted from the cells that had been transfected with the plasmid expressing the *FAK* or HK shRNA and reversely transcripted into cDNA, which was used as the template for PCR. Using the primer design software, Primer Express 2.0, the specific primers for each gene were synthesized by Beijing Saibaisheng Gene Technique Co., Ltd. and the following primers were generated: *FAK*-Forward 5'-ACTTGGACGCTGTATTGGAG-3', *FAK*-Reverse 5'-CTGTTGCCTGTCTTCTGGAT-3' (833 bp amplicon); Collagen type I -Forward 5'-TACAGCACGCTTGTGGATG-3', Collagen type I -Reverse 5'-TTGAGTTTGGGTTGTTGGTC-3' (256 bp amplicon); Collagen type Ⅲ-Forward 5'-ATGGTGGCTTTCAGTTCACC-3', Collagen type Ⅲ-Reverse 5'-TGGGGTTTCAGAGAGTTTGG-3' (425 bp amplicon); MMP-13-Forward 5'-GCGGGAATCCTGAAGAAGTCTAC-3', MMP-13-Reverse 5'-TTGGTCCAGGAGGAAAAGCG-3' (424 bp amplicon); TIMP-1-Forward 5'-TCCCCAGAAATCATCGACAC-3', TIMP-1-Reverse 5'-ATCGCTGAACAGGGAAACAC-3' (329 bp amplicon); *GAPDH*-Forward 5'-GAGGACCAGGTTGTCTCCTG-3', *GAPDH*-Reverse 5'-GGATGGAATTGTGAGGGAGA-3' (298 bp amplicon). Reaction system: 10 μL 2.5 × real master Mix, 1.25 μL 20 × SYBR solution, 0.5 μL upstream primer, 0.5 μL downstream primer and 2 μL DNA template were brought up to 25 μL with purified water. Reaction conditions: 93℃ 5 min, 1 cycle; 93℃ 45 s, 55℃ 1 min, 10 cycles; 93℃ 30 s, 55℃ 45 s, 30 cycles. The PCR reactions were subjected to 93℃ for 5 min, 1 cycle; and then 10 cycles of 93℃ 45 s, 55℃ 1 min, and 30 cycles of 93℃ 30 s, 55℃ 45 s. The size and quantity of amplified prod-

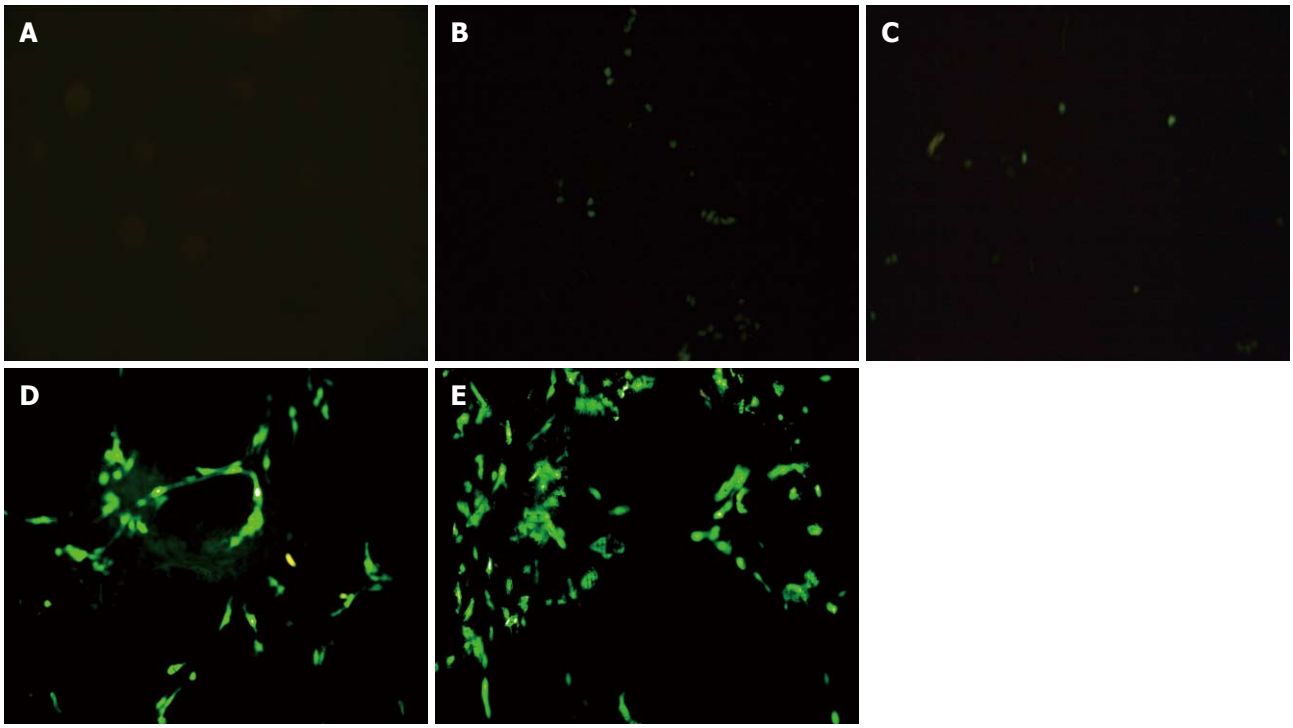

Figure 1 Expression of enhanced green fluorescent protein at 48 h after treatment (fluorescent images, original magnification × 200). A: Control group; B: Fibronectin group; C: Sofast group; D: HK group; E: Focal adhesion kinase (FAK) short hairpin RNA group. *FAK* short hairpin RNA plasmids were successfully transfected into hepatic stellate cells. The results from fluorescence microscopy and flow cytometry showed that the transfection efficiency was 40% at 48 h.

ucts were confirmed by 2% agarose gel electrophoresis. Fluorescent quantitative analysis was performed with the thermal cycler's software package to calculate the ΔCt value. The expression levels of *FAK*, type I collagen and type Ⅲ collagen, *MMP-13* and *TIMP-1* were calculated by the $2^{-\Delta\Delta Ct}$ analysis. The $2^{-\Delta\Delta Ct}$ was presented as the relative expression of the gene expression[12].

Western blotting

At 24 or 48 h after transfection of *FAK* shRNA, HSCs were harvested, washed with phosphate-buffered saline (PBS), and lysed in the improved RIPA buffer (50 mmol/L Tris-HCL, pH 7.5; 100 mmol/L NaCl; 1% NP-40; 0.5% sodium deoxycholate; 2 μg/mL leupeptin; 1% SDS; 2 mmol/L EDTA; 1 mmol/L PMSF; 50 mmol/L HEPES; 1 mmol/L sodium orthovanadate). The supernatant was collected and the protein concentration was determined using comassie brilliant blue assay. Cell extracts containing equal quantities of proteins (100-110 μg) were electrophoresed in 8% or 10% polyacrylamide gel. Subsequently, the separated proteins were transferred to nitrocellulose membrane. The membrane was blocked for non-specific binding for 30 min (5% skimmed milk in PBS), and then incubated overnight at 4℃ with rabbit anti-FAK polyclonal antibody (1:400), rabbit anti-MMP-13 polyclonal antibody (1:200), rabbit anti-TIMP-1 polyclonal antibody (1:200) or mouse anti–GAPDH monoclonal antibody (1:100). The membrane was subsequently incubated at room temperature for 2 h with goat anti-rabbit IgG (1:2000). Blots were developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology Inc.), exposed on Kodak Xdmat blue XB-1 film and quantified by Bandscan 5.0 software using GAPDH as internal control. Densitometry is reported using the integral optical density value (IOD). The results were represented in the form of IOD ratio of the target protein to GAPDH.

Statistical analysis

All the data were expressed by mean ± SD and analyzed with SPSS 13.0 software. The comparison of mean variability among all groups was conducted by one-way ANOVA analysis and two group comparison with LSD test. Student's *t* test was carried out for independent samples. Statistical significance was considered at $P < 0.05$.

RESULTS

Expression of FAK effectively down-regulated by FAK shRNA in HSC

FAK shRNA plasmids were successfully transfected into HSC. The results from fluorescence microscopy and FCM showed that the transfection efficiency was 40% at 48 h (Figure 1). The levels of *FAK* mRNA transcripts and protein expression were determined by real-time Q-PCR and Western blotting analysis. The expression of *FAK* mRNA and FAK protein in the FN group was significantly higher than that of the control group, $P = 0.000$ and $P = 0.024$, respectively. There was no difference between the FN group, Sofast group and HK group. In comparison with the HK group, the expression of *FAK* mRNA and FAK

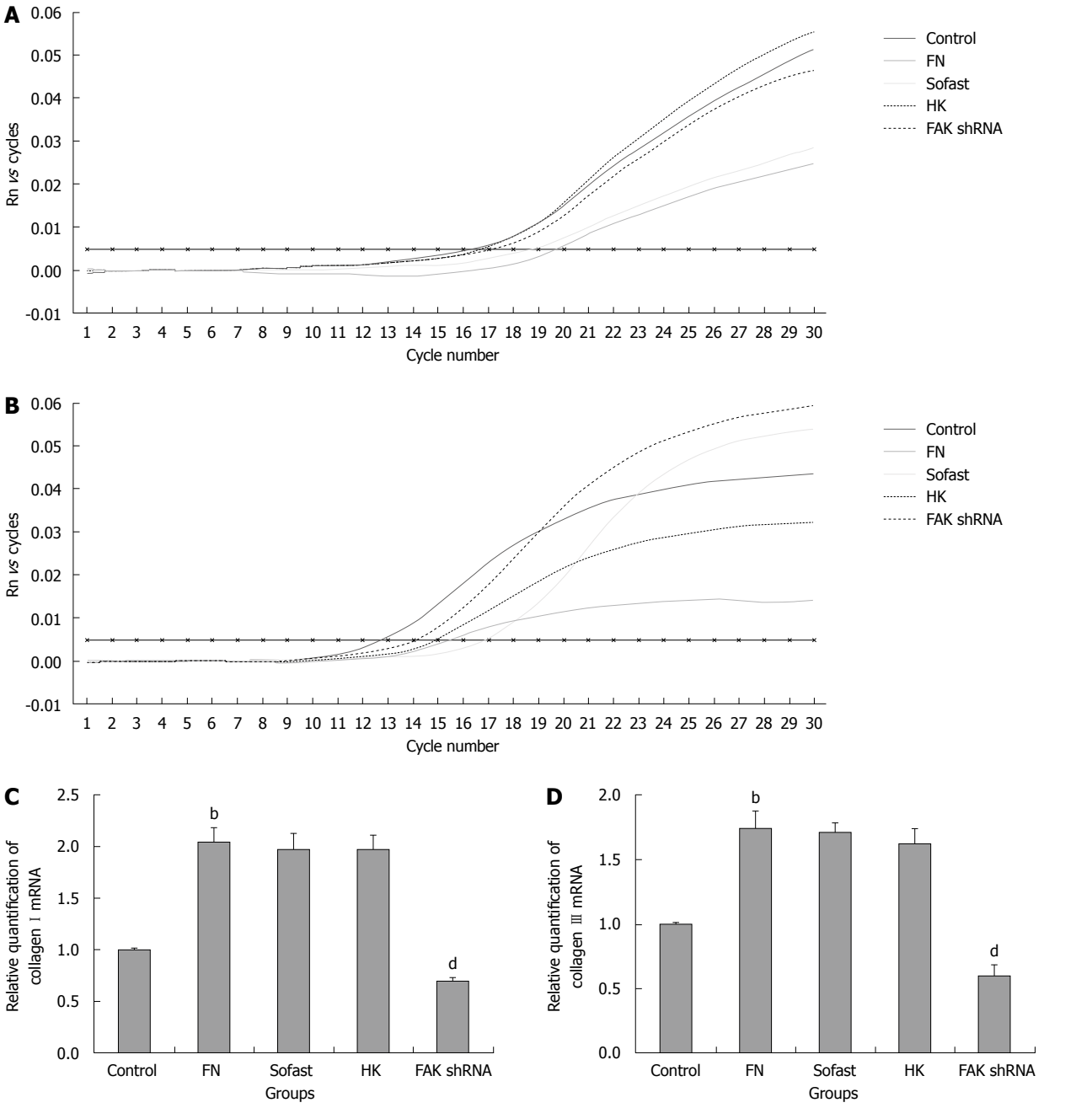

Figure 2 Focal adhesion kinase short hairpin RNA selectively inhibited the expressions of collagen Ⅰ mRNA and collagen Ⅲ mRNA in hepatic stellate cells after focal adhesion kinase short hairpin RNA transfection. A, B: Real-time polymerase chain reaction SYBR Green Ⅰ fluorescence history *vs* cycle number of target gene 1 (collagen Ⅰ, A) and target gene 2 (collagen Ⅲ, B) in sample cDNA. The cycle threshold (Ct) is shown by the darker horizontal line; C, D: The relative quantification of collagen Ⅰ mRNA (C) and collagen Ⅲ mRNA (D) are calculated according to $2^{-\Delta\Delta C(t)}$, [$\Delta\Delta$Ct = ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) experimental group - ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) control group] and shown in the bar graphs (n = 3, ^{b}P < 0.01 *vs* control, ^{d}P < 0.01 *vs* HK). It showed that the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in fibronectin (FN) group was significantly higher than in the control group. FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

protein in the *FAK* shRNA plasmid group was significantly decreased (0.37 ± 0.03 *vs* 1.59 ± 0.06, P = 0.000; 0.77 ± 0.03 *vs* 2.24 ± 0.20, P = 0.000), and the rates of down-regulation were 70.51% and 72.53%, respectively.

Effects of FAK by shRNA on the collagen synthesis in HSC

Investigation was carried out in the influence of disruption of FAK expression mediated by *FAK* shRNA on ECM synthesis in HSC. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts were determined by real-time Q-PCR. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in FN group were significantly higher than that of the control group. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased compared with the HK group (0.69 ± 0.03 *vs* 1.96 ± 0.15, P = 0.000; 0.59 ± 0.07 *vs* 1.62 ± 0.12, P = 0.020) and the down-regulated rates were 64.80% and 63.58%, respectively (Figure 2).

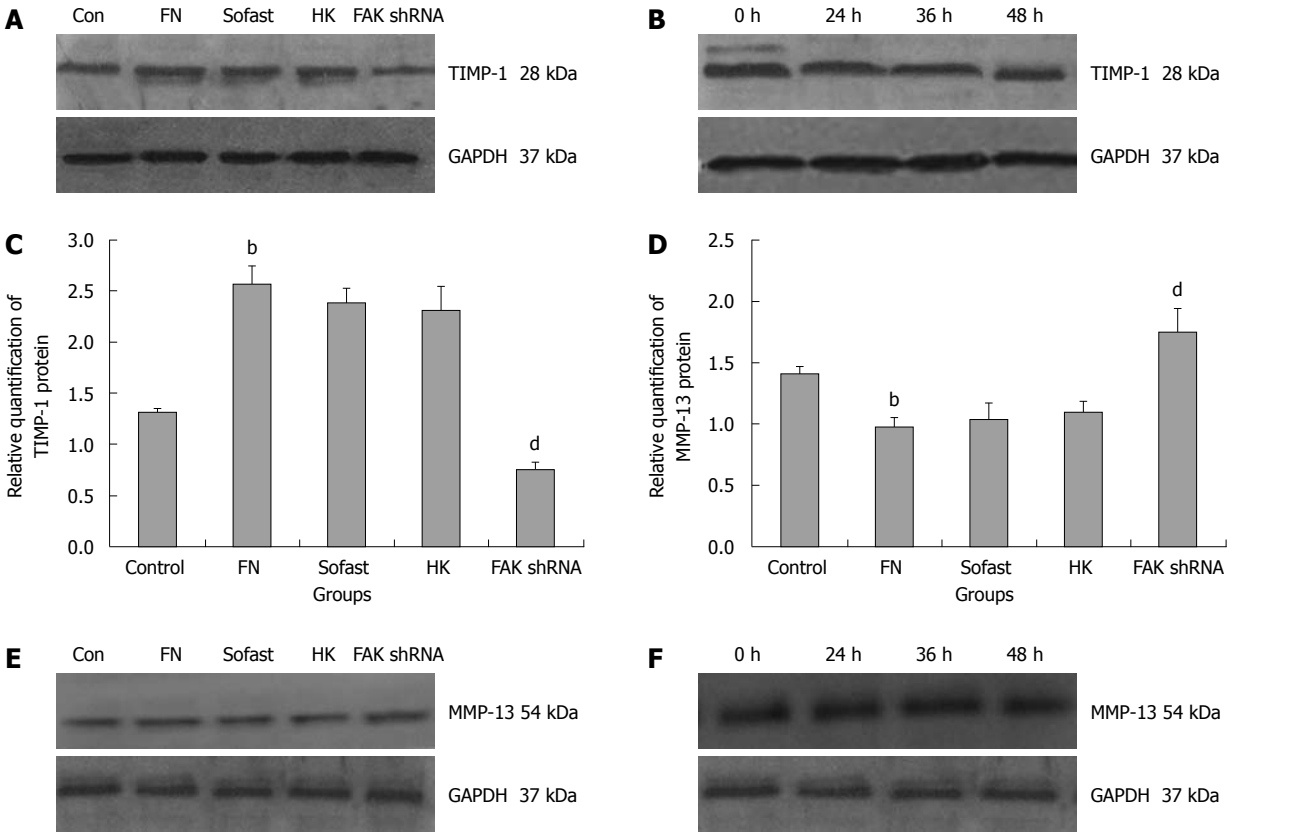

Figure 3 Focal adhesion kinase short hairpin RNA specifically inhibits the expressions of tissue inhibitors of metalloproteinases-1 protein and promotes the expressions of matrix metalloproteinases-13 protein in hepatic stellate cells. A: Cells were harvested, lysed and total protein extracts were separated by SDS-PAGE and analyzed by Western blotting with polyclonal anti-tissue inhibitors of metalloproteinases-1 (TIMP-1) antibody. GAPDH served as a loading control; B: Western blotting analysis was used to detect the expressions of TIMP-1 at different time points; C: TIMP-1 expression levels obtained from scanning densitometry were expressed as a ratio of integral optical density value (IOD) TIMP-1/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); D: Matrix metalloproteinases-13 (MMP-13) expression levels obtained from scanning densitometry were expressed as a ratio of IOD MMP-13/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); E, F: Western blotting analysis was carried out at different groups (E) and different time points (F) using polyclonal anti-MMP-13 antibody and monoclonal anti-GAPDH antibody. FN: Fibronectin; FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

Effects of FAK by shRNA on the collagen degradation in HSC

To further explore the effects of *FAK* shRNA on the ECM degradation in HSC, the levels of MMP-13 and TIMP-1 were determined by real-time Q-PCR and Western blotting analysis. The transfection of HK shRNA did not modulate the levels of MMP-13 and TIMP-1, the cells expressing HK shRNA were similar to that in FN group and Sofast group, $P > 0.05$. However, the knockdown of FAK expression by the *FAK* shRNA significantly reduced the levels of *TIMP-1* mRNA and TIMP-1 protein (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$), and the down-regulated rates were 69.78% and 67.10%, respectively (Figure 3A-C). The results of real-time Q-PCR and Western blotting analysis showed that the levels of MMP-13 of FN group were significantly down-regulated compared with that of control group. Compared with the HK group, the expression of MMP-13 mRNA was significantly up-regulated by 56.96% at 36 h after transfection of *FAK* shRNA plasmids into HSC (1.24 ± 0.04 *vs* 0.79 ± 0.03, $P = 0.020$), and the expression of MMP-13 protein could be increased by 59.63% at 48 h after transfection (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$) (Figure 3D-F).

DISCUSSION

The current knowledge on the pathophysiology of liver fibrogenesis refers to the increased synthesis and decreased degradation of ECM, mainly type I collagen and type Ⅲ collagen, thereby ECM was overproduced and deposited in the liver. Although several hepatic cell types can synthesize ECM proteins, HSCs are the major source of increased ECM in chronic liver diseases. They can undergo a proliferative and phenotypic change. Excessive deposition of ECM, mainly type I collagen and type Ⅲ collagen, results in liver fibrosis; and the up-regulation of TIMPs blocks activity of MMPs and inhibits the degradation of ECM, thereby aggravating liver fibrosis.

The interaction of HSC and ECM mainly lies between integrins, and FAK plays an integral role in the integrin signal pathway. Activated FAK has been implicated in a diverse array of cellular behaviors, such as cell proliferation[4,5], apoptosis, cell migration[6], collagen metabolism[7,8] and the transfer of tumor cells. It is closely related to numerous fibrotic diseases and it plays a vital role in the occurrence and development of liver fibrosis[13]. This is consistent with our previous studies, which indicated that FAK phosphorylation could promote collagen synthesis

of HSC *in vivo*. Furthermore, using *in vitro* cell culture techniques, we found that the synthesis of total collagen and type I collagen in HSC could be inhibited by the endogenous inhibitor FRNK[9]. We hypothesized that FAK gene silencing may represent a novel method for the treatment and reversal of liver fibrosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into HSC transiently to test our hypothesis, and the expressions of FAK mRNA and FAK protein were significantly decreased, the down-regulation rates being 70.51% and 72.53%, respectively. We have found that *FAK* shRNA can effectively and specially suppress the expression of FAK.

A substantial change in liver fibrosis or liver cirrhosis is the deposition of ECM, which is mainly composed of type I collagen and type Ⅲ collagen, covering approximately 80%-90% of the increased total collagen. The increase of type I collagen and type Ⅲ collagen is an important symbol of liver fibrosis or liver cirrhosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of type I collagen mRNA and type Ⅲ collagen mRNA was significantly down-regulated by 64.80% and 63.58%, respectively. These data show that *FAK* shRNA can effectively suppress the synthesis of collagen and *FAK* gene silencing may, therefore, represent a novel direction for the treatment and reversal of liver fibrosis.

Furthermore, we attempted to assess the role of FAK in the regulation of collagen metabolism in HSC. In the liver, ECM is regulated by MMPs and their specific inhibitors, TIMPs. A principal feature of hepatic fibrosis is a disturbance in the balance between MMPs and TIMPs. Collagenases such as MMP-1 and MMP-13 are able to degrade fibrillar collagens, mainly type Ⅰ, Ⅱ and Ⅲ collagen. These may be responsible for key events in the degradation of ECM. MMP-13 is the interstitial collagenase in rats and its specific inhibitor is TIMP-1. Although the expression of MMP-13 was increased in the liver tissues of CCl_4-induced rat liver fibrosis models, fibrosis still occurred as there was also a corresponding increase in the expression of TIMP-1[14]. This strongly suggests that a disruption in the balance between MMP-13 and TIMP-1 is possibly an important factor in liver fibrogenesis[15]. According to some studies, FAK is closely related to the expression of TIMP-1 and MMP-13[16,17]. In this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of MMP-13 mRNA and MMP-13 protein was significantly up-regulated by 56.96% and 59.63%. Correspondingly, the levels of TIMP-1 mRNA and TIMP-1 protein were significantly down-regulated by 69.78% and 67.10%, respectively. *FAK* shRNA inhibited the ratio of TIMP-1/MMP-13 expression in mRNA and protein levels in HSC after transfection. The data indicate that *FAK* shRNA regulated the collagen metabolism in HSC by disturbing the balance between MMP-13 and TIMP-1.

In summary, we have effectively disrupted the expression of FAK by *FAK* shRNA. The knockdown of FAK expression significantly reduced the synthesis of type I collagen and type Ⅲ collagen, which may be related to the up-regulation of MMP-13 and down-regulation of TIMP-1. These data support the hypothesis that *FAK* disruption by shRNA may be an efficient and specific approach for treatment of liver fibrosis. Future studies will address the signal transduction pathway by which FAK regulates the collagen metabolism in HSC.

COMMENTS

Background

Focal adhesion kinase (FAK) plays an essential role in the activation of hepatic stellate cells (HSCs) which are the major source of collagens and matrix metalloproteinases in the fibrotic liver. Liver fibrosis results from excessive deposition of extracellular matrix components, composed of mainly type Ⅰ collagen produced by HSC.

Research frontiers

The central events in the liver fibrogenesis have been proved to be the activation, proliferation and migration of HSC, and their proliferation and collagen synthesis are promoted by phosphorylation of FAK, a non-receptor protein tyrosine kinase. In the area of knockdown or inhibition of FAK with various molecular biological technologies, an area of intense research is to establish a method to knockdown or inhibit FAK expression thoroughly so as to enhance the collagen metabolism.

Innovations and breakthroughs

Recent reports have highlighted the importance of HSC including activation, proliferation and migration in pathogenesis of liver fibrosis. The collagen metabolism in HSC, particular in activated HSC, is currently an area of intense research. This is the first study to report that shRNA-mediated disruption of *FAK* expression can attenuate extracellular matrix (ECM) synthesis and promote ECM degradation. This represents a potential target for novel anti-fibrosis therapies.

Applications

The results of this study indicated that suppression of *FAK* expression may represent a novel method and direction for the treatment and reversal of hepatic fibrosis.

Peer review

The study focuses on modification of hepatic stellate cell metabolism by shRNA mediated inhibition of FAK, a non-receptor protein tyrosine kinase involved in proliferation and collagen synthesis. The authors demonstrate that FAK inhibition is associated with a decrease in collagen synthesis by HSCs.

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S- Editor Wang JL **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4107

World J Gastroenterol 2010 August 28; 16(32): 4107-4111
ISSN 1007-9327 (print)

CASE REPORT

Development of fatal acute liver failure in HIV-HBV coinfected patients

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Received: April 1, 2010 Revised: May 31, 2010
Accepted: June 7, 2010
Published online: August 28, 2010

Abstract

Coinfection with hepatitis B virus (HBV) is not uncommon in human immunodeficiency virus (HIV)-infected individuals and patients with HIV-HBV coinfection are at high risk for progression of liver disease. Current guidelines regarding the treatment of HIV infection recommend that patients who are coinfected with HIV and HBV receive highly active antiretroviral therapy (HAART) with activity against hepatitis B. While HIV-HBV coinfected patients often experience liver enzyme elevations after starting antiretroviral therapy, acute liver failure (ALF) is rare and typically occurs with older antiretroviral agents with known potential for hepatotoxicity. We describe two cases of fatal ALF in the setting of HIV-HBV coinfection after initiation of HAART. These cases occurred despite treatment with antiretrovirals that have activity against HBV and highlight the challenges in distinguishing drug hepatotoxicity and HBV immune reconstitution inflammatory syndrome. HIV-HBV coinfected patients should be monitored closely when initiating HAART, even when treatment includes agents that have activity against HBV.

Key words: Hepatitis B virus; Human immunodeficiency virus; Liver failure

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Anderson AM, Mosunjac MB, Palmore MP, Osborn MK, Muir AJ. Development of fatal acute liver failure in HIV-HBV coinfected patients. *World J Gastroenterol* 2010; 16(32): 4107-4111 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4107.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4107

INTRODUCTION

It is estimated that 10% of human immunodeficiency virus (HIV)-infected patients in the United States and Europe are chronically coinfected with hepatitis B virus (HBV)[1,2]. In other regions, rates of HBV coinfection in HIV-infected patients may be even higher[3]. HIV-HBV coinfected patients have higher rates of liver-related morbidity and mortality compared to patients infected with either virus alone[4,5]. Therefore, current guidelines suggest that HIV-infected patients with HBV infection should be treated with a highly active antiretroviral therapy (HAART) regimen that is also active against HBV[6]. However, HIV-HBV coinfected patients are particularly susceptible to certain complications of HAART. The vast majority of antiretroviral medications have been associated with some degree of hepatotoxicity and the presence of HBV infection is an independent risk factor for the development of clinically significant hepatotoxicity[7-9]. Additionally, coin-

fected patients are at risk for HBV immune reconstitution inflammatory syndrome (IRIS), which is characterized by a paradoxical hepatitis flare corresponding to an initial improvement in plasma HIV RNA level and CD4+ T-cell count on HAART[10]. Overall, liver enzyme elevations in HIV-HBV coinfected patients after starting HAART are not uncommon, but acute liver failure (ALF) is rare[11]. Reported cases have typically involved treatment with older thymidine analogue drugs such as stavudine and didanosine[12]. We describe two cases in which fatal ALF occurred in patients with HIV-HBV coinfection after beginning HAART regimens which did not include thymidine analogues but which did have activity against HBV.

CASE REPORT

Patient A

A 42-year-old African-American male with longstanding HIV/HBV coinfection was seen in clinic. He had been diagnosed with HIV infection over 10 years previously but had been on antiretrovirals for only short periods since diagnosis. As a result, his CD4+ T-cell count had reached a nadir of 5 cells/μL (1%) with a plasma HIV RNA level of 51 230 copies/mL. His plasma HBV DNA level was 147 million IU/mL. Both hepatitis B endogenous antigen (HBeAg) and anti-HBe antibody were negative. He was started on a new regimen of ritonavir-boosted atazanavir, lamivudine, and abacavir. At that time, his alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were both slightly elevated at 94 U/L (normal range 17-63 U/L) and 73 U/L (normal range 10-42 U/L), respectively, with a normal bilirubin level. The patient had never undergone a liver biopsy. Eight weeks after starting therapy, he returned to clinic with nausea, vomiting, and jaundice. The ALT level had increased to 1352 U/L and the AST was 1765 U/L. Total bilirubin was 14.1 mg/dL, direct bilirubin was 8.9 mg/dL, and prothrombin time was 18.9 s (INR 1.62). HIV RNA level had decreased to 100 copies/mL. He was admitted to the hospital for further workup. The patient did not drink alcohol and acetaminophen level was undetectable. Hepatitis C virus (HCV) RNA was undetectable, hepatitis D virus (HDV) antibody was negative, and hepatitis A virus (HAV) IgM was negative. HBV DNA had decreased to 4.42 million IU/mL. Antinuclear antibody screen was negative. All medications were held for 48 h. His ALT and AST levels decreased to 992 U/L and 1505 U/L. The appearance of the liver was normal on computed tomography of the abdomen with no suggestion of cirrhosis or portal hypertension. He was then discharged after starting a new regimen of ritonavir-boosted fosamprenavir, emtricitabine, and tenofovir. Ten days later he was re-admitted to the hospital with nausea, vomiting, and abdominal pain. ALT and AST levels had risen to 1214 U/L and 1992 U/L. Total bilirubin was 29.5 mg/dL, direct bilirubin was 18.3 mg/dL, and prothrombin time was 35.4 s (INR 3.19). HBV DNA level had decreased to 83 100 IU/mL. The ritonavir and fosamprenavir were discontinued and he was continued on emtricitabine/tenofovir. A liver biopsy showed marked septal fibrosis with nodule formation that was consistent with cirrhosis (Figure 1A and B). There was a severe mixed inflammatory infiltrate in portal and periportal areas containing lymphocytes, plasma cells and scant eosinophils (Figure 2A). The liver eosinophils were readily identified and no other special stains such as sirius red were used. Peripheral blood eosinophil count was within normal limits. Severe piecemeal necrosis was present diffusely around most of the portal tracts linking some of them together in so-called bridging necrosis (Figure 2B). After 10 d, his ALT and AST levels had come down to 405 U/L and 758 U/L, but total and direct bilirubin remained elevated at 28.8 mg/dL and 13.6 mg/dL. Due to concern for creating HIV resistance, he was switched from emtricitabine/tenofovir to adefovir. After 1 wk, he developed worsening renal failure and was switched from adefovir to emtricitabine and telbivudine. However, his condition continued to deteriorate. After 3 wk in the hospital, he became progressively encephalopathic, thrombocytopenic with a platelet count of 33 000/μL, and coagulopathic with prothrombin time of 37.7 s (INR 3.41). He developed hematemesis and became increasingly unresponsive with an ammonia level of 96 μmol/L (normal range 11-35 μmol/L). The patient was thought to be too unstable to undergo liver transplantation. After discussions with his family, he was placed on comfort care and died.

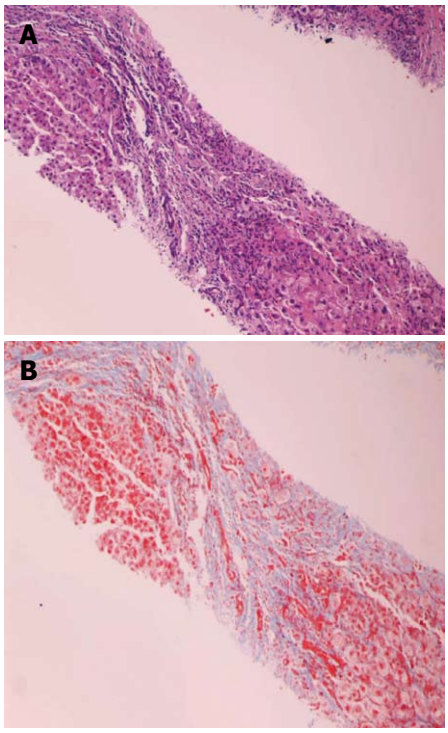

Figure 1 Liver biopsy, low power magnification (100 ×). A: Hematoxylin and eosin stain showing a low power view of two cirrhotic nodule surrounded by fibrosis. The edges of the nodules and the fibrous septi contain intense chronic inflammatory infiltrate; B: Trichrome stain delineating fibrous septa that surround liver nodules in blue color.

Patient B

A 46-year-old African-American male came to clinic with

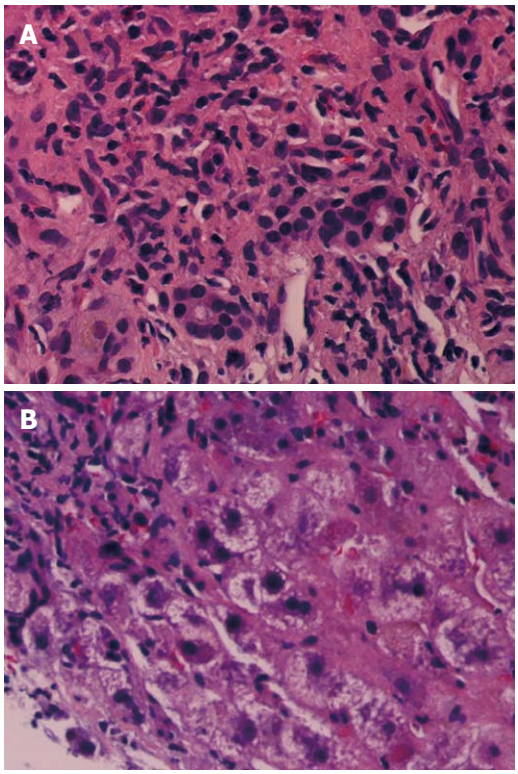

Figure 2 Liver biopsy, high power magnification (hematoxylin and eosin stain, 600 ×). A: Marked portal inflammatory infiltrate composed of plasma cells and a few eosinophils; B: High power view of a cirrhotic nodule composed of markedly swollen hepatocytes, some with granular eosinophilic cytoplasm and intense inflammatory infiltrate at the interface between parenchyma and fibrosis.

a new diagnosis of HIV infection. CD4+ T-cell count was 44 cells/μL (8%) with a plasma HIV RNA level of 9620 copies/mL. He was also diagnosed with hepatitis B infection with HBV DNA level > 500 million IU/mL. Duration of HBV infection was not known but HBV core IgM was negative. HCV antibody was negative, HBeAg was negative, anti-HBe antibody was positive, and HDV antibody was negative. ALT level was normal at 62 U/L and AST was slightly elevated at 53 U/L. He was started on a regimen of once daily ritonavir-boosted darunavir and tenofovir/emtricitabine. Five weeks after starting this regimen, he presented with nausea, vomiting, and jaundice. ALT was 1195 U/L and AST was 1396 U/L. Total bilirubin was 13.5 mg/dL, direct bilirubin was 7.7 mg/dL, and prothrombin time was 27.4 s (INR 2.42). HBV DNA level had decreased to 342000 IU/mL. HIV RNA level was undetectable at < 50 copies/mL and CD4+ T-cell count was 52 cells/μL (10%). HAV IgM was negative and HCV antibody was negative on recheck. Both serum ethanol and acetaminophen levels were undetectable. The patient reported no sexual activity for over 1 year and he never used injectable drugs. Magnetic resonance imaging of the abdomen showed fibrotic changes throughout the liver and portal hypertension evidenced by splenomegaly, a recanalized umbilical vein and minimal perigastric and perisplenic varices. After all medications were held for 72 h, he was restarted on a regimen of ritonavir-boosted fosamprenavir and tenofovir/emtricitabine. Over the next week, his ALT and AST levels trended down to 803 U/L and 773 U/L and his platelets remained within normal limits. However, over that period of time his prothrombin time increased to 42.5 s (INR 3.88) and he became profoundly encephalopathic with an ammonia level of 137 μmol/L. The patient was thought to be too unstable for liver transplantation. After a total of 10 d in the hospital he developed cardiac arrest and died.

DISCUSSION

The development of elevated liver enzymes is not uncommon in HIV-HBV coinfected patients after starting HAART[13]. In the majority of cases, these elevations are mild and do not require modification of treatment[11]. The development of more severe hepatotoxicity (liver enzymes > 10 times the upper limit of normal) is not common and ALF is rare[9]. Among HIV-infected patients in general, those who develop ALF while on HAART have experienced very high rates of mortality[14]. Therefore, an HIV-HBV coinfected patient who develops ALF after starting HAART may be at particularly high risk for mortality, given the presence of underlying liver disease and potentially impaired reserve.

The most appropriate management of such patients is not completely clear, and the optimal management of HIV-HBV coinfected patients who develop liver enzymes > 10 times the upper limit of normal after starting HAART but who do not have evidence of decompensated liver disease may be even more difficult to delineate. One question is whether it is possible to determine if the hepatic injury is from drug toxicity or HBV IRIS and whether this changes management of the patient. Liver biopsy may be helpful in identifying an opportunistic infection, such as mycobacterial disease or cytomegalovirus, that may contribute to liver disease. Liver biopsy may also indicate the presence of cirrhosis. The latter was present in the case of patient A and would have also been found in the case of patient B given radiographic findings. In patient A, liver biopsy showed severe inflammation with lymphocytes, plasma cells, and scant eosinophils. Many cases of HAART-induced ALF in patients without HBV show only mild hepatic inflammatory cell infiltration on biopsy[15]. However, the presence of portal plasma-lymphocytic infiltrate is nonspecific and can be found in chronic viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis and even some medication reactions[16]. The presence of eosinophils may be more specific for a drug reaction[17], though these were scant in this case. Overall, distinguishing between HBV IRIS and drug hepatotoxicity in such cases of ALF may not change management, particularly because it is not clear if anti-inflammatory medications such as corticosteroids are beneficial in HBV IRIS. Corticosteroids have been found to increase HBV replication[18] and this has led some to recommend against the use of corticosteroids in the setting of HBV IRIS[19].

The patients in our series met criteria for ALF according to the American Association for the Study of Liver

Diseases guideline for liver failure[20]. In this guideline, it is recommended that "all non-essential medications" be discontinued in patients with ALF. Due to the fact that the majority of antiretrovirals, including protease inhibitors[8], have been associated with hepatotoxicity, it is advisable to hold HAART at least in the short term. However, HBV flares have been associated with the discontinuation of HAART regimens that contain anti-HBV activity[21]. This theoretically could contribute to ALF. At the same time, continuing single or dual therapy with anti-HBV agents such as lamivudine or tenofovir would create an environment in which HIV resistance might develop. This has led some to recommend stopping HAART and considering treatment of HBV with agents that do not have activity against HIV in cases of significant hepatitis flares on HAART in the setting of hepatitis B cirrhosis[22]. Adefovir is an option, but this drug has less potency against HBV than other agents[23]. Interferon α-based therapies might be considered, but are contraindicated in patients with decompensated cirrhosis. Entecavir was initially thought to have no significant anti-HIV activity, but has subsequently been shown to be associated with the development of HIV resistance mutations when used in the absence of other antiretrovirals[24]. One report suggests that telbivudine may also have activity against HIV[25]. Overall, we believe that the effort to help the patient survive an episode of ALF overrides the preservation of one particular antiretroviral class for future use and that at least one agent with anti-HBV activity should be given in such a scenario. In general, it appears that patients who develop liver failure on HAART, whether coinfected with HBV or not, have a very poor prognosis. These patients should be considered for liver transplantation evaluation, as emerging data show that HIV-HBV coinfected patients have excellent outcomes with liver transplantation[26].

It is prudent to closely monitor the clinical status and liver enzymes of HIV-HBV infected patients after initiation of HAART in order to identify cases of hepatotoxicity early on. Additionally, two new antiretroviral classes have been introduced in recent years. These include the integrase inhibitor class currently represented by raltegravir and the CCR5 antagonist class currently represented by maraviroc. Based on published reports to date, these new agents appear to have minimal hepatotoxicity[27]. While they have only been available since 2007 and toxicities have yet to be fully described, these agents hold promise for HIV-infected patients with chronic liver diseases such as HBV. Perhaps most importantly, efforts should be made to prevent the acquisition of chronic HBV infection, and the HBV vaccine is recommended for all patients with HIV infection[28].

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i32.4112

World J Gastroenterol 2010 August 28; 16(32): 4112-4114
ISSN 1007-9327 (print)

CASE REPORT

Successful recanalization of acute superior mesenteric artery thrombotic occlusion with primary aspiration thrombectomy

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Received: April 12, 2010 Revised: May 16, 2010
Accepted: May 23, 2010
Published online: August 28, 2010

Abstract

Prompt revascularization of the superior mesenteric artery (SMA) thrombotic occlusion can prevent intestinal infarction and decrease necrosis of the bowel segment. Herein, we describe two cases who underwent successful endovascular recanalization for acute SMA thrombosis using a primary aspiration thrombectomy because of possible consequent laparotomy for survey of bowel viability. The two patients had dramatic pain relief immediately after the procedure and remained symptom-free during the follow-up period.

Key words: Superior mesenteric artery; Thrombosis; Aspiration thrombectomy

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Yang HJ, Cho YK, Jo YJ, Jung YY, Choi SA, Lee SH. Successful recanalization of acute superior mesenteric artery thrombotic occlusion with primary aspiration thrombectomy. *World J Gastroenterol* 2010; 16(32): 4112-4114 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i32/4112.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i32.4112

INTRODUCTION

Acute mesenteric ischemia (AMI) is a fatal vascular emergency, representing 1%-2% of acute abdominal emergencies, and develops from a sudden decreased perfusion to the intestines caused by occlusive or non-occlusive impairment of arterial or venous blood flow. The reported overall mortality of AMI is 60%-80%, and its incidence is increasing[1-3]. Superior mesenteric artery (SMA) embolism is the most frequent cause of AMI, which is responsible for approximately 40%-50% of cases[4]. Among the diagnostic tools, conventional angiography shows a complete obstruction of the proximal SMA, 1-2 cm away from the origin of SMA without collateral circulation, which correlates with acute obstruction, and needs relevant treatment[5,6]. We report two cases who underwent successful endovascular thrombolysis of AMI with different methods, despite the presence of early changes in bowel ischemia.

CASE REPORT

Case 1

A 72-year-old man sought evaluation in the emergency room (ER) because of sudden aggravation of abdominal pain and rebound tenderness 3 d ago. He had a 2-year history of diffuse, dull postprandial abdominal pain. The attending physician suspected a surgical abdomen. The patient was on medication for hypertension during the last 16 years. Laboratory data showed no significant abnormalities.An initial abdominal computed tomography (CT) showed a segmental, occlusive acute thromboembolism in the mid-portion of the main stem, and jejunal and colic branches of SMA with circumferential bowel wall

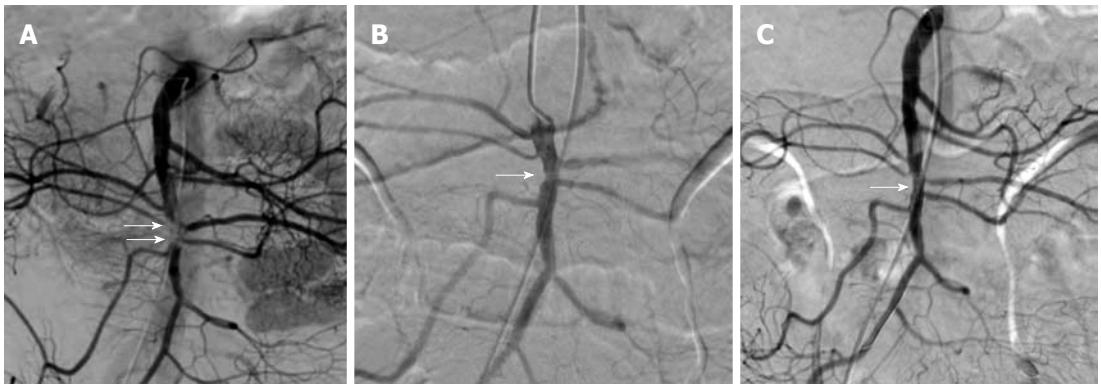

Figure 1 Successful treatment of focal thrombotic occlusion on mid-trunk of superior mesenteric artery with primary aspiration thrombectomy and additional stent implantation. A: Superior mesenteric artery (SMA) angiography showing acute thromboembolism in the mid-trunk of SMA and the origin of jejunal and colic branches (arrows); B: Completely resolved thrombi and focal severe stenosis in the mid-portion of SMA main stem which was considered the leading cause of thromboembolism (arrow) after repetitive primary aspiration thrombectomy using a 6F aspiration catheter; C: A markedly improved stenosis and luminal blood flow of SMA without residual or migrated thrombi after implantation of a 6 mm x 18 mm balloon expandable stent (arrow).

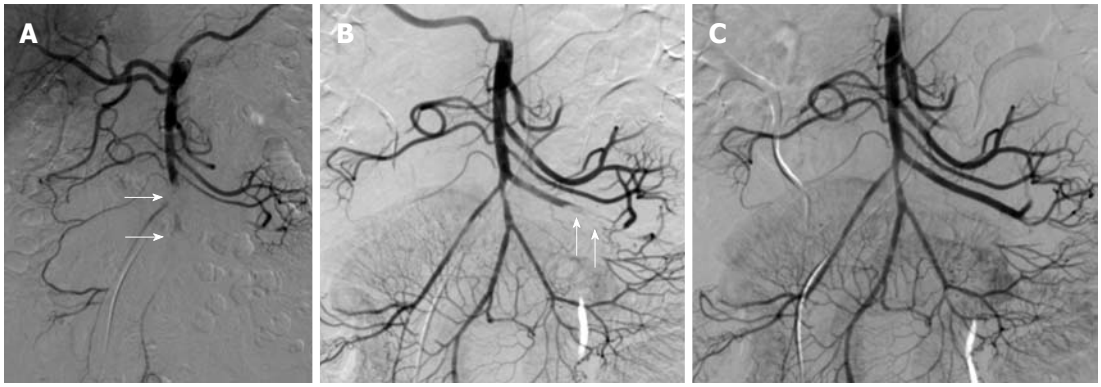

Figure 2 Successful treatment of segmental complete thrombotic occlusion on mid-trunk of superior mesenteric artery with primary aspiration thrombectomy. A: Superior mesenteric artery (SMA) arteriography showing a complete thrombotic occlusion of the mid-portion of the main stem and the origin of ileocolic and right colic branches of SMA due to a large amount of thromboembolism (arrows); B: Complete removal of thromboembolism in the main stem of SMA but an intraluminal filling defect and flow occlusion at the distal portion of jejunal branch of SMA due to migrated thrombi during the procedure (arrows) after multiple courses of aspiration thrombectomy with a 6F aspiration catheter; C: A completely recanalized main stem and jejunal branch of SMA.

thickening and decreased enhancement of mucosal layer in the right colon.

Initial SMA angiography confirmed an acute occlusive thromboembolism in the mid-trunk of SMA and the origin of jejunal and colic branches of SMA (arrows) (Figure 1A). Initially, we performed primary aspiration thrombectomy using a 6F aspiration catheter (Cordis, Johnson and Johnson, San Diego, CA, USA) after IV infusion of 5000 IU heparin. Angiography after repetitive thrombectomy showed nearly complete thrombi removal from the SMA main trunk and its branches and residual focal severe stenosis in the mid-portion of SMA main stem (arrow) (Figure 1B), which was considered the leading cause of thromboembolism. Final angiogram showed completely restored perfusion to the ischemic bowel through the patent main stem, and jejunal and colic branches of SMA after implantation of a 6 mm × 18 mm balloon expandable stent (arrow) (Genesis; Cordis, Johnson and Johnson, San Diego, CA, USA) (Figure 1C). The patient's symptoms improved almost immediately after the operation, and he no longer complained of postprandial abdominal pain. He was instructed to continue taking warfarin after discharge.

Case 2

A 67-year-old man complained of acute abdominal pain 2 d after admission. The patient's symptom was localized at the periumbilical area, and direct tenderness was present at physical examination. The patient had a history of atrial fibrillation and myocardial infarction, which was treated with coronary angioplasty 2 years ago.

Echocardiography showed a 30% ejection fraction and no evidence of thrombi in the left atrium. Laboratory data showed an elevated hs-CRP level of 14.22 mg/dL (normal range, 0-0.3 mg/dL).

Emergent CT angiography showed complete occlusion of the mid-portion of the main stem and origin of the ileocolic and right colic branches of SMA due to thromboembolism, with circumferential bowel wall thickening at the cecum and ascending colon. Surgical laparotomy was later considered because of heart failure.

The initial SMA arteriography showed complete thrombotic occlusion of the mid to distal SMA trunk and the origin of ileocolic and right colic branches of SMA (arrows) (Figure 2A). Initially, we performed primary aspiration thrombectomy using a 6F aspiration catheter (Cordis, John-

son and Johnson, San Diego, CA, USA) after Ⅳ infusion of 5000 IU heparin. After repetitive aspiration thrombectomy, follow-up angiography showed that although most thrombi were removed from the SMA main trunk, small thrombi had a migrated and occluded distal portion of the jejunal branch of SMA during aspiration thrombectomy (arrows) (Figure 2B). Finally, most thrombi in the main stem and jejunal branch of SMA were completely removed immediately after an additional aspiration thrombectomy, and the lumen of SMA was completely recanalized (Figure 2C). After the procedure, the patient's symptoms dramatically improved and the hs-CRP level decreased to 2.2 mg/dL, and he did not have recurrent symptoms during the 12-mo follow-up period.

DISCUSSION

AMI is caused by embolism (40%-50%), SMA thrombosis (20%-25%), non-occlusive mesenteric ischemia (20%), and mesenteric venous thrombosis (5%)[1-4]. Embolic or thrombotic occlusion of SMA frequently occurs on a background of generalized atherosclerotic changes in the involved arteries.

In our two cases, acute mesenteric thrombosis was managed with aspiration thrombectomy without any pharmacologic thrombolysis due to consequent laparoscopic survey to bowel.

Unfortunately, the non-specific symptoms are a frequent cause of delayed diagnosis, and most AMIs and CMIs can be asymptomatic for a long time. Contrast-enhanced CT can shows an arterial occlusion of SMA, frequently accompanied with severe stenosis of the celiac artery, and findings suggestive of bowel ischemia, including pneumatosis intestinalis, bowel wall thickening, ileus, and bowel dilatation[7]. Angiography can define the location and origin of the arterial occlusion and provide the potential for intervention if mesenteric ischemia is diagnosed prior to ischemic bowel necrosis[5,6]. Bloody diarrhea or signs of peritonitis, including abdominal rigidity and rebound tenderness, are signs of advanced bowel ischemia and bowel infarction, requiring urgent surgery. An area of transmural bowel necrosis in AMI can appear within 15 min after onset and after 6 h, and irreversible gangrene of the affected segments may ensue[8]. In our cases, the two patients were diagnosed with AMI by CT examination soon after the onset of symptoms. Although we performed endovascular treatment despite the presence of early ischemic bowel changes, such as bowel wall thickening and engorgement of the vasa recta, on initial or follow-up CT, we obtained good results as reflected by the dramatic improvement in symptoms.

Because of its favorable results, endovascular thrombolysis of acute SMA occlusion has gained popularity and has become one of the most used methods with or without combined open surgery[9]. Pharmacologic thrombolysis with or without mechanical aspiration thrombectomy is a good treatment option in terms of high survival, bowel preservation rates and low complication rate regardless of the nature of thromboembolism, period of occlusion, presence of collateral vessels and severe atherosclerotic disease.

The effect of primary aspiration thrombectomy for acute SMA thrombosis without pharmacologic thrombolysis remains a matter of debate and requires better definition. If consequent laparotomy is considered for assessment of bowel viability and use of anticoagulant is restricted due to a bleeding tendency, aspiration thrombectomy can be performed as the primary procedure. Despite contraindication or strict use of anticoagulant, a small amount of local anticoagulant use is mandatory for prevention of distal migration of small thrombi or recurrent thrombus formation.

Treatment of the underlying stenosis with angioplasty and stent implantation after intra-arterial thrombolysis has been described with at least good short-term results, but the higher restenosis rate of stents during follow-up has been reported in the recent literature[10]. We had a good short-term outcome, despite early ischemic changes, and continued long-term follow-up for recurring symptoms and restenosis of stents with CT angiography.

In conclusion, if the diagnosis of AMI is made early and there are no signs of advanced bowel ischemia, such as peritonitis, endovascular treatment for AMI. Primary aspiration thrombectomy can be performed for rapid and sufficient revascularization of acute thrombotic SMA occlusion in limited cases, consequent laparotomy must be planned to survey for bowel viability or if there is a contraindication to use an anticoagulant.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2010 August 28; 16(32): I
ISSN 1007-9327 (print)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2010 August 28; 16(32): I
ISSN 1007-9327 (print)

Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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World J Gastroenterol 2010 August 28; 16(32): I-IV
ISSN 1007-9327 (print)

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CSSN
ISSN 1007-9327 (print)
CN 14-1219/R

Indexed and Abstracted in
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

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