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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1321 experts in gastroenterology and hepatology from 67 countries.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Epigenetics in hepatocellular carcinoma: An update and future therapy perspectives

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Core tip: Hepatocellular carcinoma (HCC) is a global health concern; molecularly targeted therapeutics remains limited to sorafenib. New targets and drugs are urgently needed to broaden the limited treatment options for HCC. Many lines of evidence suggest that epigenetics is associated with the initiation and development of HCC. Here, we review the current state of knowledge on epigenetic deregulation in HCC, and potential therapies that can be exploited for interventions.

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Abstract

Hepatocellular carcinoma (HCC), the predominant form of adult liver malignancies, is a global health concern. Its dismal prognosis has prompted recent significant advances in the understanding of its etiology and pathogenesis. The deregulation of epigenetic mechanisms, which maintain heritable gene expression changes and chromatin organization, is implicated in the development of multiple cancers, including HCC. This review summarizes the current knowledge of epigenetic mechanisms in the pathogenesis of HCC, with an emphasis on HCC mediated by chronic hepatitis B virus infection. This review also discusses the encouraging outcomes and lessons learnt from epigenetic therapies for hematological and other solid cancers, and highlights the future potential of similar therapies in the treatment of HCC.

INTRODUCTION

Liver cancer is a global health concern. It is the fifth most frequently diagnosed cancer and second most frequent cause of cancer death in men worldwide. Liver cancer is endemic particularly in East Asia and Southeast Asia, where more than half of total cases globally are diagnosed. In addition, Middle Africa, West Africa and South Africa are among the top five regions affected[1]. In the United States, the incidence rate of hepatocellular carcinoma (HCC) (the most common histological subtype of liver cancer) has tripled in the past decade, with the rise being related to increased hepatitis C virus (HCV) infection[2,3]. Risk factors for HCC are well characterized, including chronic hepatitis B virus (HBV) infection, HCV infection, excessive alcohol consumption, diabetes, non-alcoholic fatty liver disease, and dietary exposure to aflatoxin[4].

EPIGENETICS AND HCC

Epigenetics is defined as heritable states of gene expression without altering DNA sequences. Epigenetic mechanisms encompass genomic DNA modifications (methylation of DNA cytosine bases), chemical modifications of histone tails, and non-coding miRNA regulation. During cell division, these epigenetic modifications are passed down faithfully to daughter cells to maintain "cellular memory"[5]. DNA methyltransferases (DNMTs) catalyze the addition of methyl groups (CH3) to the 5' cytosine nucleotides. Mechanistically, DNA methylation leads to transcriptional gene silencing in two ways. First, methylation at CpG sites sterically hinders accessibility of transcription factors to their cognate binding sites on respective gene promoters[6]. The second mechanism involves direct binding of methyl CpG binding domain (MBD)-containing proteins to the methylated DNA, causing transcription repression[7]. Gene silencing mediated by DNA methylation is observed in many cancer types. Cancers often present with features of global hypomethylation; by contrast, promoters of tumor suppressor genes, in particular, are hypermethylated[8]. Several lines of evidence suggest that changes in the epigenome are associated with liver cancer initiation and progression[9].

DNA METHYLATION CHANGES IN HCC

Thus far, three independent genome-wide methylation profiling studies demonstrated that HCC tumors display differential DNA methylome patterns compared to the respective adjacent normal liver tissues[10-12]. Hernandez-Vargas *et al*[10] used a bead array to analyze 1505 CpG sites in 30 patients with either HBV- or HCV-associated HCC, and observed that HCC tumors exhibit specific DNA methylation signatures that are correlated with major risk factors and tumor progression stage, implying potential clinical applications in early diagnosis and prognosis. Specifically, a panel of hypermethylated gene promoters (*APC*, *RASSFIA*, *CDKN2A* and *FZD7*) were able to discriminate HCC tumors from paired surrounding non-tumor liver tissues. Another set of hypermethylated genes (*e.g.*, *NAT2, CSPG2* and *DCC*) were exclusively associated with HBV-related HCC[10]. In particular, promoter methylation of *DNMT1* was found to significantly correlate with poor tumor differentiation. By using the latest Illumina Methylation450 BeadChip, which allowed measurement of DNA methylation levels at 485577 loci across 99% of RefSeq genes (including 96% of the known CpG islands), Song *et al*[11] measured DNA methylation levels in HCC tissues or adjacent normal liver tissues from 27 HCC patients, and found significant enrichment of promoter CpG island DNA methylation loci in the signaling networks of cellular development, gene expression, cell death, and cancer. The genes *BMP4*, *CDKN2A*, *GSTP1,* and *NFATC1* were among the top of the gene list. Shen *et al*[12] carried out a genome-wide methylation study using plasma DNA from a cohort consisting predominantly of HBV^+ (79%) HCC patients, and found that the top five hypermethylated genes were *DAB2IP*, *BMP4*, *ZFP41*, *SPDY1* and *CDKN2A*, whereas the top five hypomethylated genes were *CCL20*, *ATK3*, *SCGB1D1*, *WFDC6* and *PAX4*.

Functional consequences of methylation-mediated silencing of tumor suppressor genes (TSGs) were addressed by recent studies by Nishida *et al*[13] and Revill *et al*[14]. Using combined genome-wide methylation profiling and "epigenetic unmasking" approaches in primary HCC and gene re-expression in cell lines, Revill *et al*[14] narrowed down 13 relevant candidate TSGs to SMPD3 and NEFH. Overexpression of SMPD3 and NEFH led to repression of cell proliferation, with NEFH causing a lesser effect. Nishida *et al*[13] identified a panel of eight TSGs that are highly predictive of progression from chronic HCV infection to HCC. However, the biological functions of these TSGs may vary depending on the cellular context, and their roles in HCC remain to be validated in HCC arising from other risk factors. DNA methylation is therefore a significant mechanism in the silencing of these TSGs.

The above studies provide strong evidence that aberrant gene methylation is closely associated with disease stage and clinical outcome in HCC, and suggest that methylation profiling (in particular, using patient plasma) may be a feasible approach for early diagnosis and prognosis of HCC. However, specific gene methylation signatures remain to be validated.

HISTONE MODIFICATIONS IN HCC

Within the chromosome, DNA is packaged into chromatin where the DNA coils around an octamer of histones. One hundred and forty-five base pairs of DNA are wrapped around the histone octamer, comprising H2A, H2B, H3 and H4, forming the repeating unit of chromatin, the nucleosome[15]. Histone tails protruding out of the nucleosome are targets of post-translational modifications, including acetylation and methylation of lysine (K) and arginine (R) residues, phosphorylation of serine (S) and threonine (T) residues, and ubiquitination of lysine residues[16]. These modifications can turn transcription of genes on or off, and are therefore key players in establishing the gene expression patterns of cells by adjusting the tightness of DNA bound to histones, thereby affecting accessibility of transcription factors[17].

Histone acetylation is controlled by two families of enzymes: histone acetyltransferases (HATs) that "write" the acetyl mark. Acetylation counteracts the positive charge of histones, thereby loosening the tight interaction between histones and DNA. Conversely, histone deacetylases (HDACs) "erase" the acetyl group, resulting in tight coiling of DNA around the histones, leading to the transcriptionally inactive or closed chromatin state[18]. In contrast, histone methylation is associated with either transcriptionally active or closed chromatin, depending on which histone or which lysine residue is modified. For example, histone 3 lysine 27 trimethylation (H3K27me3) is associated with transcriptional repression, whereas trimethylation of lysine 4 of histone

3 is indicative of gene activation[19,20].

Evaluation of histone methylation status in HCC remains limited to correlative studies with clinicopathlogical features of HCC, using semi-quantitative methods of protein detection such as immunohistochemistry or Western blotting. High levels of trimethylated histone H3 lysine 4 (H3K4me3) were correlated with reduced overall survival and poor prognosis in HCC[21]. Another study showed that high levels of H3K27me3 predicted worse prognosis, and were additionally closely correlated with aggressive tumor features, including vascular invasion, large tumor size, multiplicity of tumors, and poor differentiation[22]. Further studies using more precise detection methods, such as ChIP-sequencing, will be required to analyze these specific DNA-protein modifications in order to fully understand their roles in HCC.

EPIGENETIC CHROMATIN MODIFIERS IN HCC

Polycomb-group proteins are chromatin-modifying complexes mediating heritable gene silencing. Polycomb repressive complexes (PRCs) function in the maintenance of cell lineage commitment and stem cell pluripotency. The PRC1 complex comprises the core protein BMI, and RING1A and RING1B, which work as ubiquitin ligases for H2AK119. The other associated protein CBX7 binds to H3K27 *via* its chromodomain[23]. The polycomb repressive complex 2 (PRC2) complex consists of SUZ12, EZH1/2, EED1, and RbAp48. EZH2 is a methyltransferase that mediates gene silencing by trimethylating H3K27[24]. During embryonic stem cell development, the $Suz^{-/-}$ and $Ezh2^{-/-}$ cells exhibit distinct defects during gastrulation. Loss of Suz12 destabilizes Ezh2, causing a global loss of H3K27me3[25].

Elevated expression of EZH2 has been reported in breast[26,27] and prostate[28] cancers. EZH2 mRNA transcript[29] and protein[30,31] levels were consistently elevated in HCC in comparison to non-tumor liver tissues. Specifically, clinicopathological analysis of paired resected tumor and non-tumor tissues showed that high levels of EZH2 were strongly associated with aggressive and metastatic features (including portal vein invasion and lack of tumor encapsulation)[29], and with poor prognosis[30], although no significant differences were observed in either disease free survival[29] or cumulative survival rate between high and low EZH2 expression groups[31].

Detailed mechanistic studies have further elucidated the biological roles of EZH2 in HCC pathogenesis, which support the above clinical correlations. For example, EZH2 was shown to silence WNT antagonists, thereby activating Wnt/β-catenin signaling to promote cancer progression[32]. In contrast, knockdown of EZH2 in liver cancer cell lines reduces the repressive H3K27me3 marker, leading to re-expression of a distinct subpopulation of tumor suppressor miRNAs (miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b), which control motility and adhesion[33]. Another study showed that knockdown of EZH2 profoundly inhibited proliferation of Dlk^{+} hepatic progenitor cells, promoting their differentiation into hepatocytes[34]. Additionally, Wang *et al*[35] reported that c-Myc together with EZH2 silences the tumor suppressive miRNA-101, which in turn targets the PRC2 complex in a double negative feedback loop fashion to account for the overexpression of EZH2 in HCC. Similarly, overexpression of EZH2 resulting from aberrant genomic loss of miR-101 was also reported in prostate cancer[36]. Taken together, these findings show the combined regulatory effects of chromatin-modifying activities and miRNA expression in promoting HCC progression, and provide evidence for an essential role of EZH2 in hepatic progenitor cell homeostasis.

In another study, the role of the viral HBx encoded by HBV was studied for its contribution to hepatocyte transformation. HBx is weakly oncogenic, and essential in the HBV life cycle[37,38]. HBx activates mitogenic pathways and increases polyploid cells (> 4 N), which causes genetic instability[39]. Moreover, HBx activates mitotic polo-like kinase (PLK) 1[40], which likely downregulates the PRC2 component Suz12 *via* phosphorylation. Elevated PLK1 and reduced protein levels of Znf198 and Suz12 are also observed in human HCC cell lines, as well as in liver tumors from X/c-myc bi-transgenic mice and woodchucks infected with the woodchuck hepatitis virus[41]. Importantly, loss of Suz12 results in de-repression of a subset of PRC2 target genes, specifically those with elevated expression in hepatic cancer stem cells, including EpCAM, BAMBI, DKK2, and DLK1[41,42]. These findings suggest that chronic HBV infection may give rise to a small population of cells with hepatic cancer stem cell properties, which ultimately could contribute to the proliferation and progression of HCC.

To summarize, PRC2 subunits SUZ12 and EZH2 have distinct roles during HCC pathogenesis. Overexpression of EZH2 is consistently found in advanced HCC. This elevated expression was associated with late stage features such as invasion and metastasis. In contrast, in the setting of chronic HBV infection, the HBx protein modulates SUZ12 protein levels, thereby maintaining the "stemness" of a subpopulation of hepatocyte stem/progenitor cells. Depending on how the risk factors interact with the host DNA and epigenetic players, each specific epigenetic modifier component may play a distinct role at different HCC stages.

MICRORNAS IN HCC

MicroRNAs (miRNAs) are non-coding small RNA (ncRNA) molecules that are 20-23 nucleotides in length. They play important regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression. More than 1000 miRNAs have been identified to date. Through their roles in post-transcriptional gene regulation, miRNAs regulate diverse cellular functions including proliferation, differentiation, apoptosis, cell fate, and plasticity[43]. Pri-miRNAs are transcribed by RNA

polymerase Ⅱ either from their own gene or are located in introns of protein-coding genes. The pri-miRNA is then cleaved in the nucleus to form an approximately 60-70 nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA, which is further exported from the nucleus to the cytoplasm by Ran-GTP and the export receptor exportin-5. In the cytoplasm, further processing by Dicer, another RNase Ⅲ endonuclease, generates the 5' phosphate and approximately 2 nt 3' overhang characteristic of an RNase Ⅲ and produces an siRNA-like imperfect duplex that comprises the mature miRNA. One strand of the duplex is incorporated into the RNA-induced silencing complex, forming a complementary complex with the 3'-untranslated region of the target mRNA, and resulting in mRNA degradation or inhibition of mRNA translation, and hence gene expression silencing[44].

It has been increasingly recognized that aberrant miRNA expression profiles are linked to liver cancer development and progression[45]. MiRNAs play a role in virus-host interaction, and provide an anti-viral defense mechanism, such as against the HCV and the primate foamy virus type 1[46,47]. In a miRNA library screen, miR-141 was shown to repress HBV expression and replication in HepG2 cells, *via* direct suppression of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α. PPAR-α regulates HBV gene expression through interactions with HBV promoter regulatory elements[48]. Another miRNA of interest is miR-122, the most abundant miRNA expressed in the liver, making up as much as 72% of total liver miRNAs[49]. Interestingly, miR-122 exerts opposite functions in HBV and HCV replication[50,51]. It can bind directly to a region of the HBV pre-genomic RNA and negatively regulates HBV replication. An inverse correlation was observed between miR-122 and HBV genome copies in peripheral blood mononuclear cells obtained from HBV^+ samples[50]. On the other hand, miR-122 is essential for the stability, propagation and replication of HCV RNA[51]. Indeed, an antisense oligonucleotide (Miravirsen) with locked nucleic acid (LNA)-modified DNA that sequesters mature miR-122 has shown high efficacy in reducing HCV RNA levels in human clinical trials[52]. However, miR-122 is frequently downregulated in HCC[53,54], suggesting its tumor suppressor role[55]; therefore, attempts to restore miR-122 expression may inhibit HCC, or theoretically promote HCV replication and HCC, and have to be carefully considered depending on the viral status of HCC patients.

In contrast to miR-122, miR-1 over-expression increases HBV replication *via* induction of the hepatic nuclear receptor farnesoid X receptor, which then enhances transcription of the HBV core protein. This is accompanied by cell cycle arrest in G_1 phase and differentiation of hepatocytes, thereby providing a favorable environment for HBV replication[56]. Furthermore, in cultured liver cancer cells, the HBx protein represses p53-mediated expression of miRNA-148a, which in turn down-regulates the hematopoietic pre-B cell leukemia transcription factor-interacting protein (HPIP). In HBV-mediated HCC, expression of miR-148a is reduced, whereas that of HPIP is elevated. These observations support that down-regulation of miR-148a has a role in liver cancer pathogenesis[57]. Other miRNAs with reported roles in HCC are listed in Table 1, and are discussed in other comprehensive reviews[58,59].

MIRNAS AS TUMOR SUPPRESSORS IN HCC

Profiling of differentially expressed miRNAs and their targets at different disease stages also suggests that miRNAs are associated with disease pathogenesis. In HBV-associated HCC, using TaqMan low-density miRNA arrays, Wang *et al*[60] found that miR-138 and miR-199a-5p expression was deregulated. In several studies, miR-138 levels were down-regulated in HCC tissues. MiR-138 exerts tumor suppressor function by directly targeting cyclin D3 and resulting in cell cycle arrest. It was also demonstrated that the use of a miR-138 mimic significantly reduced xenograft tumor growth in nude mice[61]. Differential expression levels of miR-199a-5p were also reported in HCC tissues and cell lines[62,63]. In particular, miR-199a-5p inhibits cell invasion by targeting discoidin domain receptor 1, a tyrosine kinase involved in signaling pathways that mediate cell invasion[62].

In a cohort of HCC patients characterized with a background of HBV infection (approximately 90%), miR-26a and miR-26b expression in non-liver tissues was higher in women than in men, suggesting that they have a protective role[64]. Moreover, miR-26a and miR-26b expression was down-regulated in tumors compared to paired non-tumor tissues. Patients with low miR-26 expression were associated with shorter survival, but were more likely to respond to interferon α therapy, making it an ideal candidate for predicting therapy response[64]. Additional miRNAs with a role in HCC are described below. Another miRNA that is suppressed in human liver cancer is miR-125b, which possesses tumor-suppressive functions such as arresting cell cycle progression, and inhibiting migration and invasion by directly targeting the oncogene LIN28B2[65]. MiR-140-5p expression is also decreased in HCC as well as six HCC cell lines. MiR-140-5p suppresses tumor growth and metastasis by targeting TGFBR1 and FGF9[66]. Genome-wide miRNA and mRNA profiling during mouse liver development implicates miR-302b and miR-20a in repressing transforming growth factor-β signaling[67]. MiR-122, in addition to its anti-HBV role, also functions as a tumor suppressor. Mice lacking the gene encoding miR-122a are viable but develop temporally controlled steatohepatitis, fibrosis, and HCC[68]. Restoration of miR-122 in a hepatic cell line reverses its migration and invasion properties[69].

MIRNAS AND LIVER CANCER-INITIATING CELLS

The hypothesis of liver cancer stem cells (LCSCs) is sup-

Table 1 MicroRNAs in hepatocellular carcinoma and their characteristics

Role	miRNA	Characteristics	Ref.
Viral replication	miR-141	Represses HBV expression and replication	[48]
	miR-122	Inhibits HBV replication	[50]
	miR-122	HCV RNA stabilization, propagation and replication	[51]
	miR-1	Increases HBV replication	[56]
Tumor supressor	miR-138	Down-regulated in HCC tissues. miR-138 can directly target cyclin D3	[61]
	miR-26a and miR-26b	Down-regulated in tumors compared to paired non-tumor tissues	[64]
	miR-125b	Arrests cell cycle progression, and inhibits migration and invasion by directly targeting the oncogene LIN28B2	[65]
	miR-140-5p	Suppresses tumor growth and metastasis by targeting TGFBR1 and FGF9	[66]
	miR-122a	Mice lacking the gene encoding miR-122a are viable but develop temporally controlled steatohepatitis, fibrosis, and HCC	[68]
Target tumor initiating cells	miR-181	Maintains the stemness of liver cancer stem cells, target liver differentiation transcription factors CDX2 and GATA6	[78]
	miR-150	Overexpression led to reduction of $CD133^+$ cells	[80]
	miR-548c-5p	Ectopic overexpression inhibited proliferation, migration, and invasion of $CD90^+$ HepG2 cells by down-regulating the expressions of β-catenin, Bcl-2, Tg737, Bcl-XL, and caspase 3	[81]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; miRNAs: MicroRNAs.

ported by the identification of subpopulations of cancer cells with antigenic markers, which contribute to cancer origin and chemoresistance[70,71]. To date, the three main LCSC markers are epithelial cell adhesion molecule (EpCAM)[72,73], CD133[74,75] and CD90[76,77]. Cells bearing these markers demonstrate cancer stem cell properties including: (1) ability to form tumorspheres in anchorage-independent assays; (2) ability to initiate tumor growth *in vitro* and *in vivo*; and (3) ability to self-renew. MiR-181 is highly expressed in EpCAM-positive HCC cells isolated from α-fetoprotein-positive tumors, and is important in maintaining the stemness of LCSCs by directly targeting hepatic differentiation transcription factors CDX2 and GATA6[78]. MiR-181 levels are transcriptionally induced by the Wnt/beta-catenin signaling pathway[79]. In addition, over-expression of miR-150 leads to significant reduction of $CD133^+$ cells, as well as inhibition of cell proliferation and tumorsphere formation[80]. MiR-548c-5p, miR-198, miR-375, and miR-874 levels are decreased, whereas miR-155, miR-198, and miR-1289 levels are increased in $CD90^+$ in comparison to $CD90^-$ HepG2 cells. Transfection with exogenous miR-548c-5p inhibited proliferation, migration, and invasion of $CD90^+$ HepG2 cells by down-regulating the expressions of β-catenin, Bcl-2, Tg737, Bcl-XL, and caspase 3[81].

LONG NON-CODING RNAS IN HCC

Long non-coding RNAs (lncRNA) are transcripts longer than 200 nt, constituting a subpopulation of ncRNAs. They exert molecular regulatory functions *via* diverse modes of mechanisms[82]. Accumulating evidence indicates that lncRNAs are implicated in many cellular functions and play a role in carcinogenesis of multiple cancer types[83]. It has been shown that 20% of lncRNAs are associated with PRC2, through which they recruit and guide chromatin modifying complexes to specific genomic regions to regulate gene expression[84]. They behave like transcription co-activators/repressors by directly binding with various interaction partners; for example, lncRNA *TERRA* can directly bind to human telomerase and inhibit telomerase activity[85]. Alternatively, they act as decoys competing for miRNAs to modulate the expression of target genes[86].

LncRNA *HULC* (highly up-regulated in liver cancer) was the first lncRNA with highly specific up-regulation detected in the blood of HCC patients[87]. Du *et al*[88] further revealed that HBx could regulate the promoter of HULC to promote hepatoma cell proliferation *via* down-regulating the tumor suppressor p18. HBx was also found to downregulate an lncRNA termed lncRNA-Dreh, which can inhibit HCC growth and metastasis *in vitro* and *in vivo*, and acts as a tumor suppressor in the development of HBV-HCC[89]. The downregulation of lncRNA-Dreh additionally correlated with poor survival of HCC patients.

LncRNA MALAT-1 (metastasis-associated lung adenocarcinoma transcript 1) was initially reported to be closely associated with non-small cell lung cancer (NSCLC) metastasis[90,91]. Likewise, lncRNA MALAT-1[92] and lncRNA HOTAIR (HOX antisense intergenic RNA)[93] have been shown to be upregulated in large cohorts of HCC patients. In particular, lncRNA HOTAIR was implicated as a prognostic biomarker for tumor recurrence after liver transplantation. Both studies demonstrated that siRNA-mediated reduction of lncRNA MALAT-1 and lncRNA HOTAIR suppressed cell viability and cell invasion, sensitized tumor necrosis factor (TNF)-α induced apoptosis, and increased the chemotherapeutic sensitivity of cancer cells to cisplatin and doxorubicin[93]. LncRNA MVIH (microvascular invasion in HCC) is elevated in HCC, and as its name suggests, was associated with microvascular invasion in HCC[94]. Another lncRNA overexpressed in HCC, lncRNA HEIH (high expression in HCC), was found to be significantly associated with recurrence and is an independent prognostic factor for survival[95]. Additionally, lncRNA-HEIH was found to physically interact with

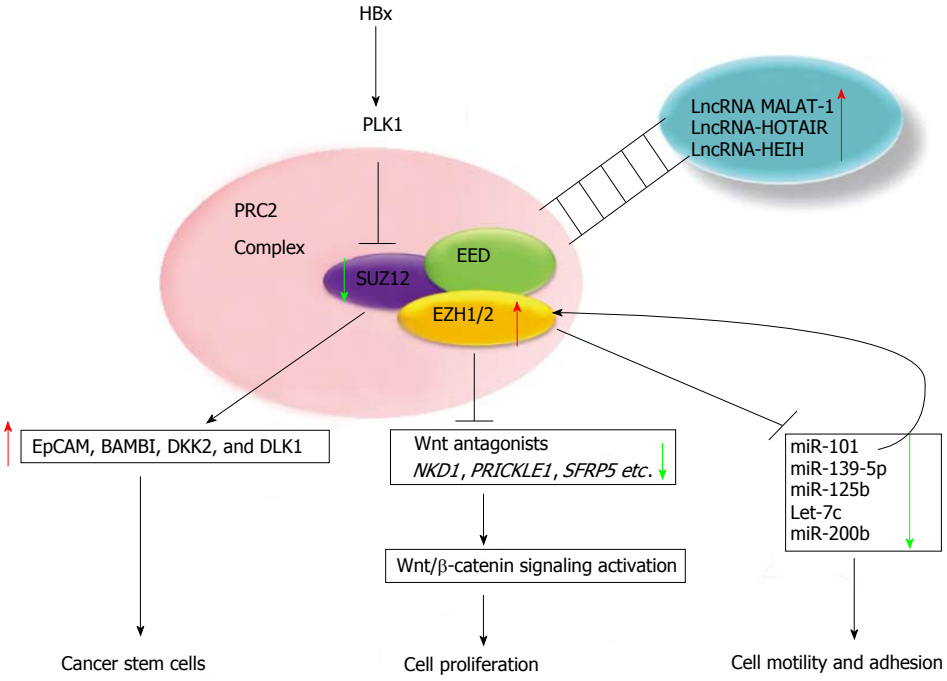

Figure 1 Epigenetic regulatory networks in hepatocellular carcinoma. The diagram depicts the composition of polycomb repressive complex 2 (PRC2), which consists of core components EZH1/2, EED, and SUZ12. The PRC2 complex intersects multiple layers of epigenetic regulators to maintain cancer stem cell features, cell proliferation, cell motility and adhesion. The red arrows indicate upregulation of genes, and the green arrows indicate downregulation of genes. LncRNA: Long non-coding RNAs.

EZH2 - an interaction which is fundamental for repressing target genes such as p16 (lncRNA-HEIH can enhance the binding of EZH2 and H3K27me3 levels across the p16 promoters)[95].

Given the key regulatory functions of lncRNAs in cancers including HCC, and the evidence of dysregulated lncRNA expression in HCC, the targeting of lncRNAs offers a novel exciting opportunity to treat HCC. In principle, targeting of lncRNA can be achieved using the following approaches: (1) siRNA-mediated silencing; (2) functional block using small molecules or oligonucleotide inhibitors to prevent interactions of lncRNAs with proteins such as PRC2; and (3) structure disruption using small molecules or oligonucleotide inhibitors to change or mimic their secondary structure to compete for their binding partners. Since targeting lcnRNAs is still in its infancy, no investigational agents are currently available[96]. The complex epigenetic regulatory networks in HCC are summarized in Figure 1.

CHALLENGES IN HCC MANAGEMENT

HCC is a heterogeneous disease whose management requires a multidisciplinary approach. One of the major clinical challenges is the inability to detect HCC at its early stages; patients are often diagnosed at advanced stages, which limits therapeutic options and leads to poor prognosis and unfavorable outcome[97]. Current treatment strategies for HCC include: (1) surgical removal of the tumor and liver transplantation; (2) minimal invasive surgery with application of radiofrequency ablation or cryoablation; and (3) chemoembolization by injecting drugs directly into the liver[98]. However, these approaches are limited by shortage of organ donors, small percentage of patients suitable for surgical removal, high post-operative recurrence rate, and underlying complications such as cirrhosis, HBV and HCV infections[99].

To date, only a number of molecularly-based therapeutics are available in the clinical management of HCC. In 2007, sorafenib (Nexavar) was approved by the United States Food and Drug Administration (FDA) for treatment of advanced primary HCC. This multi-tyrosine kinase inhibitor works by interfering with vascular endothelial growth factor signaling pathways in tumor angiogenesis. Clinical trials showed modest prolonged median survival and time to progression of 3 mo[100]. Similarly, in a cohort of patients from the Asia-Pacific region, the median overall survival increased from 4.2 to 6.5 mo[101]. Ongoing clinical trials are underway to test the efficacy of sorafenib in combination with other drugs[102]. Studies are also in progress for other drugs targeting the epidermal growth factor receptor, hepatocyte growth factor/c-Met, platelet-derived growth factor receptor, and mammalian target of rapamycin, all involved in molecular pathways of growth[103]. These drugs all show variable outcomes in the treatment of HCC.

EPIGENETIC DRUGS FOR TREATING HEMATOLOGICAL CANCERS

In contrast to conventional or molecularly-targeted therapies for inhibiting dysregulated genes or signaling pathways, epigenetic drugs provide an alternative approach by reversing the methylation status and histone modi-

Table 2 List of drugs targeting epigenetic modifications in hepatocellular carcinoma

Epigenetic modification	Targets	Drug(s)	Cell line/Animal model/Clinical trial phase	Results	Ref.
DNA methylation	DNA methyltransferase	Zebularine	Huh7 and KMCH cell lines Human xenograft models	Zebularine-sensitive cell lines (Huh7 and KMCH) showed preferential demethylation of genes for tumor suppression, apoptosis, and cell cycle regulation *In vivo* inhibition of tumor growth in xenograft model	[110]
	DNA methyltransferases	Zebularine	HepG2 cell line	Zebularine treatment inhibited cell proliferation and induced apoptosis in HepG2 cell line	[109]
	DNA methyltransferases	5-aza-2′-deoxycytidine	MMC-7721 and HepG2 cell lines	Inhibited telomerase activity, accompanied by reactivation of p16 and c-Myc. DAC synergized with cisplatin on growth inhibition	[111]
Histone acetylation	Histone deacetylase	Belinostat	PLC/PRF/5, Hep3B and HepG2 cell lines	Inhibited cell growth and induced apoptosis	[112]
	Histone deacetylase	Belinostat	Multi-center phase Ⅰ/Ⅱ clinical trial	Stabilized tumor in non-resectable advanced HCC	[115]
	Histone deacetylase	Suberoylanilide hydroxamic acid	HepG2, Hep3B and SK- Hep1 cell lines	Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis	[114]
Combination	Histone deacetylase + tyrosine kinase-inhibitors	Panobinostat + sorafinib	Huh7, Hep3B and HepG2 cell lines HCC xenograft model	Induction of apoptosis Combined panobinostat and sorafenib decreased vessel density, tumor volume and increased survival in HCC xenografts	[124]

HCC: Hepatocellular carcinoma.

fications of aberrantly expressed genes[104]. There are currently four FDA-approved epigenetic drugs, including two DNMT inhibitors, 5-azacytidine and decitabine, and two HDAC inhibitors, vorinostat and valporic acid. These drugs have been successful in treating hematological cancers, specifically myelodysplastic syndrome, a blood cancer characterized by inability to generate blood cells in the bone marrow[105]. Interestingly, low doses of 5-azacytidine and decitabine show anti-tumor effects on cultured and primary leukemia cells[106,107], as well as primary cells isolated from luminal-type breast cancer[106]. More importantly, they can reduce the number of $CD34^+$ stem cells in leukemia, and mammosphere-forming breast cancer cells. Importantly, $CD34^+$ cells are the origin and cause of tumor recurrence and chemoresistance[108].

DNA METHYLATION INHIBITORS FOR TREATING HCC

Studies using cell lines[109] and pre-clinical mouse models[110] reveal promising results that may open up new avenues for the intervention and management of HCC. Drugs targeting epigenetic changes in HCC are summarized in Table 2. Andersen *et al*[110] showed that treatment with the DNMT inhibitor zebularine can prevent and effectively inhibit tumor growth in xenograft mouse models that are sensitive to this drug. Zebularine-resistant cell lines, however, showed up-regulation of oncogenic activities that instead promote liver cancer growth. These findings suggest that this drug may only benefit a specific sub-population of HCC patients. Treatment with zebularine was also shown to inhibit cell proliferation and to induce apoptosis of the HepG2 cell line. However, the DNA methylation levels of tumor suppressor genes *p53* and *p21* were not affected by zebularine treatment, while the anti-apoptotic protein BCL-2 is down-regulated, indicating that a DNA-methylation-independent pathway exists for increased p53 and p21 protein levels[109]. Additional cell-based studies show that 5-aza-2'-deoxycytidine exerts anti-tumor effects by inhibiting telomerase activity, accompanied by reactivation of p16 and c-Myc expression[111].

HDAC INHIBITORS FOR TREATMENT OF HCC

The use of HDAC inhibitors in HCC has been investigated in preclinical and clinical studies. In preclinical studies, belinostat inhibited cell growth in a HCC cell line[112], while suberoylanilide hydroxamic acid sensitized HCC cells to acetylation of p53[113] and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[114]. In a multi-center phase Ⅰ/Ⅱ clinical trial, belinostat was found to stabilize unresectable advanced HCC[115]. Another important aspect of this clinical trial is the identification of HR23B as a potential biomarker for predicting the response to belinostat[115]. The combination of suberoylanilide hydroxamic and dihydroartemisinin significantly halted the growth of liver cancer tumor xenografts[116]. Of note, treatment with HDAC inhibitors was observed to induce cell death while simultaneously activating tumor-progression genes[117]. These observa-

tions indicate that a more in-depth understanding of epigenetic mechanisms is needed to obtain further insights into the *in vivo* determinants of responses to epigenetic drugs.

A recent addition to the family of epigenetic drugs is GSK-1, the first chemical inhibitor synthesized for targeting histone demethylases JMJD3/UTX (KDM6A/KDM6B) in a selective and potent manner[118]. These histone demethylases (JMJD3/UTX) catalyze removal of the trimethylation marker of H3K27. JMJD3 is induced *via* nuclear factor-κB exclusively in macrophages upon lipopolysaccharide (LPS) stimulation, providing an important link between inflammation and epigenetic reprogramming[119]. This small molecule specifically inhibits the LPS-induced pro-inflammatory response by sustaining the repressive effect of H3K27 on the TNF-α gene, as well as by inhibiting recruitment of RNA polymerase Ⅱ during transcription, thereby reducing TNF-α expression[118]. Chronic inflammation due to chronic HBV and HCV infection, or obesity, gives rise to liver injury that slowly progresses to HCC, with elevated levels of pro-inflammatory cytokines TNF-α and interleukin (IL)-6 in HCC[120,121]. By suppressing TNF-α expression, GSK-1 may serve as a potential candidate drug for HCC.

MIRNA-BASED TREATMENT OF HCC

MiRNAs with tumor suppressor roles in HCC, such as miR-26a, are feasible anti-cancer agents. Specifically, reduced expression levels of miR-26a have been reported in human HCCs[64], whereas systemic restoration of miR-26a expression by an adeno-associated virus suppresses cancer cell proliferation in a liver-specific Myc transgenic mouse model[122]. MiR-26a exerts these effects by directly targeting and down-regulating cyclins D2 and E2, consequently inducing cell cycle arrest. Importantly, as reported in that study, the cytotoxic effect of this miRNA is minimal in major organs. In another mouse model, in which liver cancer was induced by administration of the chemical hepatocarcinogen diethylnitrosamine, restoration of miR-124 by systemic injection significantly reduced liver tumor size[123]. Likewise, no cytotoxic effects on vital organs were detected.

COMBINATION OF EPIGENETIC DRUGS WITH EXISTING THERAPEUTIC MODALITIES IN HCC

Aberrant expression of several HDACs and copy number gains of HDAC3 and HDAC5 were detected in HCC patients[124], providing the rationale for treating HCC with HDAC inhibitors. In preclinical models of HCC, the combination of the pan-HDAC inhibitor panobinostat and sorafenib significantly decreased tumor growth and improved survival in murine xenograft models, compared to either drug used alone. Detailed molecular mechanisms include induction of apoptosis, acetylation of histone 3, down-regulation of BIRC5, or up-regulation of CDH1. This observation implies that such combination treatment may also achieve favorable clinical outcome for HCC patients.

Besides reversing aberrant epigenetic modifications in diseased conditions, epigenetic drugs can also induce host immunogenicity by increasing tumor antigen presentation. Systemic administration of the DNMT inhibitor 5-aza-2'-deoxycytidine induces the cancer/testis antigen (CTA) and augments adoptive immunotherapy by making cancer cells more visible to immunotherapy[125]. Under the inflammatory microenvironment of melanoma, melanocytes exhibit cell plasticity by converting between differentiated and undifferentiated status caused by TNF-α. Gradually, cells acquire resistance to adoptive cell transfer therapy[126]. The immunomodulatory activity of 5-aza-2'-deoxycytidine *in vivo* suggests its clinical use to design novel strategies of CTA-based chemo-immunotherapy for melanoma patients[127]. Such a concept may also be extended to the treatment of HCC, especially since HCC routinely occurs on a background of inflammation resulting from chronic HBV or HCV infection, where increased levels of IL-6[128] and TNF-α[121] are readily detected.

CONCLUSION

Like other types of cancers, HCC is associated with multiple genetic mutations and epigenetic aberrations. Whereas gene mutations are not easily amenable for therapy, epigenetic aberrations that appear frequently in HCC may serve as new targets[129]. To date, global DNA hypomethylation, promoter methylation, aberrant expression of miRNAs, and dysregulated expression of other epigenetic regulatory genes such as EZH2 are the best-known epigenetic abnormalities in HCC. Epigenetic drugs targeting these abnormalities may reverse the expression of dysregulated genes that are important in apoptosis and cell cycle arrest, thereby controlling the development and/or progression of HCC. Concerns of using epigenetic drugs are their off-target effects, which may activate genes having the opposite effects. Detailed mechanisms of action should be further investigated in multiple cancer types. Future research should aim at understanding how best to identify patient groups that would benefit most from the prescribed therapy, depending on the clinical setting, for example, disease stage, disease background, which drugs will give the best outcome, and when they should be administered. Useful biomarker(s) should provide guidance in identifying patients who may have optimal responses and reduced likelihood of relapse. Last but not least, most epigenetic drugs are used mainly in advanced tumors. Whether these drugs can be used as a preventative measure or in high risk patients who may benefit from future therapy remains to be explored.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Complementary and alternative medicines in irritable bowel syndrome: An integrative view

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Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with a high incidence in the general population. The diagnosis of IBS is mainly based on exclusion of other intestinal conditions through the absence of inflammatory markers and specific antigens. The current pharmacological treatment approaches available focus on reducing symptom severity while often limiting quality of life because of significant side effects. This has led to an effectiveness gap for IBS patients that seek further relief to increase their quality of life. Complementary and alternative medicines (CAM) have been associated with a higher degree of symptom management and quality of life in IBS patients. Over the past decade, a number of important clinical trials have shown that specific herbal therapies (peppermint oil and Iberogast®), hypnotherapy, cognitive behavior therapy, acupuncture, and yoga present with improved treatment outcomes in IBS patients. We propose an integrative approach to treating the diverse symptoms of IBS by combining the benefits of and need for pharmacotherapy with known CAM therapies to provide IBS patients with the best treatment outcome achievable. Initial steps in this direction are already being considered with an increasing number of practitioners recommending CAM therapies to their patients if pharmacotherapy alone does not alleviate symptoms sufficiently.

Key words: Irritable bowel syndrome; Complementary and alternative medicine; Hypnotherapy; Cognitive behavioral therapy; Herbal therapy; Peppermint

Core tip: Irritable bowel syndrome is a prevalent gastrointestinal disorder that interferes with daily living in 5%-20% of the population. The current review summarizes the most widely used complementary and alternative medicine (CAM) approaches that have proven to be effective and have been endorsed by professional organizations. The review encourages the use of both pharmacotherapy and CAM approaches in an integrative setting to provide the best outcome and quality of life to patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is among the most common gastrointestinal disorders with a prevalence ranging from 5%-20% in the general population worldwide[1,2]. IBS is more commonly diagnosed in women than in men and in people younger than 50 years[1,3,4]. The high prevalence of diagnosis also results in a significant so-

cioeconomic burden through decreased work productivity, increased direct and indirect healthcare costs, and - depending on the severity - a reduction in quality of life for IBS patients and their caregivers[5-8]. The estimated indirect and direct healthcare costs related to IBS in the United States have been steadily increasing and amount to $1.35 billion dollar as of 2003[9]. The worldwide health costs associated with IBS are estimated to exceed $200 billion United States dollars[10]. The International Classification of Diseases (ICD) of the World Health Organization in its latest revision, ICD-10, classifies IBS as a functional digestive disorder with the ICD-10 classification 58.9 with sub-classifications as irritable colon or spastic colon[11]. This classification does not distinguish between the Rome-Ⅲ criteria and the consensus of many professional medical organizations that have divided IBS into four different subgroups based on the primary symptom presentation as constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), mixed or alternating IBS, and unspecified IBS[2,12]. The diagnosis of IBS is mainly dependent on the absence of pathophysiological and morphological indicators and therefore remains an exclusion diagnosis concentrated on symptom presentation[13]. There have been indications in recent research studies that IBS may be the result of a low-grade inflammatory process within the lower intestinal tract but definitive and validated biochemical markers have not emerged as of yet[14-17]. There also remains a gap in our understanding of the underlying pathophysiology and what causes IBS. A few hypotheses have linked genetic predisposition, post-infectious small bowel bacterial overgrowth, and certain diets with a higher incidence for developing IBS[18-20]. However, a unified understanding of the pathophysiology that may result in a feasible and causal treatment approach has not emerged. In defense of this deficit, similar knowledge gaps exist for a wide range of conditions for which symptomatic treatment to date provides the only therapeutic approach.

Because current pharmacological treatment approaches for IBS are solely based on symptom reduction, many patients remain undertreated and dissatisfied with their quality of life. In addition, many pharmacological treatment approaches are associated with side effects that result in a smaller benefit to the patient in terms of treatment outcomes[18,19]. The treatment also depends on the specific subtype of IBS. While IBS-C patients mainly suffer from abdominal pain because of slow bowel movement and less frequent bowel release, patients with IBS-D suffer from a social stigma due to the frequent bowel release that requires a bathroom in close proximity as well as bloating and increased flatulence[8,19,21,22]. Comorbidities between IBS with depressive and anxiety disorders have been well defined although it remains unknown which of these is the cause and the effect[23-26]. A common treatment for all subtypes of IBS are antidepressants which may indicate that certain depressive and anxiety disorders play a role in the pathophysiology of IBS[25,27-29]. Another emerging field of research is the investigation of the gut-brain axis also referred to as the enteric nervous system (ENS). It has been established that interconnected sensing of afferent and efferent neurons in the ENS influences gut motility based mainly on serotonergic and cholinergic nerve innervations[30,31]. The 2 major serotonin receptors present in the intestinal tract, 5-HT_3 and 5-HT_4, and a serotonin reuptake transporter are either differently expressed or even present with mutations in certain IBS populations[27,28,32-34]. This correlates well with the current pharmacological treatment approaches of using 5-HT_3 receptor antagonists and 5-HT_4 receptor agonists in IBS patients to reduce both visceral pain perception and regulate gastrointestinal motility[35-37]. Considering that the neurotransmitter and hormone serotonin is involved in both intestinal motility and mood regulation may serve as an indicator that changes in the ENS neurotransmission are involved in the comorbidity between IBS with depressive and anxiety disorders.

As mentioned, current pharmacological treatment approaches provide limited symptomatic relief to IBS patients. This has resulted in a significant increase in self-medication and the use of complementary and alternative medicines (CAM) by patients and even healthcare providers to bridge the gap and increase quality of life[38-42]. This review will summarize the current knowledge of CAM alone and in conjunction with pharmacological treatments as an integrative approach to manage patients with IBS and improve their quality of life. Although the review is not comprehensive in addressing all aspects of CAM and integrative medical approaches to treating IBS, it is intended to provide practitioners with the most commonly used and most widely recommended CAM approaches that have shown repeated success in clinical trials over the past decades. It is important to point out that pharmacological treatment should not be abandoned by patients and their providers in lieu of CAM approaches but rather an integrative approach considered that provides both maximum relief of symptoms and increased quality of life.

LITERATURE SEARCH

This article reviews current research regarding the most commonly used CAM therapies for IBS in the United States, which are single or combination herbal products, acupuncture, yoga, hypnotherapy, and cognitive behavioral therapy. The literature search covered the period from January 1996 to June 2013 using Medline and PubMed with the search terms "irritable bowel syndrome" in combination with "yoga", "hypnotherapy", "cognitive behavioral therapy", "CBT", "CAM", "acupuncture", "herbal therapy", and "integrative medicine". Out of 714 total articles retrieved, 243 were excluded because they were reviews or protocols, 215 were not in English, and 102 were duplicates or did not relate to IBS. A total of 154 articles were selected for inclusion in this review (Figure 1).

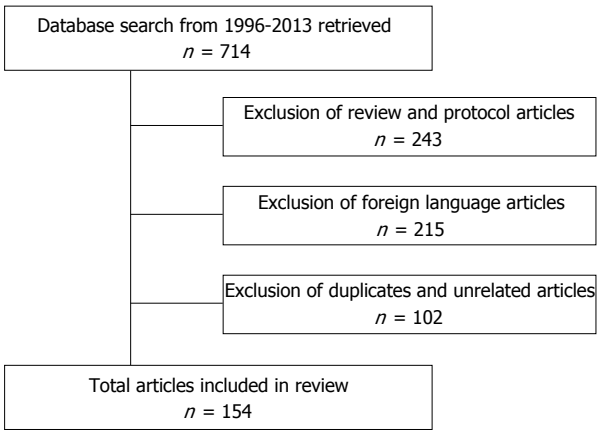

Figure 1 Flow chart illustrating the selection criteria for inclusion of articles.

PHARMACOLOGICAL TREATMENT APPROACHES

The current approach to treating IBS is symptomatic and consists of a regimen of first line pharmacological treatment options often coupled with lifestyle adjustments followed by potential off-label use of a number of other medications that are not specifically indicated for IBS if symptom management is insufficient[2,20,43]. The current leading guidelines have been developed by the Task Force on Irritable Bowel Syndrome of the American College of Gastroenterology (ACG) and the British Society of Gastroenterology[2,12]. Both associations recognize that symptomatic treatment of IBS is associated with a significant placebo effect which has been confirmed in a number of studies as well as in a now well-known unblinded study where patients were told that they were receiving placebo and still showed significant improvements in symptoms[44]. These findings support the hypothesis of an underlying connection between the brain and the gut and the potential interplay of emotions and mood disorders affecting the severity of IBS symptoms.

Considering the increased incidence of comorbid depressive and anxiety disorders in patients with IBS, the use of antidepressants, mainly tricyclic (TCA) and selective serotonin reuptake inhibitor (SSRI) antidepressants, have shown improvements in IBS symptoms and there seems to be an indication that such medication may also provide symptom relief in IBS patients without comorbid psychiatric disorders[45-48]. The effectiveness of low dose TCA and SSRI antidepressants as well as benzodiazepines is yet another indication that the enteric nervous system is influenced by mood and that this in turn affects the innervation by serotonergic and cholinergic neurons in the gastrointestinal (GI) tract[49,50].

The initial step in treating IBS is to consider supportive treatments that may help to alleviate mild gastrointestinal symptoms by increasing fiber and probiotics consumption, regular exercise regimens, and eliminating certain food items that may be linked to an allergic reaction (often lactose intolerance). These supportive treatments can be considered integrative in nature since many IBS patients will remain on a specialized diet even after initiating pharmacotherapy if their symptoms are moderate to severe[40,51,52]. Patients with moderate IBS symptoms often require a first line treatment to reduce symptoms and may also benefit from CAM therapy, especially cognitive behavior therapy (CBT) or hypnotherapy[53-57]. A number of publications indicate that the patient-practitioner relationship can have a significant influence on treatment outcomes[58,59]. Practitioners should try to communicate clearly with patients about the diagnostic process, the potential treatment options, setting realistic goals for outcomes and improvement, and providing an atmosphere that is caring and supportive.

The most commonly used pharmacological interventions for symptomatic relief of moderate to severe IBS constitute prokinetics and antispasmodics for IBS-C patients and opioid agonists, anticholinergics, and 5-HT_3 antagonists for relieving IBS-D symptoms[19,20]. Prokinetics are not specific to IBS and increase gastrointestinal motility in general by acting *via* dopamine and 5-HT_3 receptors as antagonists or 5-HT_4 receptors as agonists[60,61]. Tegaserod is to date the only Food and Drug Administration approved prokinetic drug specific for the treatment of IBS-C but has been significantly limited in its use due to an increased cardiovascular risk[62,63]. Lubiprostone, a 5-HT_4 agonist, has been recently approved to treat IBS-C in women through activation of chlorine channels leading to increased water secretion into the lumen which decreased transit time and associated visceral pain in patients[61]. The common use of 5-HT_3 receptor antagonists such as ondansetron and granisetron to reduce visceral pain perception in IBS-D patients has shown some benefits but is also limited by side effects. The best risk-to-benefit ratio has been shown for the 5-HT_3 antagonist alosetron to date[36,64,65].

The common use of antispasmodics in both IBS-C and IBS-D patients serves to reduce abdominal pain and cramping but requires close monitoring especially in IBS-C patients because of further slowing of GI motility[66-68]. All of the currently available antispasmodics are not specific to IBS and act as anticholinergics which are associated with a number of side effects including hyposalivation and cardiovascular events[20,69].

The use of opioid agonists to reduce GI motility in IBS-D patients is another off-label use that can help to improve quality of life and reduce pain perception. The most commonly used opioid agonists are diphenoxylate and loperamide although there is a risk for dependence development which needs to be monitored[70,71].

Low-grade inflammation has been observed in some IBS patients, especially those with post-infectious IBS and small intestinal bacterial overgrowth (SIBO) which has led to the use of antibiotics and glucocorticoids[72-74]. An important consideration in the treatment of low-grade inflammatory processes is the choice of agents since the anti-inflammatory effects should remain localized and not influence the systemic immune system.

So far, the salicylate derivative mesalazine which is also used to treat Crohn's disease with a known inflammatory component has shown an increase in quality of life and symptom reduction in IBS-D patients[75]. Glucocorticoids may provide benefits in IBS although this has only been shown in animal models to date and is based on the observation that patients on oral glucocorticoids show a lower incidence of IBS[76,77]. Rifaximin remains the only antibiotic that has been tested in IBS patients and has shown a moderate improvement in GI symptoms and quality of life whereas other antiinfectives such as nystatin and tetracyclines did present with unacceptable systemic side effects and a low responder rate[78-81].

A number of new targets and accompanying drugs are being developed and tested which may provide additional benefits in the treatment of IBS[19,82]. The coming years will show if they are effective and associated with less side effects compared to the currently available pharmacological options.

Aside from the pharmacological treatments which are often limited by significant side effects, up to 50% of patients are self-medicating using herbals and dietary supplements or other CAM approaches to improve their quality of life and reduce IBS symptoms[38,83,84]. Further increases in the use of CAM are a result of underdiagnosed or misdiagnosed IBS since the differential diagnosis of IBS can be complicated and delayed[40,85,86]. This review addresses the current state of CAM use in IBS and how both CAM and conventional therapeutic treatment can be used in synergy as an integrative approach to treating IBS and providing patients with the best possible quality of life.

COMPLEMENTARY AND ALTERNATIVE MEDICINES

The wide variety of CAM approaches includes basic changes in diet and lifestyle such as increased fiber intake or regular exercise as well as specific use of herbal medicines, combination products, mechanical interventions (acupuncture or massage), or behavioral therapy (cognitive behavioral therapy, relaxation techniques, and hypnotherapy)[43,84]. CAM is often used for chronic health conditions either alone or in conjunction with pharmacological treatment options. About half of IBS patients reported using at least one CAM treatment alone or in addition to their prescription medicine[42,87]. A lack of evidence-based clinical trials often limits the available knowledge about the efficacy of CAM in disease conditions, which is why the ACG Task force on IBS reports that CAM have not consistently demonstrated a strong positive outcome[2]. Recent systematic reviews, however, show indications that various CAM modalities may benefit IBS patients and increase their quality of life[42,43,84,88]. Since IBS can present with varying symptoms and severity even with daily or weekly fluctuations within the same patient, the effectiveness of CAM may at times appear inadequate or questionable by patients themselves. The most commonly used CAM interventions that have been evaluated in clinical trials, are dietary changes, use of probiotics, exercise, single herbal extracts, herbal combination products, hypnotherapy, acupuncture, and relaxation techniques. All of these approaches are discussed in more detail.

Diet and lifestyle modifications

Diet modifications are not generally considered CAM and usually are the first step in reducing IBS symptom severity even before pharmacotherapy is initiated (Table 1). However, exclusion diets can often be supplemented with CAM to reduce specific symptoms such as bloating and distension[2,89]. Exclusion diets may benefit patients with a known allergy and those in post-infectious IBS or SIBO[40,51,52] and consists of removing wheat, dairy products, eggs, coffee and caffeinated beverages, yeast, potatoes, and citrus fruits[52]. Despite the reported successes with exclusion diets, they may, at times, be hard to follow thereby resulting in bouts of increased IBS symptom severity. While dietary restrictions may benefit some IBS patients, entirely skipping meals actually has shown to worsen IBS symptoms[52,90].

Some contribution to intestinal symptoms may come from a diet that is high in fat, carbohydrates, and sugar alcohols. It has been shown that increased fat consumption is linked to increased stool numbers and diarrhea and therefore should be considered as a factor in worsening IBS-D[90,91]. Fructose-rich food and beverage items (soft drinks, baked and packaged goods, cereals) can also aggravate flatulence, abdominal discomfort, and diarrhea and should therefore be monitored in IBS patients[51,52]. Especially poorly absorbed sugar alcohols that are present in diet soft drinks and low carbohydrate foods can exacerbate GI symptoms[51,92]. Together with general restrictions on carbohydrate intake, lactose intolerance and malabsorption appear to be more prevalent in IBS patients. If lactose is not absorbed from the GI tract it is metabolized *via* the gut bacteria and leads to increased bloating, distension, and diarrhea which can aggravate IBS symptoms[93-95].

Fiber is often recommended as a dietary change to reduce global IBS symptoms but the clinical data to date are less clear. It has been shown that soluble fiber can lower GI symptoms in IBS-C although the data supporting it is highly variable both in the amount of fiber consumed (ranging from 5-30 g/d) and the duration of the trials (3-16 wk)[51,91,96,97]. Fiber, regardless soluble or insoluble, was not able to reduce pain perception in IBS patients and specifically insoluble fiber such as nuts and whole grains may exacerbate IBS symptoms overall[97].

An important lifestyle adjustment that should be recommended to IBS patients is regular exercise. Mild exercise or physical activity has been shown to reduce IBS symptoms and alleviates bloating and gas production in several studies[98,99]. Since regular exercise also helps to increase gastrointestinal motility it is beneficial in IBS-C patients with primary low GI movement and hard stools[100]. As part of exercise, yoga has been investigated due to its low impact on joints and its relatively targeted postures

Table 1 Clinical trials on diet and exercise interventions for irritable bowel syndrome

Intervention	Study design	Sample size	Outcome	Ref.
Acceptability questionnaire	Anonymous survey	256	Most acceptable were tablets (84%), diet and lifestyle changes (82%), yoga (77%); less acceptable were acupuncture (59%) and suppositories (57%)	[40]
Food elimination	Open label pilot study	20	Significant improvements in stool frequency ($P < 0.05$), pain ($P < 0.05$), and IBS-QOL ($P < 0.001$)	[89]
Diet and lifestyle	Cross-sectional study	1717	Significant difference between IBS and non-IBS participants in regards to residential type (OR = 1.27) and frequency of meals (OR = 1.69)	[90]
Diet and lifestyle	Questionnaire	983	BMI was associated with abdominal pain and diarrhea, healthier diet and physical activity were associated with fewer GI symptoms	[91]
Diet	3-way cross-over study	22	IBS-D patients showed significant increase in small bowel and mucosal permeability for mannitol and lactulose sugars compared to healthy controls	[92]
Diet	Questionnaire	1978	Potential for higher lactose intolerance incidence in patients with IBS compared to healthy patients	[93]
Diet	Case-control study	177	Symptomatic lactose intolerance more frequent in patients with IBS than healthy subjects, but incidence of lactose intolerance not different between groups	[94]
Diet	Case-control study	120	Lactose intolerance resulted in more frequent self-reported symptoms in patients with IBS-D than controls ($P < 0.001$, OR = 6.25), IBS-D patients consumed significantly less dairy products ($P = 0.04$)	[95]
Exercise	Randomized, controlled trial	56	No difference in quality of life between exercise and usual care groups, exercise group presented with significant less symptoms of constipation after 12 wk intervention	[98]
Exercise	Cross-over study	8	Gas retention during rest was associated with significant abdominal symptoms in IBS patients ($P < 0.01$), symptoms improved during exercise ($P < 0.05$) compared to rest	[99]
Exercise	Descriptive comparative study	89	Women with IBS report less physical activity ($P < 0.05$), women with IBS who were physical active reported significantly less symptoms of fatigue ($P = 0.003$) compared with the ones with IBS who were physically inactive	[100]
Yoga	Randomized cross-over study	25	Lower functional disability ($P = 0.073$) and anxiety levels ($P = 0.09$) in the yoga group compared to the waitlist group, significantly lower GI symptoms ($P < 0.01$)	[101]
Yoga	Randomized parallel design	21	Similar reductions in symptoms after 2 mo for yoga and the group receiving loperamide in IBS-D patients	[102]

IBS: Irritable bowel syndrome; QOL: Quality of life; BMI: Body mass index; IBS-D: Diarrhea-predominant IBS; GI: Gastrointestinal.

that can help to reduce GI symptoms[101,102]. Pranayama yoga administered twice daily has been shown to increase sympathetic tone and may benefit IBS-D patients that present with decreased sympathetic activity to the same degree than daily loperamide administration in the control group[102].

Herbal medicines and supplements

Aside from diet and lifestyle changes, another commonly used CAM intervention that is often self-administered is the use of herbal supplements either as single herbs or in combination products (Table 2). A few well-designed clinical studies have been performed on a number of such herbal supplements but in general the current knowledge remains limited to make a definitive judgment about their effectiveness in treating IBS symptoms. Many of the commonly used supplements have evolved from folk and traditional applications as remedies for gastrointestinal disorders.

The use of peppermint extracts has been studied in a number of clinical trials which evaluated the administration of enteric coated peppermint oil capsules to IBS patients[103-107]. The duration of the trials ranged from 4-8 wk and was not divided into specific IBS subtypes. The trials showed a significant reduction in abdominal pain and severity compared to placebo after 4 wk and a significant increase in quality of life although the effects did not last once peppermint was discontinued[108-110]. The spasmolytic effects of peppermint oil have been well known in folk medicines and mainly been attributed to the presence of mono- and sesquiterpenes[110]. Peppermint oil has also been recommended by the American Academy of Pediatricians as well as received a positive evaluation from the Task force on IBS of the ACG[2,111] although caution is advised for its use in young children due to its side effects of causing respiratory depression and heartburn[111]. Peppermint oil appears to be more potent in exerting the spasmolytic effects than aqueous extractions such as teas since it allows for a more concentrated dose of the proposed active ingredients[112].

Hydroalcoholic extracts from artichoke leaves have also been used to reduce IBS symptoms and evaluated in at least two clinical studies[113,114]. Artichoke has long been used as a digestive aid which aims to reduce bloating, abdominal pain and cramps, as well as reducing both diarrhea and constipation through normalization of GI motility[115]. Both studies were conducted as part of a post-marketing surveillance which may limit the credibility of the results due to limited influence on the study design (no double-blinding, no placebo control) and potential consumer bias. Both trials report a significant improvement in IBS symptoms, specifically in normalizing GI motility and reducing bloating as well as relieving distension and abdominal pain and cramps[113]. Since the change in IBS symptom severity was compared between baseline and follow-up, the results provide limited evidence of the effectiveness compared to placebo or standard pharmacological treatment. Given the high placebo responder rate,

Table 2 Clinical trials on Herbal medicines and supplements for irritable bowel syndrome

Intervention	Study design	Sample size	Outcome	Ref.
Peppermint oil	Randomized, double-blind, placebo-controlled study	99	Peppermint oil (Colpermin®) group showed significant symptom improvement ($P < 0.05$) compared to placebo group after 1 mo	[104]
Peppermint oil	Randomized, placebo-controlled study	18	Peppermint oil significantly reduced GI symptoms ($P < 0.01$) after 3 wk compared to placebo	[106]
Peppermint oil	Randomized, double-blind. Placebo-controlled study	57	Total IBS severity score was significantly decreased after 4 wk of treatment ($P < 0.009$) and after 2 mo ($P < 0.01$) in the peppermint oil group compared to placebo	[108]
Peppermint oil	Randomized, double-blind, placebo-controlled study	90	Significant reduction in IBS symptoms, no abdominal pain in more patients in the peppermint oil group compared to placebo ($P < 0.001$), less severe abdominal pain in peppermint oil group ($P < 0.05$) in peppermint oil group after 2 mo	[109]
Peppermint oil	Randomized, double-blind, placebo-controlled study	65	Significant reduction in abdominal pain in peppermint oil group compared to placebo group ($P < 0.001$), but pain score increased 2 wk after completion of trial	[110]
Artichoke leaf	Post-marketing surveillance	279	Significant reduction ($P < 0.05$) in overall IBS symptoms after 6 wk of treatment	[113]
Artichoke leaf	Post-marketing surveillance in IBS with concomitant dyspepsia	209	Significant reduction in Nepean Dyspepsia Index after 2 mo ($P < 0.001$) and normalization of bowel pattern ($P < 0.001$)	[114]
Turmeric	Partially blinded, randomized, two-dose pilot study	207	Reduction in IBS prevalence in both treatment groups (1 or 2 tablets) compared to baseline ($P < 0.001$) after 2 mo intervention, no significant differences between groups	[116]
Curcuma and fumitory	Randomized, double-blind, placebo-controlled study	106	No significant differences between curcuma, fumitory, and placebo groups in abdominal pain ($P = 0.81$) and distension ($P = 0.48$) after 3 mo	[117]
STW5	Randomized, double-blind, placebo-controlled study in patients with dyspepsia	137	Significant decrease in gastrointestinal symptom score between STW5 and placebo ($P < 0.001$)	[118]
STW5	Randomized, double-blind, placebo-controlled multicenter study in patients with functional dyspepsia	315	Significant decrease in gastrointestinal symptom score between STW5 and placebo ($P < 0.05$) after 2 mo intervention	[119]
STW5	Randomized, double-blind, placebo-controlled multicenter study	203	Significant reduction in abdominal pain scores for STW5 ($P = 0.009$) and STW5-Ⅱ ($P = 0.005$) and IBS-SSS ($P = 0.001$ for STW5 and $P = 0.0003$ for STW5-Ⅱ) compared to placebo after 4 wk intervention	[120]
Padma Lax	Randomized, double-blind, placebo-controlled pilot study	61	Significant improvement in global IBS symptom scores compared to placebo ($P < 0.05$) following 3 mo intervention	[123]
TCM	Randomized, double-blind, placebo-controlled study	119	No significant improvements in IBS global symptom score between TCM and placebo group at week 8 ($P = 0.38$) and week 16 ($P = 0.62$)	[129]

IBS: Irritable bowel syndrome; TCM: Traditional chinese medicine; SSS: Symptom severity scale.

artichoke leaf extracts will require additional trials that are more rigorous in terms of study design.

Turmeric, a spice traditionally used in Asian cuisine where it often provides both for taste and color improvement, was evaluated in IBS patients and indicated decreases in IBS symptoms and increased quality of life if given in two different doses of 72 and 144 mg per day over 8 wk[116]. However, this study again lacks a double-blinded and placebo-controlled design which reduces the strength of the presented data. Another double-blind placebo controlled study compared curcuma extract from which turmeric is derived with a placebo and a fumitory extract[117]. Both curcuma and fumitory extracts did not show any significant improvements in abdominal pain and distension compared to the placebo group.

All single herbal supplements discussed have a strong folkloric and traditional background as digestive aids and it is therefore not surprising that to date trials with such herbal supplements are focused on these single extracts.

Combination products of herbal extracts add to the wide range of available supplements used in the self-treatment of IBS. The first combination product which has received some interest from patients and healthcare providers alike is Iberogast®, a mixture of nine herbal plant extracts that was originally mainly used for functional dyspepsia in Germany[118,119]. The product has been on the market for more than 30 years. Iberogast® for use in IBS symptoms was investigated in 208 patients with various IBS subtypes in the United States[120]. This study adheres to the clinical trial standards by utilizing a randomized, double-blind, placebo-controlled study protocol over a 4 wk period. The extract consists of liquid extracts from chamomile flowers, bitter candytuft, angelica root, caraway fruits, milk thistle, lemon balm leaves, greater celandine, licorice root, and peppermint leaves[121]; all of which have been used in folklore and traditional medicines to aid in digestive disorders. The study indicates that Iberogast® significantly improves quality of life and reduces abdominal pain in IBS patients[122] which appears to be mediated through influences on serotonin, acetylcholine, and opioid receptors in the GI tract[121]. Despite the positive outcome, further research is required to support the findings of this

Table 3 Clinical trials on mechanical complementary and alternative medicines interventions for irritable bowel syndrome

Intervention	Study design	Sample size	Outcome	Ref.
Physical activity	Randomized study	75	Significant decrease in IBS-SSS between physical activity and placebo group (P = 0.003)	[133]
Reflexology	Randomized, single-blind, placebo-controlled study	34	No significant difference between foot reflexology and non-reflexology massage group	[134]
Acupuncture	Randomized, single-blind, placebo-controlled study	230	Acupuncture and sham acupuncture significantly improved IBS-GIS scores compared to waitlist group (P = 0.001), no difference between acupuncture and sham acupuncture during 3 wk intervention	[143]
Acupuncture	Randomized, single-blind, placebo-controlled study	43	Significant improvements (P = 0.022) in quality of life for both acupuncture and sham acupuncture compared to baseline after 10 intervention sessions (5 wk), no differences between acupuncture and sham acupuncture	[144]
Acupuncture/ moxibustion	Randomized, single-blind, placebo-controlled study	29	Significant reduction in IBS-SSS in acupuncture/moxibustion group after 4 wk compared to sham acupuncture/moxibustion group (P = 0.01)	[146]
Yoga	Observational pilot study (adolescents)	20	Decrease in pain frequency (P = 0.031 for 8-11 yr old and P = 0.004 for 12-18 yr old) and pain intensity (P = 0.015 in 8-11 yr old) after 10 yoga sessions compared to baseline, decrease in pain frequency was maintained for 3 mo following intervention (P = 0.004 for 8-11 yr old)	[149]

IBS: Irritable bowel syndrome; GIS: Global improvement scores; SSS: Symptom severity scale.

study and a few case reports from patients and healthcare providers alike. Iberogast® has been recognized by the ACG task force on IBS as a potential complementary treatment to reduce certain IBS symptoms[2].

A Tibetan preparation of twelve different plant extracts, commonly referred to and marketed as Padma Lax, was tested in IBS-C patients over the course of three months in a randomized and double-blinded observational study and showed significant improvements over a placebo in reducing constipation, abdominal pain, and flatulence[123]. The dose had to be adjusted for some of the patients since they developed loose stools that bordered on diarrhea indicating that this combination herbal product may have significant potential as a laxative. The ingredient list includes the known laxatives rhubarb root, cascara bark, and nux vomica seeds which have been used as strong laxatives in severe constipation in traditional medicines across the world[124]. It has been proposed that some of the herbs exert the laxative effect through activity on cholinergic receptors as antagonists to reduce GI contractility[124,125].

Traditional Chinese medicine (TCM) has provided a range of different treatment approaches over the centuries, among them a number of TCM herbal mixtures that are specifically formulated based on the patients' symptoms[126]. Such individualized medicine is not uncommon but has the obvious limitation of resisting standardization and fitting the rigorous clinical trial design that is used as a major determinant of effectiveness in Western medicine. *Tong Xie Yao Fang* (TXYF) is one such TCM that has been studied in a few clinical trials but often adjustments were made to the herbal combination based on the predominant IBS symptom presentation[127-129]. A review of 12 studies with modified TXYF preparations reached the general conclusion that the extracts improved IBS symptoms, in particular abdominal pain, distension, flatulence, and diarrhea[128]. However, the study design and end points were diverse between these studies complicating a direct comparison of outcomes. A more streamlined and standardized approach to TXYF trials is warranted.

Overall, the use of single and combination herbal supplements appears promising but should be approached with caution given the lack of rigorous larger clinical trials. The strongest evidence for the use of herbal medicines is currently available for peppermint oil preparations and the herbal combination product Iberogast®.

Mind-body therapies

Since there is evidence of the involvement of the enteric nervous system or brain-gut axis in the pathophysiology of IBS, the use of mind-body interventions as CAM treatments may provide benefits in relieving symptoms. Mind-body therapies are interventions that primarily "focus on the interactions among the brain, mind, body, and behavior with the intent to use the mind to affect physical functioning and promote health"[130]. Yoga, Tai-chi, meditation, hypnotherapy, deep-breathing exercises, relaxation techniques, and acupuncture all fall under this definition. While yoga, relaxation techniques, and acupuncture are commonly used as CAM therapies, they involve both a physical and psychological component with a focus on influencing physical functions through mechanical interventions. Meditation, hypnotherapy, and CBT do not involve a mechanical component but rather seek to change physiological function through psychological reprogramming entirely[131,132]. Mind-body therapies have been evaluated for their potential application as CAM in IBS. Clinical evidence supporting the use of yoga, relaxation, acupuncture, hypnotherapy, and cognitive behavior therapy is indicating that these CAM interventions show improvement in IBS symptoms and overall quality of life.

Mechanical CAM interventions

Mechanical interventions are based on direct body interventions such as massage, acupuncture, yoga, and physical exercise (Table 3)[131]. While some interventions may

not benefit patients with IBS because of the significant physical involvement that may cause a temporary worsening of symptoms, low impact physical exercise such as aerobic training, bike riding, and muscle strengthening have shown benefits in maintaining GI function and reducing flatulence and gas production[90,99,100,133]. One study indicated that strenuous exercise shows an inverse relationship with IBS symptoms[91] while another study pointed to reduction of constipation in IBS-C patients with mild exercise levels[98]. Johannesson and colleagues evaluated the impact of regular exercise on IBS symptoms severity scores compared to a control group and found a significant reduction in symptoms over the course of 12 wk[133]. It is therefore important to emphasize that a patient should start off with low impact and light exercise regimens that are not exhausting or causing increases in abdominal pain or overall IBS symptoms. As such, mild physical exercise may be considered a lifestyle change rather than an actual CAM intervention, but guided assistance may benefit IBS patients who seek counseling and advice on respective exercise regimens for their condition.

To date there is little information about the potential impact of massage and reflexology treatments on IBS symptoms. One study compared the use of foot reflexology massage to non-reflexology foot massage and could not find any significant difference in the small study of 34 patients[134]. However, case reports have indicated that gut-directed massage may relieve specific symptoms such as bloating and chronic constipation although such reports were not specific to IBS patients[135-137]. It therefore remains unknown if reflexology or massage techniques can provide benefits to IBS patients.

A number of trials investigated the effect of gut-directed and general acupuncture on symptom relief in IBS patients. It is well known that acupuncture can affect physiological functions in a number of conditions through regulating various neurotransmitter systems. It has been shown that application of acupuncture in IBS patients targets serotonergic, cholinergic, and glutamatergic pathways, can lower blood cortisol levels related to stress, and can increase the concentration of endogenous opioids to reduce visceral and global pain perception[138-140]. A complicating factor in the study of acupuncture effects are adequate comparison groups. One commonly used comparison group is sham acupuncture which uses needles as well so to suggest to patients that they are being treated when the practitioner does not utilize the specific acupuncture points and only superficially applies needles[141,142]. What appears to be a contributing factor to the effectiveness of acupuncture is the patient-practitioner relationship, especially in IBS patients where an underlying psychological contribution to IBS symptoms can be suspected[58,59]. In one study, a sample of 230 IBS patients were randomly assigned to receive either acupuncture, sham acupuncture, or remain on a waitlist for the duration of the trial[143]. Initially both the true acupuncture and sham acupuncture groups received only sham acupuncture for 3 wk followed by 3 wk of true acupuncture in half of these patients while the other half continued receiving sham acupuncture. Both groups showed significant improvement in global IBS symptoms compared to the waitlist control group, but the patients receiving true acupuncture did not differ from the sham acupuncture group thus complicating interpretation of results related to the actual acupuncture points being used. Other studies using sham acupuncture as a comparator have also found that improvements in quality of life and IBS symptoms did not differ from true acupuncture points thus potentially indicating that acupuncture for IBS is primarily a placebo response[144,145]. When combined with moxibustion, acupuncture has shown significant improvements in IBS symptoms with reduced abdominal pain, gas and bloating being reported in one study including 29 subjects over a 4 wk, eight session intervention[146]. A Cochrane meta-analysis of studies including acupuncture suggests that further studies are warranted to confirm the beneficial effects of such treatments in IBS patients[147].

A mechanical intervention that has been studied in IBS is yoga as a specific form of exercise and focused relaxation technique combined[148]. Yoga consists of different poses accompanied by a specific breathing pattern to focus attention on muscle contraction and relaxation. There are certain poses that can be utilized to focus on GI tract motility and abdominal pain perception. There are only few trials conducted with yoga as the intervention but a number of indicators suggest that yoga may provide relief of IBS symptoms. One study in 22 male patients with confirmed IBS-D compared yoga poses and breathing techniques to conventional treatment over the course of 8 wk[102]. Overall GI symptoms were reduced in both groups at the end of the study but the yogic intervention group showed a higher parasympathetic reactivity leading to improved IBS symptom outcomes whereas the control group presented with increased gastric activity. Another small clinical trial compared the use of yoga in adolescents to a wait list group over the course of 8 wk[101]. In the first 4 wk, the yoga intervention group received instructions on daily yoga practices and continued the poses after the first 4 wk. The control wait list group received yoga intervention after 4 wk for another month at which point both groups were compared. This preliminary study indicates that yoga intervention over the course of a 2 mo period improves GI symptoms in adolescent IBS patients and is well received by youth. Another small yoga intervention study conducted in 20 children aged 8-18 years with IBS or functional abdominal pain indicates that yoga does decrease both pain frequency and pain intensity at the end of intervention and that this effect also persisted for at least another 3 mo after the intervention has ended[149]. In addition, adolescents in this trial reported increased quality of life throughout the intervention and during follow-up. Although not many studies have been conducted using yoga as a CAM intervention, the current data suggests that it may provide benefits in IBS patients by alleviating both pain and GI symptoms.

Table 4 Clinical trials on psychological complementary and alternative medicines interventions for irritable bowel syndrome

Intervention	Study design	Sample size	Outcome	Ref.
Hypnotherapy	Pre- and post-assessment	23	Normalized hypersensitivity pain threshold in hypersensitive group (P = 0.04) after 12 wk of treatment, no significant change in hyposensitive and normosensitive groups	[154]
Hypnotherapy	Randomized controlled trial in children with functional abdominal pain or IBS	53	Significant reduction in pain scores in hypnotherapy group (P < 0.001) compared to standard medical therapy at 1-yr after intervention	[159]
Hypnotherapy	Questionnaire	83	69% of patients were either satisfied or very satisfied with hypnotherapy following 12 wk intervention, overall improvement in quality of life and GI symptoms	[160]
Hypnotherapy	Randomized, placebo-controlled study	138 in two studies (90 and 48)	Significant reduction in IBS symptoms in hypnotherapy groups (P < 0.05) compared to supportive therapy after 3 mo of intervention	[161]
Hypnotherapy	Randomized, placebo-controlled study	90	Significant improvement in overall IBS symptoms in gut-directed hypnotherapy and medical treatment group compared to medical treatment group alone (P = 0.046) after 12 wk; improvement remained up to 12 mo after intervention in hypnotherapy group (P = 0.004) compared to medical treatment alone	[162]
Hypnotherapy	Pre- and post-assessment	75	Group hypnotherapy decreased symptom severity significantly (P < 0.01) at 3, 6, and 12 mo post-intervention	[163]
Hypnotherapy	Retrospective analysis	208	Significantly higher use of hypnotherapy (P < 0.001) by initial responders *vs* non-responders at 2-7 yr follow-up, in total 87% of participants reported hypnotherapy to be useful	[164]
Cognitive behavior therapy	Randomized-comparator-controlled study in patients with functional bowel disorders	431	CBT was more effective than education (P = 0.0001) and desipramine was more effective than placebo (P = 0.01) after 12 wk of treatment as assessed by treatment satisfaction	[170]
Cognitive behavior therapy	Randomized, placebo-controlled study in patients with functional bowel disorders	397	No significant differences between treatment arms for desipramine, cognitive behavior therapy, and placebo groups	[171]
Cognitive behavior therapy and mindfulness training	Randomized controlled trial	195	Internet-delivered cognitive behavior therapy resulted in adequate relief of IBS symptoms that was significant compared to internet-delivered stress management at 6 mo follow-up (P = 0.004)	[173]
Cognitive behavior therapy	Randomized controlled trial	149	Significant reduction in symptom severity scores in CBT plus mebeverine group compared to mebeverine alone at post-treatment and 3, 6, and 12 mo follow-up (regression P = 0.001)	[174]
Psychotherapy [cognitive behavior therapy]	Randomized controlled trial	50	Rome-Ⅱ scores significantly decreased (P = 0.001) in patients receiving CBT in conjunction with standard medical care compared to standard medical care alone after 2 mo intervention	[175]
Cognitive behavior therapy	Randomized controlled trial	28	Psychosocial functioning was significantly improved (P = 0.004) in patients receiving CBT in addition to standard medical care compare to standard medical care alone at 3 mo follow-up	[176]
Cognitive behavior therapy	Randomized controlled trial	76	Cognitive behavior therapy presented with significant improvements compared to stress management and attention control groups in reducing visceral sensitivity (P < 0.0001) compared to baseline at 3 mo follow-up	[177]
Cognitive behavior therapy	Randomized, placebo-controlled study	85	Internet delivered CBT reduced several IBS symptom parameters (total pain, diarrhea, bloating primary symptoms all P < 0.001) after 10 wk of intervention compared to discussion board control group; quality of life and visceral sensitivity were also significantly improved (P < 0.001) after 3 mo follow up	[179]
Mindfulness training	Randomized controlled trial	75	Women in mindfulness training group showed significant reduction (P = 0.006) in IBS symptom severity compared to support control group after 8 wk of intervention which remained significant at 3 mo follow-up (P = 0.001)	[180]
Cognitive behavior therapy	Retrospective analysis	75	Long-term follow-up after 15-18 mo of original intervention resulted in lasting significant reductions in visceral sensitivity (P < 0.05), increase in quality of life (P < 0.05), and gastrointestinal symptoms (P < 0.05)	[181]

IBS: Irritable bowel syndrome; CBT: Cognitive behavior therapy; GI: Gastrointestinal.

Psychological CAM interventions

While mechanical interventions can provide IBS symptom relief, compliance can often be an issue as well as limited physical ability to follow the treatment (especially with exercise and yoga). Other mind-body CAM approaches are solely based on psychological interventions with hypnotherapy and cognitive behavior therapy showing the most clinical evidence of effectiveness (Table 4).

Hypnotherapy is based on initiating a suggestive state for the patient similar to sedation but without loss of consciousness that allows for heightened senses and control over body functions affecting mood, pain perception, cardiovascular responses, and gastrointestinal motility[150,151]. Hypnosis and hypnotherapy have been used for various applications foremost for the treatment of acute and chronic pain conditions but also to improve mood or change certain undesirable behaviors[152,153]. It has been shown that gut-directed hypnotherapy can alleviate IBS symptoms comparable to current pharmacological treatment approaches[154-156]. Several clinical studies and meta-analysis indicate that 8-12 weekly hypnotherapy sessions can improve pain, GI motility, mood (improving depressive and anxiety disorders), and overall quality of life of IBS patients significantly even in the absence of pharmacological treatment[157-161]. Interestingly, in a number of studies during follow-up the beneficial effects of hypnotherapy remained for at least 10 mo even if patients did not continue therapy[55,155,162-164]. A Cochrane database review examined available studies and found a positive effect associated with hypnotherapy although the effect size could not be determined due to the heterogeneity of study designs and the relatively small sample sizes[54]. Although there is clinical evidence for the use of hypnotherapy in treating IBS symptoms, further research utilizing a homogenous study design needs to be emphasized to support its benefits.

In contrast to hypnosis where the patient is being influenced and subjected to a subconscious suggestive state that influences physiological processes, CBT takes a more direct approach in influencing the conscious awareness of IBS symptoms and attempts to enable the patient to overcome the negative attitudes they may have towards their chronic condition[165-167]. Similar to hypnosis, patients are enabled to change a negatively perceived situation into a positive outlook which then affects the actual symptoms. CBT has been shown to be effective in improving symptoms in a number of chronic disorders such as obesity, chronic pain, insomnia, or depressive disorders[166,168,169]. While hypnosis can be utilized to directly affect perception of symptoms, CBT is more geared towards enabling patients themselves to change their behavior and thought processes about their condition. In IBS patients, symptom aggravation may often occur when patients worry about the condition and focus their thoughts around them[170,171]. CBT has been shown to improve both the quality of life and reduce symptom severity in IBS patients, especially in regards to pain perception and comorbid depressive and anxiety disorders[170,172,173]. Comparing the use of CBT as a CAM approach to treating IBS with conventional pharmacological treatment indicates that it does not only improve the direct IBS symptoms such as pain and GI dysmotility, but in addition also benefits mood and coping with the condition[174-177]. Although the effects of CBT lasted past the intervention period in some of the trials, the beneficial effects seemed to wane over time which may indicate a long-term supportive treatment with CBT at least in frequent intervals following the initial treatment period. Currently, an ongoing irritable bowel syndrome outcome study evaluates the long-term effects of self-versus clinician-administered CBT on IBS symptoms and the economic benefit of this intervention[178].

Closely related to CBT as a CAM intervention is mindfulness exercises that promote acceptance instead of control of IBS symptoms[179,180]. This technique is often delivered in conjunction with CBT and is more patient-centered to increase quality of life through coping mechanisms. In conjunction with CBT, mindfulness exercises have shown a reduction in IBS symptoms beyond the intervention period up to 3 years[173,181].

INTEGRATIVE APPROACHES AND CLINICAL IMPLICATIONS TO TREATING IBS

Most of the clinical trials that have been discussed focus on the comparison between CAM approaches and standard care with pharmacological therapies in treating IBS symptoms. This separation often creates a painful choice for patients that need the immediate relief with medication but also seek long-term alleviation of the symptoms through a pro-active approach. Integrating both conventional pharmacological care and CAM treatments to provide the best symptom relief and highest quality of life to IBS patients should therefore be considered by healthcare providers. A number of clinical trials - although small in sample sizes and number - have shown that a combination of pharmacotherapy with a CAM treatment is superior to either treatment alone. As long as the CAM treatment does not interfere or interact with the pharmacological treatment, both can coexist in the treatment of IBS symptoms.

A study evaluating the use and cost of CAM by patients with functional bowel disorders (including IBS patients) reported a number of interesting results[85]. The most common types of CAM were ginger, massage therapy, and yoga with a median yearly cost of $200, which was considered significantly less compared to standard pharmacotherapy. Furthermore, the use of CAM was associated with female gender, higher education, and comorbid anxiety disorders. Although most of the patients using CAM (35% of 1012 participating patients) were not referred by their healthcare provider, those who received a recommendation from their primary care physician followed the advice. This indicates the important role of the healthcare provider as a mediator and facilitator for patients to seek CAM treatments. Several of the CAM approaches discussed in this review have been recommended or given at least positive consideration by current professional organizations (ACG and the British Society for Gastroenterology) to be considered in the treatment of IBS in addition to pharmacotherapy[2,12]. Another important point that the study reveals is that CAM use is not based on dissatisfaction with pharmacotherapy but seems to be rather linked to higher comorbidity with de-

pressive and anxiety disorders as well as somatization of their condition[85]. CAM users in this study also showed a higher symptom severity on the IBS-SSS as well as gastrointestinal distension. The authors of the study conclude that CAM use can benefit patients with various functional bowel disorders and should be made more widely available potentially through providing insurance coverage. Medical professionals should recommend CAM approaches such as hypnotherapy, yoga, cognitive behavior therapy, or herbal supplements that have shown benefits to IBS patients that do not experience adequate symptom relief with pharmacotherapy alone.

In a study that evaluated a gap in effectiveness between current treatment approaches and treatment outcomes conducted in the United Kingdom, 32% of general practitioners reported an effectiveness gap for IBS patients in their practice[86]. The most common reasons given for the effectiveness gap were lack of effective treatments, adverse effects of current treatments, and unacceptability of available treatments by patients. These findings are supported by other studies which indicate a lack of treatment effectiveness in a significant percentage of IBS patients[40,43,182]. The use of CAM has been shown in randomized controlled trials as well as in systematic reviews to decrease or close the effectiveness gap thereby increasing quality of life and treatment outcomes for IBS patients. The authors of this study conclude that there is a discrepancy between available evidence for the effectiveness of CAM in various chronic conditions and its recommendation by current practice guidelines which often omit such approaches. In addition, patients appear to be more favorable towards trying CAM approaches than practitioners thereby most referrals are initiated by patients themselves or without including the healthcare provider when considering CAM treatments in addition to conventional care[86,183,184].

The integrative medical approach has gained significant traction in the last decade with the growth of the Consortium of Academic Health Centers for Integrative Medicine[185] to which many well respected United States institutions belong (Yale University, Stanford University, University of California, Johns Hopkins University, among others). Integrative medicine as a subdivision of medical practice is seeking to emphasize on patient-centered care and improve wellness and quality of life rather than limiting the treatment outcome to a specific disorder. The definition of integrative medicine remains somewhat elusive to date ranging from simply adding CAM treatments to conventional care to a more holistic healthcare approach overall. While the conventional approach to treating IBS is primarily founded on evidence-based clinical trials and extensive knowledge, the CAM approach to treating IBS also claims a long tradition of use and an increasing body of research supporting the benefits of CAM treatments in IBS. The debate over integrative medicine continues with defenders of conventional medicine indicating that such a definition should not exist because treatments with proven benefits will eventually become the standard of care no matter where they originated from. However, proponents of integrative medicine argue that excluding CAM or preventing patients from seeking CAM treatments in addition to conventional care will result in reduced quality of life and worse treatment outcomes. As mentioned above, this has been supported by various studies. It has been argued that even if a CAM treatment is not supported by sufficient evidence-based clinical trials, as long as it does not cause adverse effects or interfere with conventional therapy it should not be denied to patients seeking such treatments. Instead, physicians and healthcare providers should seek training or knowledge in integrative medicine to best support their patients. This applies especially to chronic conditions such as IBS that present with a high placebo response and where a number of CAM treatments - herbal therapies, probiotics, dietary changes, acupuncture, yoga, hypnotherapy, and cognitive behavior therapy - have shown benefits.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Diagnostic utility of faecal biomarkers in patients with irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a common functional gastrointestinal (GI) disorder characterized by unspecific symptoms. In clinical practice it is crucial to distinguish between non-inflammatory functional problems and inflammatory, malignant or infectious diseases of the GI tract. Differentiation between these involves the use of clinical, radiological, endoscopic, histological and serological techniques, which are invasive, expensive, time-consuming and/or hindered by inaccuracies arising from subjective components. A range of faecal markers now appears to have the potential to greatly assist in the differentiation of inflammatory bowel disease (IBD) and IBS. Faecal markers of neutrophil influx into the mucosa are reliable indicators of intestinal inflammation and their role has been mainly studied in discriminating IBD from non-IBD conditions (including IBS) rather than organic from non-organic diseases. Phagocyte-specific proteins of the S100 family (S100A12, calprotectin) are amongst the most promising faecal biomarkers of inflammation. Faecal leukocyte degranulation markers (lactoferrin, polymorphonuclear elastase and myeloperoxidase) have also been suggested as diagnostic tools for the differentiation of IBD and IBS. More recently, additional proteins, including granins, defensins and matrix-metalloproteases, have been discussed as differential diagnostic markers in IBD and IBS. In this review, some of the most promising faecal markers, which have the potential to differentiate IBD and IBS and to advance diagnostic practices, will be discussed.

Key words: S100A12; Calprotectin; Lactoferrin; M2-pyruvate kinase; Polymorphonuclear elastase; Defensins; Granins; Irritable bowel syndrome

Core tip: Faecal markers of intestinal inflammation represent a practicable, non-invasive, inexpensive and objective diagnostic tool to differentiate organic [inflammatory bowel disease (IBD)] and functional [irritable bowel syndrome (IBS)] gastrointestinal diseases. Faecal markers have the potential to be incorporated into standard clinical practice for the routine assessment of IBS and IBD. Neutrophil-derived faecal biomarkers show a high diagnostic accuracy in the differentiation of IBD *vs* IBS. They can provide reassurance to the physicians that the clinical diagnosis of IBS is correct. Future progress in our knowledge about the biology of these proteins and the underlying pathogenesis of IBS will help translate IBD/IBS research into patient care.

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IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal (GI) disorders, with a reported prevalence of approximately 10% to 15% worldwide[1]. The exact pathogenesis of IBS is only partially understood but seems to be multifactorial. There is evidence that heritability and genetics, environment and social learning, dietary or intestinal microbiota, low-grade inflammation and disturbances in the neuroendocrine system of the gut play a central role[2]. There is no medical therapy established to alter the natural history of IBS and most traditional therapies (*e.g.*, bulking agents, antidiarrheals, antispasmodics) focus on improving individual symptoms. However, these symptom-based therapies have limited efficacy and as such novel and emerging therapies have been developed based upon the evolving understanding of the pathophysiology of IBS[3,4].

Though a variety of GI and extraintestinal symptoms and presentations are associated with IBS, it is primarily characterized by symptoms of abdominal pain or discomfort associated with an altered bowel function in the absence of any organic cause. Patients commonly report abnormal defecation ranging from diarrhoea to constipation, including a combination of the two, the degree of which can vary in both severity and duration[5,6]. Four subtypes of IBS were recognized: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M), and unsubtyped IBS (IBS-U). IBS presents a challenge to gastroenterologists, with several groups having attempted to define a set of standardized symptom-based criteria for the diagnosis of IBS. Although no symptom-based criteria have ideal accuracy for diagnosing IBS[7], the third iteration of the Rome criteria (Rome Ⅲ) and the Manning criteria are widely used by clinicians to diagnose IBS[8,9].

Since many GI disorders present with symptoms similar to IBS, it is important to exclude other causes. The diagnosis of IBS should be made using symptoms based on clinical criteria rather than excluding underlying organic disease by exhaustive investigation. Routine laboratory studies are normal in IBS and thus only a limited number of diagnostic studies are used to rule out other likely conditions. However, patients with alarm symptoms (*e.g.*, fever, weight loss, blood in stools, nocturnal of progressive abdominal pain), laboratory abnormalities, abnormal physical findings, and/or a family history of inflammatory bowel disease (IBD) or colorectal cancer (CRC) require more extensive evaluation (*e.g.*, imaging studies and/or colonoscopy)[2,3,10]. Otherwise, a limited number of diagnostic studies can rule out organic illness in the majority of patients and a sizeable number require no testing at all. However, whilst alarm symptoms ("red flags") may have a relatively modest predictive value for identifying organic disease, their presence as exclusion criteria would result in many missed cases of IBS[11]. It is this large symptomatic overlap between functional and organic disease, in conjunction with the current lack of a biochemical, histopathological, or radiological diagnostic tests for IBS, which engenders the need for more definitive diagnostic tools[2].

FAECAL MARKERS OF INTESTINAL INFLAMMATION

A simple, reliable, reproducible and non-invasive test, with the ability to differentiate IBD from other GI condition, such as IBS, would be of substantial clinical utility. Serological markers (*e.g.*, C-reactive protein, erythrocyte sedimentation rate) reflect the presence and intensity of a (systemic) inflammatory process and are not specific for intestinal inflammatory disease. Radiological and endoscopic techniques are invasive, time-consuming and/or expensive. Clinical disease (activity) scores are hindered by inaccuracies arising from subjective components. Faecal markers, however, offer a non-invasive approach to objectively measuring intestinal inflammation with the ability to differentiate organic and functional GI diseases. Stool markers are inexpensive, easily measured and therefore suitable for extensive use. Faecal markers include a heterogeneous group of substances that either leak from or are generated by the inflamed intestinal mucosa. The inflamed hyper-permeable gut mucosa is associated with increased protein cytokines and markers of neutrophil activation in faecal samples. Faecal markers of neutrophil influx into the mucosa are promising indicators of intestinal inflammation and their role has been mainly studied in discriminating IBD from non-IBD conditions (including IBS) rather than organic from non-organic diseases (Figure 1). Lactoferrin, polymorphonuclear (PMN) elastase and myeloperoxidase (MPO) are faecal markers of neutrophil degranulation. Of the proteins stored in neutrophilic granules, lactoferrin is the most accurate marker of intestinal inflammation. Importantly, lactoferrin, MPO and PMN elastase are not only expressed in neutrophils and show limited stability in stool samples at room temperature. Other faecal markers including alpha 1-antitrypsin, tumour necrosis factor alpha, lysozyme, and markers of eosinophil degranulation (*e.g.*, eosinophil protein X, eosinophil cationic protein) have also been described as markers of intestinal inflammation but their clinical utility and/or diagnostic accuracy is inferior and data on their role in differentiating IBD from IBS are lacking or very limited[12-20]. The utility of other faecal markers [*e.g.*, granins, defensins, matrix-metalloproteases (MMP)] in differentiating organic from functional disease has not been widely studied. More recently, neutrophil-derived S100 proteins have been identified as faecal markers for differentiating IBD and IBS. Proteins of

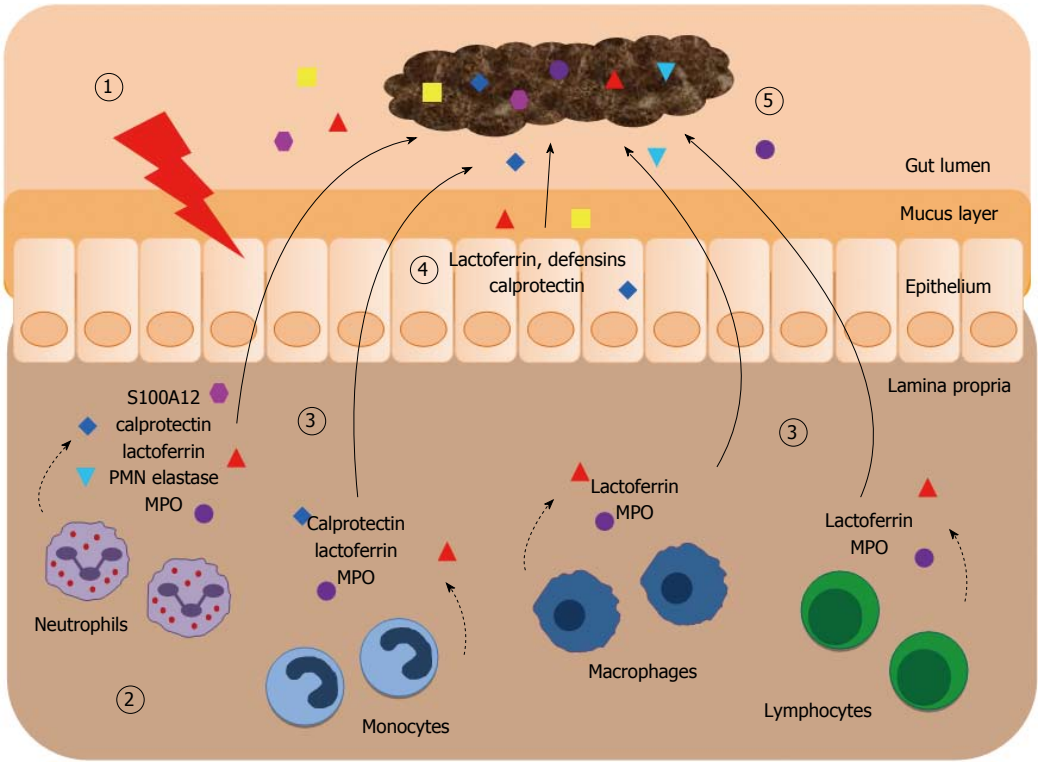

Figure 1 Faecal markers of intestinal inflammation. (1) Initially, unidentified triggers affect the epithelium and lead to an activation of the intestinal immune system; (2) The initiated immune response involves the influx of different innate immune cells (*e.g.*, granulocytes, monocytes, macrophages) and cells of the adaptive immune system (*e.g.*, T cells) into the affected mucosa. These cells actively secret inflammatory mediators or release granule proteins by cell degranulation. The contents of neutrophil granules [▲ lactoferrin, ▼ polymorphonuclear (PMN) elastase, ● myeloperoxidase (MPO)] have antimicrobial properties. The cytosol is the source of the damage associated molecular pattern proteins S100A8/A9 (◆ calprotectin) and S100A12 (●); (3) During early stages of intestinal inflammation these released proteins spill over from the mucosa into the gut lumen; (4) Some of these factors (including ■ defensins) are also released from the epithelium and the mucus layer; (5) In direct contact with the intestinal mucosa, the faecal stream contains the specific proteins of mucosal disease. The detection of these markers in faeces indicates the presence and degree of intestinal inflammation.

the S100 family [S100A8/A9 (calprotectin), S100A12] are molecules released from the cytosol by activated or damaged cells under conditions of cell stress, followed by pro-inflammatory activation of pattern recognition receptors. S100 proteins are remarkably resistant to degradation by faecal bacteria, making them suitable markers for gut wall inflammation[14]. Faecal S100A12 and calprotectin are highly sensitive and specific markers of intestinal inflammation and exert a strong influence upon the pathogenesis of IBD[21]. In this review, some of the most promising faecal markers, which have the potential both to differentiate IBD and IBS and to advance diagnostic practices, will be discussed (Figure 1).

CALPROTECTIN

S100A8 [also known as calgranulin A and myeloid-related protein 8 (MRP8)] and S100A9 (calgranulin B, MRP14) are members of the S100 calcium-binding protein family. Both proteins are linked to the innate immune system and expressed in granulocytes, monocytes/macrophages and epithelial cells (Figure 1)[14]. The two proteins exist in multiple isoforms, the most abundant of which is the S100A8/S100A9 heterodimer ("calprotectin")[22,23]. Calprotectin constitutes 60% of cytosolic protein in neutrophils and the influx of these neutrophils into the GI mucosa during inflammation is therefore proportional to the amount of measured faecal calprotectin[24,25]. Furthermore, calprotectin has not only shown resistance to degradation in faeces and stability at room temperature, but has also been reported to correlate well with ^{111}Indium-labelled granulocyte scintigraphy[24,26]. It is these favourable characteristic prerequisites for the validity of a faecal biomarker that have witnessed the emergence of calprotectin as one of the most studied faecal biomarkers for intestinal inflammation[27].

Elevated faecal calprotectin levels have been reported in multiple organic GI diseases when compared with functional GI diseases (Table 1). In a large-scale study, Tibble *et al*[28] determined that at a cutoff value of 10 mg/L, faecal calprotectin had a sensitivity of 89% and a specificity of 79% for detecting organic disease, which performed better than the respective values for a positive Rome I criteria diagnosis (85% and 71% respectively). Following this, Costa *et al*[29] discussed the value of setting a cutoff point determined by the collective results of complete GI investigations on all patients with chronic abdominal pain and diarrhoea. For example, by using a cutoff of 60 μg/g they were able to produce their optimal diagnostic accuracy, with a sensitivity of 81% and a specificity of 88%[29]. In another study, for patients presenting with lower GI symptoms, D'Incà *et al*[30] reported

Table 1 Studies investigating faecal markers in the differentiation of inflammatory bowel disease or healthy controls *vs* irritable bowel syndrome

Study	Marker	Cutoff value	Se	Sp	PPV	NPV	Subjects (*n*)	CD (*n*)	UC (*n*)	IBS (*n*)	HC (*n*)	Other (*n*)	Other diagnosis (*n*)	Verification
Kaiser *et al*[35] 2007	S100A12	> 0.8 mg/kg	86%	96%	98%	76%	195	32	27	24	24	88	Bacterial (65) and viral (23) enteritis	Endoscopy/histology; immunohistochemistry
	CP	> 50 mg/kg	63%	86%	90%	51%								
Sidler *et al*[42] 2008	S100A12	> 10 mg/kg	97%	97%	97%	97%	61	30	1	14	0	16	Reflux esophagitis (6), juvenile polyp (2); eosinophilic GI disorder (3), others (5)	Endoscopy/histology
	CP	> 50 mg/kg	100%	67%	75%	100%								
Tibble *et al*[26] 2000	CP	> 30 mg/L	100%	97%	-	-	276	31	0	159	56	30	Microscopic colitis (6), polyps (3), CRC (2), diverticulosis (19)	Radiology and/or colonoscopy
Tibble *et al*[28] 2002	CP	> 10 mg/L	89%	79%	76%	89%	602	102	87	339	0	74	Coeliac disease (12), diarrhea (14), CRC (7), colitis (6), small bowel enteropathy (21), diverticulosis (14)	Radiology and/or colonoscopy
Carroccio *et al*[32] 2003	CP	> 50 μg/g	66%	84%	83%	68%	158	18	0	55	20	65	Cow's milk/food intolerance (22), coeliac disease (23), CRC/polyps (3), diverticulosis (4), colitis (2), CD (9), giardiasis (2)	Endoscopy/histology (in selected patients only)
		> 100 μg/g	46%	93%	90%	59%								
Fagerberg *et al*[33] 2006	CP	> 50 μg/g	95%	93%	95%	93%	36	10	7	5	0	14	Indeterminate colitis/IBD (3), polyps (1), proctitis (1), food intolerance (4), others (5)	Endoscopy/histology
Sydora *et al*[47] 2012	CP	> 150 μg/g (desk top device)	56% -100%	100%	-	-	42	7	9	7	19	0	-	Mayo clinic or harvey bradshaw index
Dolwani *et al*[91] 2004	CP	> 60 μg/g	100%	79%	60%	100%	138	25	0	24	26	63	Symptoms of diarrhea and/or abdominal pain (63)	Barium follow through
Costa *et al*[29] 2003	CP	> 50 μg/g	83%	82%	90%	71%	239	49	82	48	34	26	Intestinal neoplasms (26)	Colonoscopy and/or radiology
Canani *et al*[31] 2006	CP	> 95 μg/g	93%	89%	93%	89%	45	17	10	8	0	10	Food allergy (5), infectious enterocolitis (4), familial Mediterranean fever (1)	Endoscopy, histology and radiology
Summerton *et al*[40] 2002	CP	> 50 mg/kg	82%	73%	-	-	134	4	10	7	28	85	CRC (8), upper-GI lesions (44), diverticulosis (15), polyps (12), colon adenoma (6)	Upper or lower endoscopy
Dai *et al*[92] 2007	LF	> 24 μg/g	100%	100%	-	-	177	18	59	25	34	41	Bacteria infectious bowel disease (41)	Colonoscopy
Walker *et al*[70] 2007	LF	> 7.25 μg/mL	84%	97%	99%	55%	170	79	62	7	22	0	-	Endoscopy and/or radiology (in selected patients only)
Kane *et al*[68] 2003	LF	> 4 μg/g	86%	100%	100%	87%	271	104	80	31	56	0	-	Clinical, radiographic, endoscopic, and histological criteria, as appropriate
Sidhu *et al*[71] 2010	LF	> 7.25 μg/g	67%	96%	87%	87%	465	104	126	137	98	0	-	Colonoscopy (in selected patients only), questionnaires
Schoepfer *et al*[25] 2008	CP	> 50 μg/mL	83%	100%	100%	74%	136	36	28	30	42	0	-	Endoscopy/histology
	LF	> 7 μg/mL	87%	96%	98%	77%								
D'Inca *et al*[30] 2007	CP	> 50 mg/kg	78%	83%	86%	-	144	31	46	20	0	47	CRC (8), polyps (26), diverticulosis (11), CD (2)	Colonoscopy/histology
	LF	> 0.04 OD	80%	85%	87%									
Otten *et al*[41] 2008	CP	> 50 mg/kg	96%	87%	65%	99%	114	6	5	91	0	12	Unspecified colitis/IBD (12)	Colonoscopy/sigmoidoscopy
	CP FRT	> 15 mg/kg	100%	95%	82%	100%								
	CP FRT	> 60 mg/kg	61%	98%	88%	91%								
	LF	> 7.25 mg/mL	78%	90%	67%	94%								
	LF FRT	> 128 ng/mL	78%	99%	95%	95%								
Schröder *et al*[38] 2007	CP	> 24 μg/g	93%	100%	100%	91%	76	25	20	31	0	0	-	Endoscopy/histology; clinical disease activity indices
	LF	> 8.9 μg/g	82%	100%	100%	80%								
	PMNE	> 19 ng/g	84%	87%	91%	79%								
Langhorst *et al*[36] 2008	CP	> 48 μg/mL	82%	84%	-	-	139	43	42	54	0	0	-	Clinical disease activity indices; endoscopy
	LF	> 7.05 μg/mL	87%	77%										
	PMNE	> 0.062 μg/mL	77%	77%										

Langhorst *et al*[37] 2009	HBD2	Median UC: 107 μg/g IBS: 76 μg/g HC: 30 μg/g	-	-	-	-	100	0	30	46	24	0	-	Endoscopy/histology; immunohistochemistry; faecal CP and LF
Ohman *et al*[87] 2012	CgB	< 0.48 nmol/g	78%	69%	-	-	111	0	0	82	29	0	-	Faecal CP; rectal sensitivity; colon transit time; questionnaires
	Sg II	> 0.16 nmol/g	80%	79%										
Annaházi *et al*[86] 2013	MMP-9	> 0.245 ng/mL	85%	100%	-	-	94	0	47	23	24	0	-	Clinical and endoscopic Mayo score; faecal CP
Silberer *et al*[39] 2005	CP	> 18.6 μg/g	62%	95%	-	-	119	21	18	40	40	0	-	Endoscopy/histology
	LF	> 6.64 μg/g	33%	95%										
	PMNE	> 124 ng/g	80%	95%										
Jeffery *et al*[82] 2009	M2PK	> 4 U/mL	67%	88%	47%	94%	199	9	1	91	94	4	Collagenous colitis (1), CRC (1), stricture (1), coeliac disease (1)	Colonoscopy (n = 87) or radiology (n = 4)
	CP	> 50 μg/g	93%	92%	62%	99%								
Chung-Faye *et al*[81] 2007	M2PK	> 3.7 U/mL	73%	74%	89%	57%	131	31	50	43	0	7	CRC (7)	Endoscopy/histology
	CP	> 25 μg/g	80%	74%	87%	65%								

Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CD: Crohn's disease; UC: Ulcerative colitis; IBS: Irritable bowel syndrome; HC: Healthy control; CP: Calprotectin; LF: Lactoferrin; PMNE: Polymorphonuclear elastase; MMP: Matrix-metalloprotease; HBD: Human β-defensin; Cg: Chromogranin; Sg: Secretogranin; MPO: Myeloperoxidase; M2PK: M2-pyruvate kinase; IBD: Inflammatory bowel disease; GI: Gastrointestinal; CRC: Colorectal cancer; FRT: Faecal rapid test.

a sensitivity, specificity and diagnostic accuracy of 78%, 83% and 80% respectively for diagnosing inflammatory disease, irrespective of diagnosis. Similar results have also been obtained in the paediatric population[31-33]. Carroccio *et al*[32] reported specificities which were in line with previous studies, but the sensitivities were far lower. This was attributed to a combination of a higher potential number of referrals for possible coeliac patients (due to their hospital being a tertiary centre for food intolerance), and the reported high frequency of negative calprotectin results for patients with coeliac disease. Furthermore, they highlighted the association between false-positive results for faecal calprotectin and both nonsteroidal anti-inflammatory drug use and liver cirrhosis, believed to be due to the mucosal abnormalities associated with each[34].

In efforts to highlight the potential of faecal calprotectin to distinguish between IBD and IBS specifically, a number of further studies have been performed[26,32,35-40] (Table 1). Langhorst *et al*[37] confirmed that faecal calprotectin was significantly raised (104 μg/g) in patients with active ulcerative colitis (UC) compared to faecal levels in patients with IBS (19 μg/g). In a slightly smaller prospective study, Schröder *et al*[38] reported that faecal calprotectin had a sensitivity of 93% and a specificity of 100% when differentiating IBD from IBS (cutoff 24.3 μg/g), though the diagnostic accuracy of calprotectin was not statistically significant superior to that of faecal lactoferrin or polymorphonuclear (PMN) elastase. The distinctly high diagnostic values found in this study were potentially due to a selection bias (their hospital represents a referral centre for IBD), which was supported by the exceptionally high number of patients suffering from IBD compared to IBS[38]. The comparative diagnostic accuracies between faecal calprotectin and other faecal markers have also been studied extensively[25,30,35,36,38,39,41,42]. Silberer *et al*[39] have reported a high and similar diagnostic accuracy of faecal calprotectin and PMN elastase for the differentiation of chronic IBD and IBS, which was superior to that of other leukocyte proteins in the faeces including lactoferrin and myeloperoxidase (MPO). In another such study, Schröder *et al*[38] reported that any combination of calprotectin, lactoferrin and PMN elastase did not improve their diagnostic accuracy in distinguishing between IBD and IBS, a result supported by other studies[36,38,41].

Correlation of faecal calprotectin with endoscopically and histologically assessed disease has always been the "gold standard" to ascertain its true prognostic value. Schoepfer *et al*[25] were able to demonstrate good correlation of faecal calprotectin with endoscopically assessed severity of disease in both Crohn's disease (CD) and UC. These findings were confirmed by a recent study reporting a significant correlation of faecal calprotectin levels and endoscopic disease activity in 126 included patients with IBD and 32 patients with IBS[43]. Following from this, the role of faecal calprotectin in being able to distinguish between IBD patients in remission with or without IBS symptoms has been investigated. Faecal calprotectin tends to be increased in subgroups of IBS-positive patients with IBD in remission, regardless of diagnosis[44,45]. Keohane *et al*[45] reported that both CD and UC patients with IBS-like symptoms had significantly higher faecal calprotectin levels than those without, most likely indicating the presence of ongoing subclinical inflammation rather than coexisting functional disease. Berrill *et al*[46] have reported that there is no statistical difference between the faecal calprotectin levels of patients with IBD

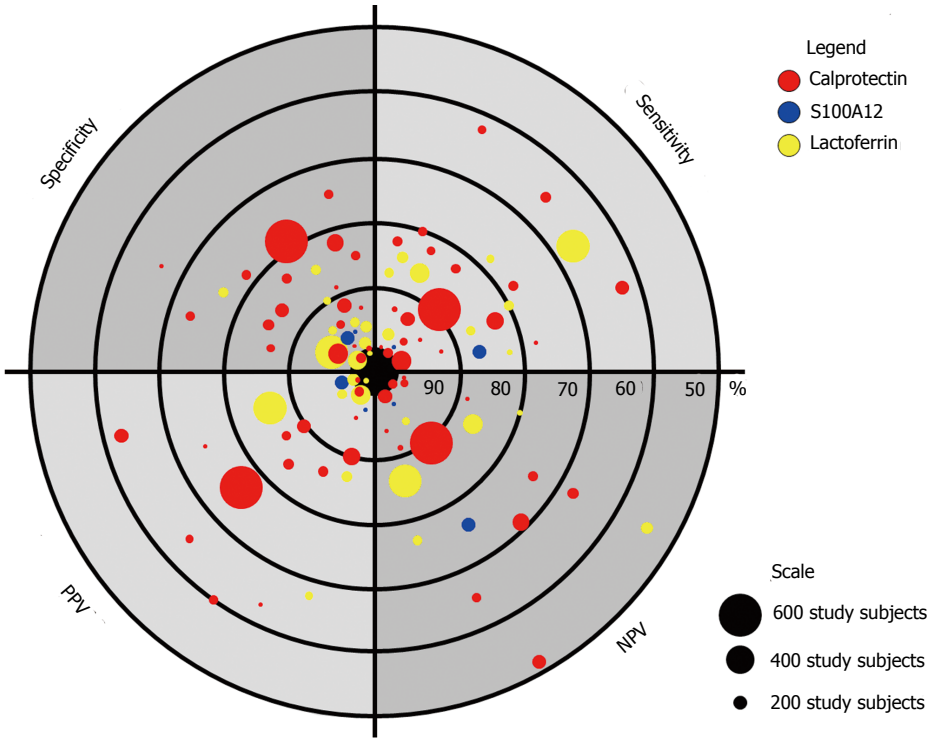

Figure 2 Diagnostic accuracy of faecal markers in the differentiation of organic gastrointestinal disease *vs* irritable bowel syndrome. The figure illustrates statistical measures of the diagnostic performance of different studies on the role of faecal markers in the diagnosis of irritable bowel syndrome. Sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) of different biomarker studies are represented with highest values close to the center of the "dartboard" (*i.e.*, 100%). Each dot represents a biomarker study and different colors represent the type of the faecal marker (see legend). The size of each dot represents the number of included study subjects (see scale).

Table 2 Overall diagnostic accuracy of faecal markers in the differentiation of inflammatory bowel disease *vs* irritable bowel syndrome in relation to the size of study cohorts

	Se	*n*	Sp	*n*	PPV	*n*	NPV	*n*	Ref.
Calprotectin	85%	2984	85%	2984	81%	2274	82%	2130	[25,26,28-33,35,36,38-42,47,81,82,91]
S100A12	89%	256	96%	256	98%	256	81%	256	[35,42]
Laktoferrin	78%	1811	94%	1811	91%	1376	82%	1232	[25,30,36,38,39,41,68,70,71,92]
M2-PK	69%	330	82%	330	64%	330	79%	330	[81,82]

Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; n: Number of study subjects; M2PK: M2-pyruvate kinase.

in clinical remission with IBS-type symptoms compared with those without. While faecal calprotectin may be useful as a noninvasive marker to distinguish patients with IBD in need of intensified follow-up, the utility of faecal calprotectin as an aid to discriminate between inflammatory and functional symptoms in IBD patients remains uncertain.

There have also been some interesting results of other faecal calprotectin analysis techniques. Otten *et al*[41] reported that faecal rapid testing of calprotectin had an associated sensitivity and specificity of 100% and 95% respectively, at a cutoff value of 15 mg/kg. Interestingly, these results outperformed those of the standard enzyme-linked immunosorbent assay (ELISA) faecal calprotectin test (Table 1)[41]. Similarly, Sydora *et al*[47] found that "desk top" faecal analysis devices reported sensitivities of 56%-100% and specificities of 100% when differentiating between IBD and IBS (cutoff 150 μg/g). However, these data were generated from a very small cohort (Table 1), and though showing promise should nonetheless be treated with caution at present. In addition, it was recently reported that different faecal calprotectin ELISA kits show a between-assay variability[48].

Since many disorders present with symptoms similar to IBS, it is important to exclude other causes like IBD. Overall, calprotectin is the most widely studied faecal marker for the differentiation between IBD and IBS and a sensitive and specific marker of inflammatory activity in the gut (Table 2, Figure 2). Because of its high diagnostic accuracy in ruling out intestinal inflammation, many clinicians use faecal calprotectin as a noninvasive screen for IBD in their patients with IBS symptoms[49].

S100A12

S100A12, also known as calgranulin C or EN-RAGE (extra cellular newly identified receptor for advanced

glycation end-products), is another member of the S100 calcium-binding protein family. In contrast to calprotectin, S100A12 is expressed almost exclusively by neutrophils (Figure 1) and does not form heterodimers with either S100A8, S100A9 or associate with the heterodimer S100A8/S100A9[50]. S100A12 was reported to function as a pro-inflammatory molecule, the binding of which to RAGE on endothelial cells, mononuclear phagocytes, and lymphocytes leads to upregulation of pro-inflammatory cytokines[51]. More recently, it was shown that S100A12 is a ligand of Toll-like receptor 4, amplifying monocyte activation and thus contributing to organ-specific as well as systemic inflammation[52]. Not surprisingly, S100A12 has been implicated in multiple inflammatory disorders[53-55]. S100A12 is strongly upregulated during chronic active IBD[21] and its release from intestinal mucosal specimens correlates to the intestinal inflammation status[56].

More recently, the value of S100A12 as a faecal biomarker of inflammatory conditions within the bowel has been investigated[42,57-62]. de Jong *et al*[62] showed that S100A12 was equally distributed in faeces, as well as being temperature stable for up to 7 d. Furthermore, in their study of 48 children, they reported that faecal S100A12 had a sensitivity of 96% and a specificity of 92% (cutoff 10 mg/kg) when distinguishing between healthy controls and the IBD group (mainly CD)[62]. In the wake of these findings we assessed the correlation between faecal S100A12 levels with endoscopic and histological findings in patients with IBD and IBS[35]. We demonstrated a sensitivity of 86% and a specificity of 96% (cutoff 0.8 mg/kg) when differentiating active IBD from IBS. Our study also showed a strong correlation between faecal S100A12 levels and endoscopically and histologically confirmed intestinal inflammation in both CD and UC. Our head-to-head comparison of faecal S100A12 and faecal calprotectin showed that faecal S100A12 was superior in distinguishing active IBD from IBS[35]. Similarly, in a prospective study of a paediatric population presenting with GI symptoms, Sidler *et al*[42] investigated the utility of faecal S100A12 compared to faecal calprotectin as a marker for intestinal inflammation. Children diagnosed with IBD (n = 31) had elevated faecal S100A12 (median 55.2 mg/kg) and faecal calprotectin (median 1265 mg/kg) levels when compared to 30 children without IBD (median S100A12 1.1 mg/kg; median calprotectin 30.5 mg/kg). The sensitivity and specificity of faecal S100A12 for the diagnosis of IBD (cutoff 10 mg/kg) were both 97%, whereas faecal calprotectin had a sensitivity of 100% and a specificity of only 67% (Table 1).

Though more recent studies into the role of S100A12 for diagnosis, prediction of outcomes and monitoring of disease responses for other GI diseases (including necrotizing enterocolitis and CRC) have been undertaken[58,59,63], further prospective studies into the role of S100A12 in distinguishing organic from functional disease are required to consolidate promising initial data (Table 2, Figure 2).

LACTOFERRIN

Lactoferrin is a multifunctional iron binding glycoprotein that is found in the secretions of most mucosal surfaces including tears, saliva, human breast milk, synovial fluid and serum[64]. Lactoferrin has been shown to exert bacteriocidal activity and is a major component of secondary granules released during the degranulation of polymorphonuclear neutrophils in response to inflammation[65,66]. In the intestinal lumen, the presence of inflammation triggers polymorphonuclear neutrophils to infiltrate the intestinal mucosa, causing a proportional increase of faecal lactoferrin levels (Figure 1)[67]. Lactoferrin demonstrates reasonable stability in faeces; it is unaffected by multiple freeze-thaw cycles, though it has been reported that after 48 h at room temperature, stool concentrations of lactoferrin declined slightly to 90% of their original levels[13,39,68].

Several studies have attempted to elucidate the utility of lactoferrin as a marker for intestinal inflammation, with variable outcomes[69]. Results were more variable when assessing the capabilities for lactoferrin as a distinguishing marker between IBS and IBD (Table 1). Compared to other proteins stored in neutrophilic granules such as PMN elastase, MPO, and human neutrophil lipocalin, Sugi *et al*[13] reported that lactoferrin was a superior faecal marker of neutrophil-derived intestinal inflammation. D'Incà *et al*[30] were able to quantify that, in colonoscopy referrals for lower GI symptoms, results of faecal lactoferrin assays yielded an overall sensitivity, specificity, positive predictive value (PPV), and diagnostic accuracy of 80%, 85%, 87% and 81% respectively in identifying intestinal inflammation. Similarly, Walker *et al*[70] reported that all of their included patients with IBS (n = 7) had normal levels of faecal lactoferrin (cutoff 7.25 μg/mL) and that the sensitivity, specificity, PPV and negative predictive value (NPV) for distinguishing individuals with IBD from those without IBD, were 84%, 97%, 99% and 55% respectively. Furthermore, in a recent meta-analysis, Gisbert *et al*[69] calculated the mean sensitivities and specificities of faecal lactoferrin in the diagnosis of IBD to be 80% and 82% respectively. Silberer *et al*[39] found that calprotectin and PMN elastase, but not lactoferrin, correlated with the severity of inflammation determined by ileocolonoscopy and were able to differentiate chronic IBD from IBS. When comparing receiver operating characteristic (ROC) curves calculated for healthy controls and patients with IBD, the areas under the curve (AUCs) for PMN elastase and calprotectin were 0.916 and 0.872 respectively, whilst that for lactoferrin was 0.693[39]. On the other hand, our recent review of studies on faecal markers of intestinal inflammation revealed that the diagnostic accuracy of faecal lactoferrin in the differentiation of IBD *vs* IBS had sensitivities and specificities between 56%-100% and 61%-100% respectively, with PPVs and NPVs of 59%-100% and 78%-99% respectively[14] (Table 1). In a more recent study, Sidhu *et al*[71] were further able to demonstrate that patients with inactive IBD had significantly higher median faecal lactoferrin levels than those with IBS. Of particular interest were the results of Otten *et al*[41] showing that new faecal rapid testing techniques for evaluating faecal lactoferrin in the primary care setting were

at least comparable to the more standard ELISA tests when testing 114 patients referred for lower GI endoscopy for investigation of abdominal complaints (bloating, change in defecation frequency or consistency, or blood and mucus in the faeces) (Table 1).

Considering these positive results, the main disadvantages of faecal lactoferrin stem from its non-specificity to any particular organic disease and by the fact that it is not solely expressed by degranulated neutrophils. Lactoferrin is secreted endogenously by several mucosal epithelial cell types and can therefore act as a non-inflammatory induced source of faecal lactoferrin[72]. Furthermore, it has been reported that the use of non-steroidal anti-inflammatory drugs may increase the amount of lactoferrin detected in faeces, probably due to an associated induced enteropathy[32,73,74].

Similarly to S100 proteins, it should be emphasized that lactoferrin itself is not a marker of any specific organic disease, but rather of neutrophilic intestinal inflammation[75]. A negative faecal lactoferrin test, therefore, should only be seen as the absence of significant neutrophilic intestinal inflammation. It has consequently been proposed that faecal lactoferrin may have a role in excluding underlying inflammatory conditions thus removing the need for colonoscopy in patients presenting undifferentiated diarrhoea with no alarm symptoms[76]. In studies designed to compare IBD patients with healthy controls or IBS, direct comparison of calprotectin and lactoferrin revealed comparable levels of diagnostic accuracy (Tables 1 and 2)[25,30,36,38,39,41]. These conclusions support the notion that although lactoferrin may be of limited use in the direct classification or diagnosis of organic disease, it may yet have utility in IBD diagnosis.

M2-PYRUVATE KINASE

The glycolytic enzyme M2-pyruvate kinase (M2-PK) is a multifunctional protein, involved in several nonglycolytic pathways influencing cellular physiology including immunological responses, cellular growth and apoptosis[77]. The dimeric isoform of M2-PK (tumor M2-PK) is present in undifferentiated and proliferating tissues and M2-PK is upregulated in a range of GI malignancy[78]. The determination of M2-PK in stool samples was proposed as a new promising screening tool for CRC[79]. The usefulness of faecal M2-PK for the detection of intestinal inflammation was also studied in patients with IBD since these patients have increased cell turnover in the GI tract. The PK stool test requires a single, small and random faecal sample whilst the enzyme is stable for two days at room temperature[80]. Czub *et al*[80] have reported that faecal M2-PK could potentially be a useful marker for IBD activity with a better correlation for UC patients. Likewise, Turner *et al*[61] showed that faecal M2-PK reflects severity of paediatric UC by having very high faecal values. Furthermore, the authors demonstrated that faecal M2-PK has, in contrast to other faecal biomarkers (calprotectin, lactoferrin, S100A12), the best ability to predict steroid refractoriness in severe paediatric UC, but is still inferior to a clinical disease activity index[61]. Importantly, it has also been shown that faecal M2-PK is able to differentiate between patients with IBD or IBS (cutoff 3.7 U/mL) and that M2-PK and faecal calprotectin are highly significantly correlated[81]. In this study 67% of included patients (n = 88) had organic GI disease and faecal M2-PK had a sensitivity of 73%, specificity of 74%, PPV of 89%, and a NPV of 57% for IBD and CRC. These results were comparable to the diagnostic accuracy of faecal calprotectin (cutoff 25 μg/g) in the same patients with a sensitivity of 80%, specificity of 74%, PPV of 87%, and a NPV of 65% (Table 1). Jeffery *et al*[82] showed that, in a setting of a low prevalence or organic bowel disease, faecal M2-PK is able to differentiate organic disease from functional bowel disease (cutoff 4 U/mL) with a sensitivity of 67%, specificity of 88%, PPV of 47% and a NPV of 94%. In this study the incidence of functional bowel disorder was much higher (87% of included patients; n = 91) than in the aforementioned study (33% of included patients; n = 43) and the results showed that M2-PK does not perform as well as calprotectin (cutoff 50 μg/g; sensitivity 93%; specificity 92%, PPV 62%, NPV 99%) (Table 1)[82]. The authors concluded that use of calprotectin and M2-PK may be particularly advantageous as a rule-out test in clinical populations with a similar disease prevalence.

POLYMORPHONUCLEAR NEUTROPHIL ELASTASE

PMN elastase is a neutral serinproteinase, which is released from leucocyte granules as a mediator of inflammation by activation of neutrophils. Elastase is stable for four days in faeces at room temperature[39]. Silberer *et al*[39] showed that faecal PMN elastase levels in patients with IBS (n = 40) were in the range of healthy persons (n = 40). Faecal PMN elastase and calprotectin correlated with endoscopically classified severity of intestinal inflammation and yielded similar AUCs when ROC curves were calculated for healthy persons and patients with IBD (n = 39). The authors concluded that faecal PMN elastase and calprotectin are able to differentiate between chronic IBD and IBS. Similarly, Langhorst *et al*[36] showed that faecal PMN elastase, calprotectin and lactoferrin differentiate IBD and IBS. Patients with IBS (n = 54) demonstrated significantly lower levels of PMN elastase in stools when compared to patients with endoscopically active IBD (n = 60) and, interestingly, when compared with endoscopically inactive IBD (n = 25). The specificity and overall diagnostic accuracy of PMN elastase in patients with IBS were each 82% and slightly lower than for faecal lactoferrin (83%), faecal calprotectin (87%), and serum CRP (91%). Schröder *et al*[38] prospectively evaluated the diagnostic accuracy of faecal PMN elastase alone (cutoff 62 ng/g) and in combination with faecal calprotectin (cutoff 15 μg/g) and/or lactoferrin (cutoff 7.3 μg/g) to detect intestinal inflammation in patients with IBD (n = 45) and IBS (n = 31)[38]. The sensitivity, specificity, PPV, and NPV

of faecal PMN elastase in distinguishing between IBD and IBS was 84%, 87%, 91% and 79%, respectively, and increased to 96%, 100%, 100% and 94%, respectively, when combined with faecal calprotectin ± lactoferrin. The odds ratio for having intestinal inflammation with an elevated faecal PMN elastase was 37 (95%CI: 12-116). However, the results of the study indicate an advantage of calprotectin over lactoferrin and PMN elastase in the detection of intestinal inflammation.

HUMAN β-DEFENSIN-2

Defensins belong to the class of protective antimicrobial peptides and play an important role in the host innate defense at the mucosal surface of the GI tract (Figure 1). Human β defensins (HBD) are expressed in the colon by epithelial cells and plasma cells. HBD-2 plays a crucial role in determining innate immune responses to bacteria in the gut. Cumulating evidence suggests a special role for HBD-2 as a marker for intestinal inflammation in IBD[83]. Interestingly, Langhorst *et al*[84] reported that elevated faecal levels of HBD-2 indicate an activation of innate immunity not only in IBD but also in IBS[37,84]. Faecal HBD-2 levels of patients with IBS (n = 46) were significantly elevated compared with health controls (n = 24) and similar to those in patients with active UC (n = 30), whereas faecal levels of calprotectin and lactoferrin did not differ between healthy controls and patients with IBS. These findings suggest a pro-inflammatory activation of the mucosal innate immune system in patients with IBS in the absence of endoscopic or histologic signs of inflammation. These results support the idea that IBS could be a (low-grade) inflammatory disorder though the functional significance remains to be established.

MYELOPEROXIDASE

MPO is another lysosomal protein that is released from granules of neutrophil granulocytes during inflammation (Figure 1). MPO produces oxygen radicals during the neutrophil's respiratory burst, which are important in the killing of bacteria. MPO is stable for at least four days in feces at room temperature[39]. To date, MPO has shown to be of only limited utility as an inflammatory marker for IBD[85]. Thus, the use of MPO in the differentiation between IBS and IBD has not been widely studied (Table 1). In addition, Silberer *et al*[39] found that MPO separated healthy controls (n = 40) and patients with IBS (n = 40) from patients with chronic IBD (n = 39) less effectively than PMN elastase or calprotectin.

MATRIX-METALLOPROTEASE 9

MMPs are a family of zinc-dependent endopeptidases capable of degradation of extracellular matrix proteins. MMPs are secreted by various cell types including tumor cells and several immune cell types. MMP-9 is released from neutrophils and elevated in colonic biopsies, urine, and blood plasma of patients with UC[86]. Annaházi *et al*[86] compared faecal MMP-9 levels in patients with UC (n = 47) with those of patients with diarrhea predominant IBS-D (n = 23) and healthy controls (n = 24). Healthy controls and patients with IBS-D showed very low faecal MMP-9 levels compared with faecal levels of patients with UC. The sensitivity and specificity of faecal MMP-9 in distinguishing between UC and IBS-D was 85% and 100%, respectively (cutoff 0.245 ng/mL). Faecal MMP-9 levels correlated significantly with faecal calprotectin levels. The authors suggested that faecal MMP-9 could be a novel marker to help in the differential diagnosis of patients with diarrhea and abdominal pain. However, this is the first published study on the diagnostic role of faecal MMP-9 in IBD and IBS and further studies are needed to confirm these findings.

GRANINS

Granins are proteins expressed by cells of the enteric, endocrine, and immune system, and may broadly reflect activity of these systems. Chromogranins (Cg) and secretogranins (Sg) are precursors of several bioactive peptides and regulate a number of cellular functions. Öhman *et al*[87] assessed the association between faecal levels of Cg and Sg with IBS. The results showed that, compared to healthy controls (n = 29), IBS patients (n = 82) demonstrated higher levels of CgA, SgⅡ, and SgⅢ, but lower levels of CgB. Thus, faecal levels of SgⅡ, SgⅢ and CgB may be used to discriminate between IBS patients and healthy individuals. However, there was no disease control group included in this study, which therefore precludes the proper evaluation of faecal granins as diagnostic biomarkers. Faecal granins are however unlikely to be specific IBS markers since other diseases (*e.g.*, coeliac disease) also manifest increased Cgs[88]. Furthermore, faecal calprotectin levels were not associated with the faecal concentrations of granins. Finally, the study design cannot differentiate whether the increased faecal levels of granins cause IBS or its symptoms, or merely reflect the phenotype of IBS. Elevation of faecal granins may serve as a marker for guiding medical treatment of IBS. However, the lack of specificity of faecal granins does not support the use of these proteins as positive biomarkers for IBS.

CONCLUSION

Extensive diagnostic tests in the evaluation of patients with typical symptoms of IBS and the absence of alarm features are not necessary[89]. A positive diagnostic strategy based on symptom-based criteria and simple blood tests is not inferior to a strategy of exclusion of organic disease with multiple unnecessary, expensive, and potentially harmful diagnostic tests and procedures[90]. Faecal surrogate markers of intestinal inflammation represent a practicable, inexpensive and objective diagnostic tool to differentiate organic and functional GI diseases. Neutrophil-derived faecal biomarkers show a high diagnostic accuracy in the differentiation of IBD *vs* IBS (Table 2) and could be useful in reducing unnecessary invasive investi-

gations. Thus, these markers can provide reassurance to physicians that their clinical diagnosis of IBS is correct. Further studies are required to more comprehensively define and compare the role of these faecal proteins in the diagnosis and pathogenesis IBS. Nonetheless, faecal biomarkers have the potential to be incorporated into standard clinical practice for the routine assessment of IBS and IBD.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Molecular basis of the irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a functional disorder characterized by abdominal pain, discomfort and bloating. The pathophysiology of IBS is poorly understood, but the presence of psychosocial basis is now known. There is an increasing number of publications supporting the role of genetics in IBS. Most of the variations are found in genes associated with the brain-gut axis, revealing the strong correlation of brain-gut axis and IBS. miRNAs, which play critical roles in physiological processes, are not well studied in IBS. However, so far there is found an involvement of alterations in miRNA expression or sequence, in IBS symptoms. IBS phenotype is affected by epigenetic alteration and environment. Changes in DNA and histone methylation are observed in patients who suffered childhood trauma or abuse, resulting in altered gene expression, such as the glucocorticoid receptor gene. Finally, diet is another factor associated with IBS, which may contribute to symptom onset. Certain foods may affect on bacterial metabolism and epigenetic modifications, predisposing to IBS.

Key words: Irritable bowel syndrome; Gastrointestinal diseases; Genetics; Epigenetics; Diet

Core tip: Irritable bowel syndrome (IBS) is a multifactorial disease, whose development and phenotype are related to both genetic and epigenetic factors. Gene polymorphisms and epigenetic modifications affect the function of brain-gut axis and are responsible for many of the symptoms of the disease. The relationship between environmental factors and IBS shows the effect of environment on gene expression alteration by epigenetic modification.

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INTRODUCTION

Irritable bowel syndrome (IBS) is amongst the most widely recognized functional gastrointestinal disorders and is remarkably prevalent in the general population, affecting as many as 5%-20% of people worldwide[1]. The prevalence of IBS is slightly higher in women, with a variable influence of age across studies. Symptom based criteria is applied to diagnose the entity. The presence of chronic or recurrent abdominal pain or discomfort, relieved by defecation and associated with an altered bowel habit, in the absence of any underlying structural or bio-

Table 1 Genetic alterations on irritable bowel syndrome

Gene	Polymorphism	Ref.
Serotonergic system		
SERT promoter	5-HTTLPR, deletion	[13-17]
	rs25531	[81]
HTR3A	-42C > T	[50]
HTR3B	386A > C	[82]
HTR3C	489C > A	[83]
HTR3E	rs62625044	[50]
Adrenergic and opioidergic system		
α_2-adrenergic receptor	α2C del 322-325, deletion	[19]
	α2A-1291C > G	[19,84]
COMT	α2A-1291 C > G	[20]
	$Val^{158}Met$	[21,22]
CNR1	(AAT)n triplet repeat	[23]
	rs806378	[24]
CRH-R1	rs7209436	[27]
	rs242924	[27]
BDNF	$Val^{166}Met$	[85]
OPRM1	118A > G	[85]
Cytokines		
IL-10	-1082 A > G	[28-30]
	396 T > G	[30]
	-819T > G	[34]
TNF alpha	-308G > A	[28]
	-238G > A	[86]
GNβ3	825C > T	[32]
TLR9$^+$	-1237T > C	[84]
	2848 G> A	[84]
IL1R	Pst- I 1970C > T	[86]
IL4	-590C > T	[84,87]
	-33T	[87]
IL6	-174G > C	[84,86]

SERT: Serotonin reuptake transporter; COMT: Catechol-*O*-methyltransferase; CNR1: Cannabinoid receptor 1; CRH-R1: CRH receptor 1; IL: Interleukin; TNF: Tumor necrosis factor.

chemical abnormalities, identifies patients with IBS[2].

The syndrome has been subdivided into different subgroups based on the predominant bowel habit; diarrhea-predominant (D-IBS), constipation-predominant (C-IBS), or a mixture of both diarrhea and constipation (M-IBS). The use of these subgroups has received acceptance by most clinical investigators, as it commonly dictates symptomatic pharmacological management[3]. However, the value of this categorization is under consideration, knowing that each IBS patient could switch from one subgroup to another over time.

There is a significant variability in the clinical presentation of patients with IBS and they could differ by predominant stool type, severity and frequency of pain/discomfort and comorbidities including psychological distress and somatic complaints[4]. Moreover, IBS symptoms can fluctuate over time. The severity and intensity of IBS symptoms vary from very mild in patients who do not seek medical attention to very severe one that may significantly affect quality of life with the same degree of impairment as major chronic disorders. Despite the fact that a minority of IBS patients chooses to consult a physician, IBS is a clinical problem of considerable cost for the health care system because of its high prevalence and the chronic or recurrent nature of symptoms[5].

The pathophysiology of IBS is largely unknown and it is generally considered a multifactorial disorder. Among the putative mechanisms involved in the pathogenesis of IBS, there is evidence to support the key role of heritability and genetics factors. It is recognized that psychological factors and stress appear to be the primary drivers of symptoms in IBS patients. There is a hypothesis that IBS patients have a certain personality with predisposition to develop the disease. Dimensions of personality that are important in clinical practice include response to stress, attitude toward illness, health and medical treatment. These constitutional features may have genetic origins that may be influenced by early environmental experiences.

GENETICS AND IBS

Gene polymorphisms

IBS, as a multifactorial disorder, is also associated with altered brain-gut axis[6]. A recent study showed that corticotrophin-releasing hormone (CRH) is involved in stress-related pathophysiology of IBS and in the inflammation of the intestinal mucosa[7]. Polymorphisms in genetic factors may influence these mechanisms, and affect brain-gut interrelations[8-10]. Polymorphisms involve the serotonergic, adrenergic and opioidergic systems, and genes encoding proteins with immunomodulatory and/or neuromodulatory features[9,10].

Serotonergic system

Serotonin [5-hydroxytryptamine (5-HT)] controls gastrointestinal secretion, motility, and visceral perception by activating at least five types of receptors[10]. Alterations in 5-HT levels and signaling are present in IBS patients which may induce diarrhea, nausea, and vomiting[11,12]. So far, only a few gene polymorphisms are associated with IBS. Polymorphisms in promoter of serotonin reuptake transporter (*SERT*) gene effect on transcription activity and influence 5-HT reuptake efficiency. In a recent study, among 9 polymorphisms in promoter region of SERT, only one polymorphism (insertion/deletion polymorphism) was associated with diarrhea in women with IBS. The deletion polymorphism decreases expression of the sodium-dependent serotonin transporter and, thus, reduces reuptake of serotonin[13]. Another study showed a lower prevalence of the SS genotype (homozygosity for deletion) in IBS and, particularly, in D-IBS, but this was only observed in male patients[14] (Table 1).

This polymorphism is also correlated with behavioral traits and psychiatric disorders and IBS patients homozygous for the deletion present significantly higher risk for depressive episodes[15]. Another study also associated insersion/deletion polymorphism with anxiety. Long allele (insertion) in females is implicated with negative emotion but acts contrary in males[16]. This allele influences the efficacy of tegaserod treatment. IBS patients

carrying the long allele respond poorly to treatment[17].

Adrenergic and opioidergic systems

Autonomic system has an important role in gastrointestinal motility, acting *via* adrenergic receptors. Genetic variations in α_2-adrenergic receptor may change sensory and motor function in IBS[18]. α2C Del 322-325 deletion, a variation resulting in a loss-of-function phenotype, is associated with C-IBS (constipation IBS)[19]. The α2A -1291 C>G is associated with D-IBS, but no with C-IBS[20] (Table 1).

A polymorphism (Val^{158}Met) in catechol-*O*-methyltransferase, an enzyme metabolizing catecholamines, showed association with IBS[21]. Patients carrying this polymorphism have a reduced response to pain[22] (Table 1).

Alterations in cannabinoid receptor genes are also analysed and associated with IBS. A polymorphic (AAT)n triplet repeat in the 3-flanking region of the cannabinoid receptor 1 (*CNR1*) gene is related with IBS and severity of abdominal pain in IBS[23] (Table 1).

Additionally, single nucleotide polymorphisms (SNPs) in CRH receptor 1 (CRH-R1), which plays a critical role in stress-induced pathophysiology of IBS, were studied for moderating IBS phenotype and negative emotion in IBS patients (Table 1). Findings of this study showed association between SNPs and IBS moderation, but no association was found with negative emotion[24]. Genetic variation rs806378 in CNR1 is associated with colonic transit in D-IBS and sensation rating of gas[25] (Table 1). This polymorphism is also correlated with treatment effectiveness of nonselective cannabinoid receptor agonist, dronabinol[26,27].

Cytokines

Several studies have reported cytokine gene polymorphisms in IBS. Interleukin (IL)-10-1082 G/G, a high producer IL-10 genotype, correlated with lower risk for developing IBS[28,29] (Table 1). Gene SNPs of IL-8 and IL-10 were also analyzed by Romero-Valdovinos *et al*[30] and an association between alleles IL-8$^+$ 396G and IL-10-1082A and IBS was found. These findings were confirmed by other study[31] (Table 1). TNF alpha (-308 G/A) polymorphism and IBS are correlated, and G/G genotype may increase risk of IBS. G/A genotype has a protective role[28] (Table 1). A study evaluating GNβ3 825C>T polymorphism in IBS showed significant interactions between gastrointestinal infection and T allele in the development of IBS, suggesting gene-environment interactions[32] (Table 1). However, another study replicated none of these results[33]. Another IL-10 polymorphism associated with IBS is IL-10-819 T>C. The frequency of IL-10 -819 CC genotype was significantly higher in D-IBS[34] (Table 1).

miRNAs and IBS

miRNAs are small (21-23 nucleotides) single-stranded RNA molecules[35,36]. miRNAs are not translated into proteins and have regulatory function, such as translational repression of targeted mRNAs[37]. miRNAs form RNA-induced silencing complex, which can prevent the expression of proteins, either by activating endonuclease that degrades mRNAs or by blocking translation[38]. miRNAs are connected with physiological processes such as cell division and death[39], cellular metabolism[40], intracellular signaling[41], immunity[42] and cell movement[43]. Thus, altered miRNA expression can affect these critical processes, and as a result, lead to various pathological and occasionally malignant outcomes.

Cancer is one of human diseases clearly associated with miRNA regulation. miRNAs may involve in tumor development as tumor suppressors or oncogenes. They also play roles in tumor invasion and metastasis. Down-regulation of miR-15 and miR-16 is correlated with the pathogenesis of B-cell chronic lymphocytic leukemia[44]. In addition, miR-125b, miR-145, miR-21 and miR-155 expression is associated with the increased risk of breast cancer[45]. The implication of miRNAs in immune-related diseases, such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), and type I / II diabetes is also known. In MS, miR-34a, miR-155 and miR-326 are overexpressed[46]. In SLE, increased risk of disease development is associated with decreased expression of miR-46a[47]. Several studies show that miRNAs regulate critical pathways in inflammation, such as pathways correlated with nuclear factor kappa beta. miR-155 and miR-146 are the best characterized miRNAs which are implicated in immune-diseases[46,48,49].

The role of miRNAs in IBS is not well studied. The first association of miRNAs and IBS was from Kapeller *et al*[50]. This study showed that the variation c.*76G>A (rs62625044) in the 3' untranslated region (UTR) of the serotonin receptor type 3 subunit genes *HTR3E* correlates with D-IBS. This functional variation is located in the miRNA-510 target site of the gene. The co-localization of HTR3E and miR-510 in enterocytes of the gut epithelium and the presence of cis-regulatory variation show the regulation of serotonin receptor gene expression by miRNA.

Next evidence came from Zhou *et al*[51], who evaluated the miRNA expression in blood microvesicles (circular membrane fragments that are shed from the cell surfaces and accompanies cell activation) and gut tissues in D-IBS patients and IBS patients with normal membrane permeability. They found that miR-29a expression was increased in blood microvesicles in the small bowel and colon tissues of IBS patients with increased permeability. miR-29a is complementary in the 3' UTR of the glutamine synthetase gene. These results suggest the role of glutamine synthetase in the intestinal membrane permeability and the role of miR-29a in regulation of glutamine synthetase and intestinal membrane permeability.

EPIGENETICS AND IBS

Phenotype is the combination of DNA sequence, epigenetic DNA modifications and environmental factors.

The presence of epigenetic changes in monozygotic twins, leading to phenotypic alterations, suggests a potential role of epigenetics in IBS[52]. DNA methylation and histone modification are the most common epigenetic mechanisms. DNA methylation usually silences gene expression[53]. However, histone acetylation or methylation may activate or not gene transcription[54].

IBS is associated with early life trauma or abuse, and this condition leads to negative health outcomes and behaviors in adults. Childhood trauma influences somatic symptoms and neural network development and neuroendocrine system development[55-57]. In a recent study, IBS patients showed enhanced cortisol response to a visceral stressor. The hypothalamic-pituitary-adrenal (HPA) axis hyperresponsiveness to stressor is more related to early adverse life events rather than to the presence of IBS[55].

Early childhood trauma decreases glucocorticoid receptor expression by hypermethylation of glucocorticoid receptor gene[58]. Altered glucocorticoid receptor gene expression, which mediates the negative feedback of the HPA axis, reduces the capability of HPA to deal effectively with stress. In animal model of IBS, animals exposed to perinatal stress had methylation of glucocorticoid receptor promoter, decreased gene expression and prolonged elevation of corticosterone levels[59].

The impact of early adverse life events on developing IBS or other diseases is being explored lately. Gluckman *et al*[60] developed a hypothesis that epigenetic processes, including DNA methylation and histone modification, partially mediate developmental plasticity. Another group searched for a mechanism that link the social environment early in life and long-term epigenetic programming of behavior and responsiveness to stress. They took into account data suggesting that DNA methylation is a dynamic process and postmitotic cells may change methylation pattern responding to different environmental stimuli. This study showed that maternal licking and grooming in the rat triggered activation of 5-HT receptors, activation of the transcription factor nerve growth factor-induced gene A and acetylation of the promoter of the glucocorticoid receptor (mediated by a histone acetyl transferase), leading to differential epigenetic programming of the glucocorticoid receptor[61].

Alterations in acetylation motif change behavior in adult offsprings. Except maternal care, diet may also affect behavioral plasticity[62]. Maternal separation acts as a stressor and helps adult rats to develop intestinal mucosal dysfunction, increased HPA axis responses and anxiety-like behavior[63].

Finally, early life stress increases the levels of proinflammatory cytokines. In IBS patients, levels of IL-6 and IL-8 were high, as a result of epigenetic glucocorticoid alterations[64,65]. Upregulation of proinflammatory cytokines influences tryptophan metabolism, resulting on changes of 5-HT activity[66]. The kynurenine:tryptophan ratio, which shows tryptophan catabolism, is increased in IBS patients with severe symptoms, and they were more likely to have depression or anxiety.

DIET, NUTRIGENOMICS/ NUTRIGENOMICS AND IBS

It is well documented, that the interplay between genes and diet may be reflected in susceptibility to various diseases[67]. Scientific studies have demonstrated the effectiveness of dietary therapies in alleviating the symptoms and even in altering the progression of inflammatory and autoimmune disorders[68,69]. Concerning the IBS, even if many patients recognize the impact of specific diet in symptom occurrence, limited population-based studies have evaluated the importance of diet in IBS and its role remains uncertain[70-72]. Diet may contribute to symptom onset through several mechanisms such as food allergy and intolerance. Additionally, certain food may alter the composition of the luminal milieu, directly or indirectly through effects on bacterial metabolism. Diet is known also to influence the epigenetic modification of genes[73]. Finally, IBS symptoms may develop following exposure to food-born pathogens[72]. Furthermore, an increase probability of developing IBS is associated with the inheritance of a number of contributory genetic polymorphisms, as well as with the altered expression of certain genes[74]. The variant forms of genes often result in an abnormal response to normal gut bacteria that may be changed through inappropriate diet or environment. Shifts in the bacterial makeup of the human gut microbiota have been associated with gut disorders including IBS[75]. In the field of nutritional research, 2 terms have been established: nutrigenomics which aims to study how genotype determines optimal dietary requirements for health on an individual basis, and nutrigenomics which studies the effect on diet on DNA structure and gene expression[76]. However, the most of the nutrigenetic/nutrigenomic work has focused on cardiovascular disease, type Ⅱ diabetes mellitus or inflammatory bowel disease[77,78] and no study has be done on IBS. Low FODMAPs diet, that is elimination of fermentable Oligo-, Di- and Mono-saccharides, and Polyols from diet, is an area of intense investigation for symptoms' alleviation[79,80]. FODMAPs' ingestion could result in the symptomatology of these patients, because they are osmotically active, they are fermented and through bacterial overgrowth can cause bloating, pain and the sequence of symptomatology in IBS. Thus, the application of these approaches in the field of IBS research is open. It is hoped that the nutrigenomics/nutrigenomics implementation will promote the understanding of diet-gene interactions and facilitate a better characterization of individual IBS patients for further identification of nutritional patterns that allow personalized therapies.

CONCLUSION

IBS is a multifactorial disease, whose development and phenotype are related to both genetic and epigenetic

factors. Most factors involve in pathogenesis by causing changes in gene expression. Gene polymorphisms and epigenetic modifications affect the function of brain-gut axis and are responsible for many of the symptoms of the disease. IBS is one of the diseases where the environmental influence is strong. Early life incidents and diet habits play an important role in disease development. The relationship between environmental factors and IBS shows the effect of environment on gene expression alteration by epigenetic modification.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Is irritable bowel syndrome an organic disorder?

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Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is generally considered to be functional because there appears to be no associated anatomical defect. Stress and psychological factors are thought to play an important role in IBS. The gut neuroendocrine system (NES), which regulates all functions of the gastrointestinal tract, consists of endocrine cells that are scattered among the epithelial cells of the mucosa, and the enteric nervous system. Although it is capable of operating independently from the central nervous system (CNS), the gut NES is connected to and modulated by the CNS. This review presents evidence for the presence of an anatomical defect in IBS patients, namely in the gastrointestinal endocrine cells. These cells have specialized microvilli that project into the lumen and function as sensors for the luminal content and respond to luminal stimuli by releasing hormones into the lamina propria, which starts a chain reaction that progresses throughout the entire NES. The changes in the gastrointestinal endocrine cells observed in IBS patients are highly consistent with the other abnormalities reported in IBS patients, such as visceral hypersensitivity, dysmotility, and abnormal secretion.

Key words: Cholecystokinin; Dysmotility; Endocrine cells; Enteric nervous system; Ghrelin; Peptide YY; Secretion; Secretin; Serotonin; Visceral hypersensitivity

Core tip: This review presents recent observations in irritable bowel syndrome (IBS) patients that point toward the existence of an anatomical defect in the gastrointestinal endocrine cells. It includes also an argument that IBS is an organic disorder and that the abnormalities in the gastrointestinal endocrine cells can explain the visceral hypersensitivity, dysmotility and abnormal secretion reported in these patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal disorder with a reported prevalence of 5%-20% and an incidence of about 200 per 100000 of the world population[1-29]. Patients with IBS suffer from recurrent abdominal pain/discomfort and altered bowel habit, which vary in both degree and time pattern between patients: from tolerable to severe, and from daily symptoms to intervals of weeks/months, respective-

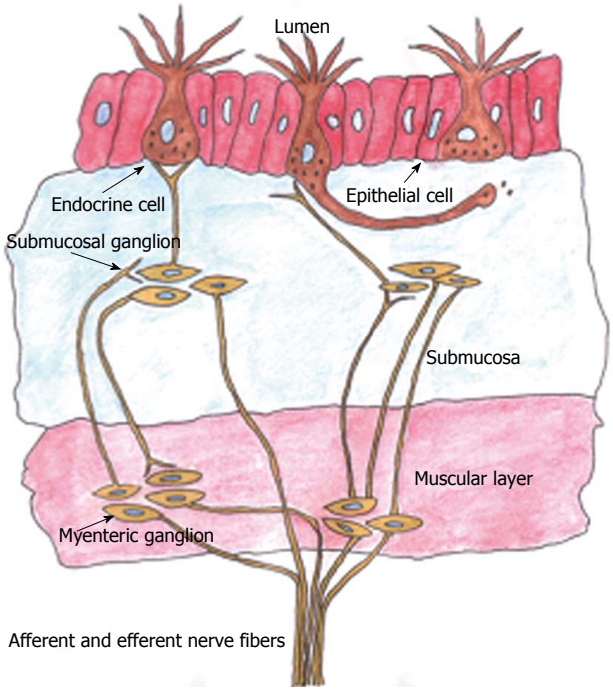

Figure 1 Schematic illustration of the gut neuroendocrine regulatory system. The endocrine cells are scattered among the epithelial cells and have specialized microvilli that project into the lumen and function as sensors of the gut contents, and they respond to luminal stimuli by releasing hormones into the lamina propria, where they exert their action locally on nearby structures. These endocrine cells interact with the enteric nervous system, which is in turn connected to and modulated by the central nervous system through afferent and efferent nerves.

ly[3,11,30-40]. IBS is more common in females than males, and in patients younger than 50 years of age[3,11,14,15,19,21,26,30,31,33-40].

IBS is not associated with the development of serious disease or with an excessive mortality rate[41,42]. However, it considerably reduces the sufferer's quality of life, interfering with their education, working ability and social life. Moreover, IBS represents an economic burden to both patients and society[23,43-49], since IBS patients visit their doctors more frequently, undergo more diagnostic tests, consume more medications, miss more workdays, are less productive at work, and are hospitalized more frequently than those without IBS[28,32,39,50-53].

There are no biomarkers for the diagnosis of IBS[54,55], which is instead based on the assessment of symptoms such as the Rome Ⅲ criteria[56,57]. IBS patients are subgrouped according to differences in the predominant bowel symptoms as IBS-diarrhea (D), IBS-constipation (C), IBS-both diarrhea and constipation (M), and non-subtyped IBS (patients with insufficient abnormality of stool consistency to meet the criteria for IBS-C, -D, or -M)[56,57]. The Rome criteria were introduced to facilitate positive diagnoses, reduce the costs due to unnecessary testing, and guide treatment; however, they fall short on these expectations and are generally neglected in clinical practice by both general practitioners and gastroenterologists[54,58-64].

IBS is considered to be a functional disorder in the absence of a known anatomic defect[65], the pathophysiology of which is incompletely understood. The pathogenesis appears to be multifactorial, with several factors suggested to play a role in the process, such as psychological factors, genetic factors, low-grade chronic intestinal inflammation, an abnormal gut neuroendocrine system (NES) and/or altered signaling in this system, dietary factors, and intestinal flora[66].

IBS patients can be roughly divided into two subsets: sporadic (nonspecific) and postinfectious (PI-IBS)/inflammatory bowel disease (IBD)-associated (IBD-IBS)[66]. Sporadic IBS includes patients who have had symptoms for a long time and without any associated events, in particular gastrointestinal or other infections. PI-IBS is defined as a sudden onset of IBS symptoms following gastroenteritis in individuals who have previously had no gastrointestinal complaints, and IBD-IBS is defined in IBD patients in remission who display IBS symptoms[66]. PI-IBS constitutes about 6%-17% of patients with IBS[67], and IBD-IBS occurs in 33%-46% of ulcerative colitis patients and 42%-60% of those with Crohn's disease[68-72].

This article summarizes the published findings on abnormalities in the gut neuroendocrine cells, discusses them in view of the currently known facts about IBS, and presents an argument for IBS being an organic gastrointestinal disorder.

GUT ENDOCRINE CELLS

The gut contains a large number of endocrine cells that are dispersed among the epithelial cells of the gut mucosa in all of the gut segments except for the esophagus[66,73-78]. These cells constitute the largest endocrine organ in the body and comprise about 1% of all epithelial cells in the gastrointestinal tract, where they are separated from one another by epithelial cells[73,74,79-81]. These cells have specialized microvilli that project into the lumen and function as sensors for the gut contents and respond to luminal stimuli by releasing hormones that, in general, target other parts of the digestive system (Figure 1)[82-94]. There are at least 15 different populations of endocrine cells in the gastrointestinal tract[60,73-76]. Some of them [including somatostatin and peptide YY (PYY) cells] have long slender cytoplasmic processes that project toward neighboring cells, increasing their paracrine effects (Figure 2)[95]. The distribution, functions, and modes of action of the most important endocrine/paracrine cells are given in Table 1[60,75,76,96-108].

Some of the different endocrine cell types are located in specific areas of the gut, while others (primarily somatostatin and serotonin cells) are found throughout the gut[73,74,76]. They secrete one or more signaling substances into the lamina propria, where they exert their action locally on nearby structures (autocrine/paracrine mode) and/or *via* an endocrine mode of action (by circulating in the blood to reach distant targets)[109]. These cells interact in an integrated manner with each other and with the enteric nervous system (ENS) and the afferent and efferent nerve fibers of the central nervous system (CNS), in particular the autonomic nervous system[60,76,99,110]. All of the cell types in the crypt/villus originate from pluripo-

Table 1 Overview of the main endocrine cells in the gastrointestinal tract

Cell content	Localization	Source of release	Actions
Serotonin (5-HT)	EC cells in the stomach, large and small intestines	Noradrenalin; acetylcholine; acidification and intraluminal pressure	Inhibits gastric emptying and stimulates colonic motility; accelerates small- and large-intestine transit activates the submucosal sensory branch of the enteric nervous system that conveys sensation from the gut to the central nervous system
Histamine	EC-like cells in the gastric oxyntic mucosa	Vagus nerve stimulation and inhibited by somatostatin	Stimulates gastric acid secretion
Somatostatin	The stomach, and large and small intestines	Mixed meal and acidification of the stomach	Inhibits intestinal contraction; inhibits gut exocrine and neuroendocrine secretion
Ghrelin	Gastric oxyntic mucosa	Protein and fat ingestion; suppressed by carbohydrate ingestion	Increases appetite and food intake; stimulates gastric and intestinal motility
Gastrin	Gastric antral mucosa	Intraluminal peptides; amino acids; calcium; amines; low pH and prostaglandins. Somatostatin inhibits release	Stimulates gastric acid secretion and histamine release; trophic action on gastric mucosa; stimulates contraction of the LES and antrum
CCK	Small intestine, especially the duodenum	Intraluminal protein and fat and inhibited by somatostatin	Inhibits gastric emptying; stimulates gallbladder contraction and intestinal motility; stimulates pancreatic exocrine secretion and growth; regulates food intake
Secretin	Small intestine, especially the duodenum	Acidification and inhibited by somatostatin	Stimulates pancreatic bicarbonate and fluid secretion; inhibits gastric emptying; inhibits contractile activity of the small and large intestines
GIP	Small intestine, especially the duodenum	Intraluminal glucose; amino acids and fat	Incretin action; inhibits gastric acid secretion
Motilin	Small intestine, especially the jejunum	Protein and fat ingestion	Induces phase-III migrating motor complex; stimulates gastric emptying; stimulates contraction of the LES
Neurotensin	Small intestine	Fat	Stimulates pancreatic section; inhibits gastric secretion; delays gastric emptying; stimulates colon motility
PYY	Terminal ileum and large intestine	Protein-rich meals	Delays gastric emptying; inhibits gastric and pancreatic secretion, stimulates the absorption of water and electrolytes; major mediator of the ileal brake
PP	Terminal ileum and large intestine	Protein-rich meals	Inhibits pancreatic secretion; stimulates gastric acid secretion; relaxes the gallbladder; stimulates motility of the stomach and small intestine
Enteroglucagon (oxyntomodulin)	Terminal ileum and large intestine	Intraluminal carbohydrates and fat	Inhibits gastric and pancreatic secretion; reduces gastric motility; has some incretin effect
Chromogranin	All gastrointestinal tract segments	Ingestion of a meal	Induces formation, sorting, and release of secretory granules of the gut endocrine/paracrine cells; an inflammatory mediator

CCK: Cholecystokinin; EC: Enterochromaffin; GIP: Gastric inhibitory peptide; LES: Lower esophageal sphincter; PP: Pancreatic polypeptide; PYY: Peptide YY.

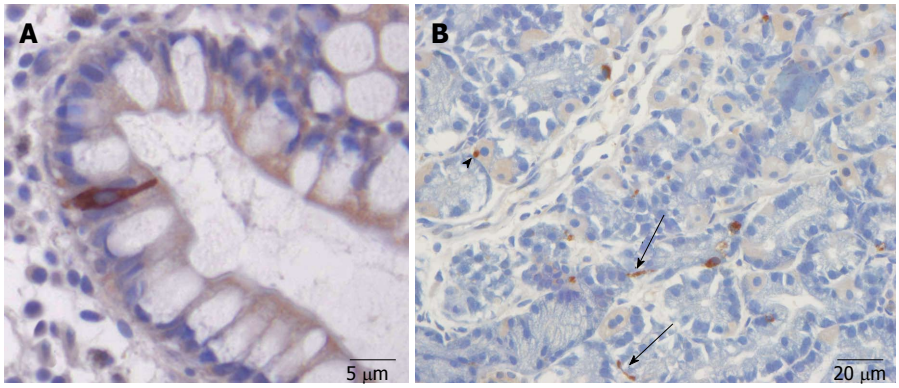

Figure 2 The gut endocrine cells. A: A chromogranin-A-immunoreactive endocrine cell in the ileum. The endocrine cell extends from the basal membrane of the mucosa that project into the gut lumen; B: Somatostatin-immunoreactive cells in the gastric oxyntic mucosa. Note the long cytoplasmic processes (arrows), which can occasionally be seen to end at the base of parietal cells (arrowhead).

tent stem cells of endodermal origin[73,74,111]. Each intestinal crypt contains four to six stem cells that differentiate into all epithelial cell types including enterocytes, goblet cells, Paneth cells, and endocrine cells[112-125]. These cells regulate several functions of the gastrointestinal tract, including sensation, motility, secretion, absorption, local

Table 2 Abnormalities in the densities of gastrointestinal endocrine/paracrine cells in patients with sporadic irritable bowel syndrome

Gastrointestinal segment	Endocrine cell type	IBS-D	IBS-M	IBS-C
Stomach				
Oxyntic mucosa	Ghrelin	High	Normal	Low
	Serotonin	High	Normal	Low
	Somatostatin	Low	Low	High
	Chromogranin A	Normal	Normal	High
Antrum	Serotonin	Normal	Low	High
	Gastrin	High	High	High
	Somatostatin	Low	Low	Low
	Chromogranin A	Normal	Low	High
Small intestine				
Duodenum	Serotonin	Normal	-	Normal
	CCK	Low	-	Normal
	Secretin	Low	-	Normal
	GIP	Low	-	Low
	Somatostatin	Low	-	Low
	Chromogranin A	Low	-	Low
Ileum	Serotonin	Low	Low	Low
	PYY	Normal	Normal	High
	Chromogranin A	Low	Low	Low
Large intestine				
Colon	Serotonin	Low	-	Low
	PYY	Low	-	Low
	Chromogranin A	Low	-	Low
Rectum	Serotonin	Normal	-	Normal
	PYY	Low	-	Low
	Enteroglucagon	Low	-	Low
	Somatostatin	High	-	High
	Chromogranin A	Normal	-	Normal

CCK: Cholecystokinin; PYY: Peptide YY; GIP: Gastric inhibitory peptide; IBS-C: Irritable bowel syndrome (IBS) with constipation as the predominant bowel symptom; IBS-D: IBS with diarrhea as the predominant bowel symptom; IBS-M: IBS with both constipation and diarrhea as the predominant bowel symptoms.

immune defense, and food intake (by affecting the appetite)[60,73,74,76,110].

ABNORMALITIES IN GUT ENDOCRINE CELLS IN IBS PATIENTS

Several abnormalities have been reported in all segments of the gastrointestinal tract of patients with IBS. As mentioned above, the endocrine cells exert their effects in part locally; however for some of them the endocrine mode of action is difficult to elucidate[99]. One example of this is the serotonin cells. The serotonin that they secrete is taken up into the blood and carried by platelets as they circulate through the gut[126-129]. Thus, the circulating serotonin is locked within the dense granules of the platelets, without any possibility of being delivered to distant targets. Therefore, summarizing and discussing abnormalities in the endocrine cells are considered separately herein relative to the various segments of the gastrointestinal tract.

Sporadic IBS

Abnormal endocrine/paracrine cells have been found in the stomach (Figure 3), proximal small intestine (duodenum), distal small intestine (ileum), colon (Figure 4), and rectum of patients with sporadic IBS[130-141]. These abnormalities manifest mostly as changes in the densities of these cells (*i.e.*, an anatomical defect). The abnormalities in the different endocrine cells in the various segments of the gastrointestinal tract of patients with sporadic IBS are summarized in Table 2. In addition to the abnormalities observed in the endocrine cells, there are alterations in the levels of serotonin transporter (SERT), which appear to be increased in the ileum and decreased in the rectum of IBS patients[130,141,142].

PI-IBS and IBD-IBS

Similar to sporadic IBS, abnormal endocrine/paracrine cell densities have been found in both PI-IBS and IBD-IBS. However, the nature of these abnormalities is different from those in sporadic IBS (Table 3)[141,143-150].

POSSIBLE ROLE OF GASTROINTESTINAL ENDOCRINE CELL ABNORMALITIES IN IBS

The mechanisms regulated by gastrointestinal endocrine cells include gut sensation, motility, and secretion. IBS patients exhibit visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion[65,107,151-153]. The degree to which the abnormalities in these cells observed in IBS patients contribute to these disturbed functions is discussed to below.

Visceral hypersensitivity

Visceral hypersensitivity has been demonstrated in the colorectal segment of IBS patients[154-161]. Hypersensitivity has also been reported in the esophagus, stomach, and small intestine[162-166]. However, visceral hypersensitivity is not present in all IBS patients, and a large prospective study found that only 20% of IBS patients showed hypersensitivity[167]. Furthermore, visceral hypersensitivity does not seem to be a panintestinal disorder[165]; IBS patients only appear to exhibit rectal hypersensitivity[159]. Whether the severity of abdominal pain is correlated with colorectal hypersensitivity in IBS remains a matter of controversy[154,156,168-172].

As mentioned above, serotonin cells have specialized microvilli that project into the gut lumen and act as sensors for the gut contents, and in particular for increased pressure. Serotonin is released in a regulated and calcium-dependent manner from serotonin cells into the surrounding tissues in response to luminal stimuli[173,174]. It activates the sensory branch of the ENS, which is localized in the submucosal plexus in the submucosa, and the myenteric plexus in between the smooth muscle fibers. These sensory branches convey sensation from the gut to the CNS through the sympathetic and parasympathetic nervous systems (Figure 1)[175-177]. The pain stimuli activate the cerebral cortex through the thalamus and permit the recognition of visceral pain[65,177]. Some studies have found IBS patients to be tolerant of somatic

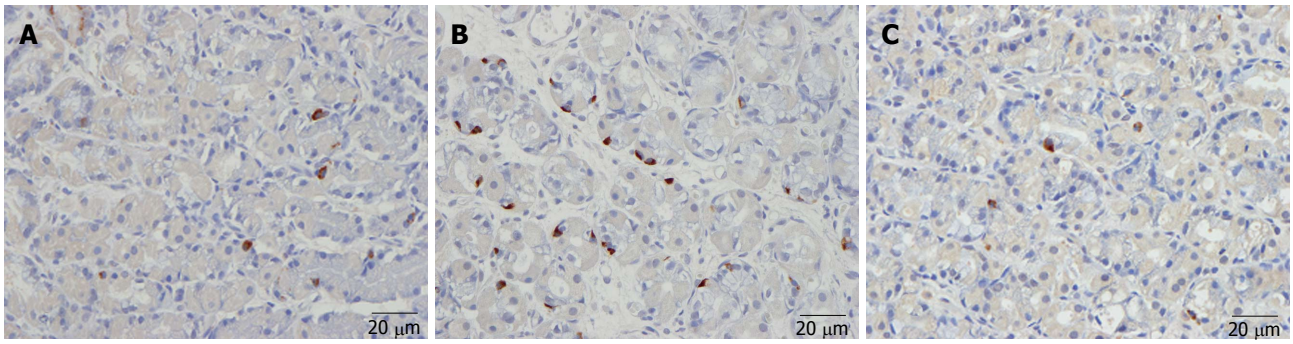

Figure 3 Ghrelin-immunoreactive cells. A: Ghrelin-immunoreactive cells in the gastric oxyntic mucosa of a healthy subject; B: In a patient with irritable bowel syndrome (IBS) with diarrhea as the predominant bowel symptom (IBS-D); C: In a patient with IBS with constipation as the predominant bowel symptom (IBS-C). The density of ghrelin cells is higher in IBS-D and lower in IBS-C than in the healthy control.

Table 3 Abnormalities in the densities of gastrointestinal endocrine/paracrine cells in patients with post infectious irritable bowel syndrome and inflammatory-bowel-disease-associated irritable bowel syndrome

Gastrointestinal segment	Endocrine cell type	PI-IBS	IBD-IBS
Small intestine			
Duodenum	Serotonin	High	-
	CCK	High	-
Large intestine			
	Serotonin	High	High/low
	PYY	High	Low
	PP	-	Low
	Enteroglucagon	-	High

CCK: Cholecystokinin; PYY: Peptide YY; PI-IBS: Post infectious irritable bowel syndrome; IBD-IBS: Inflammatory-bowel-disease-associated irritable bowel syndrome.

pain, and hence the hypersensitivity is confined to the viscera[158,165,178], while other studies found IBS patients to have a lower tolerance to somatic pain than healthy subjects[162,179,180]. Azpiroz *et al*[167] postulated that the exclusive visceral hypersensitivity experienced by some IBS patients could be attributable to abnormalities at the level of the gut, spinal cord, or brain, whereas patients with both visceral and somatic hypersensitivities have a disturbance above the gut level. Those authors also argued that a peripheral mechanism is involved in the visceral hypersensitivity in IBS.

The data presented in Table 2 suggest that none of the abnormalities in the gut endocrine cells could possibly contribute to the development of the visceral hypersensitivity seen in some sporadic IBS patients. However, it has been reported that SERT levels are increased in the ileum and reduced in the rectum of these patients[130,141,142]. The gut mucosa has a high SERT-producing capacity, since all of the epithelial cells lining the luminal surface of the gut express SERT[142,181]. A reduction in SERT results in impaired intracellular uptake and degradation in the gut epithelial cells, consequently increasing the availability of serotonin within the gut mucosa[182,183]. Considering that the serotonin cell density in sporadic IBS does not differ from that of a healthy subject, a decrease in SERT would markedly increase the amount of serotonin available at its receptors[141,142]. An increase in serotonin at the 5-hydroxytryptamine (5-HT)$_3$ receptors of the ENS sensory neurons would activate the sensory nerves, which would then transmit nociceptive information to the nervous system[99]. Conversely, duodenal and rectal serotonin cell densities are high in PI-IBS patients, possibly contributing to the development of visceral hypersensitivity.

Dysmotility

Dysmotility has been reported to occur in all segments of the gastrointestinal tract of patients with IBS, but mostly in the small and large intestines[151,153]. Some studies found lower pressures in the lower esophageal sphincter and abnormal esophageal contractions in IBS patients[184,185]; however, such esophageal motility abnormalities were not confirmed in other studies[186,187]. In addition, some authors have reported abnormal gastric emptying in patients with IBS[153,188-193], while others did not find any such abnormality in these patients[161,194-197]. It therefore seems that abnormalities of gastric emptying do not occur in all IBS patients. Furthermore, while IBS-C patients often exhibit delayed gastric emptying, rapid gastric emptying is found in IBS-D patients[155,189].

The ghrelin cell density in the gastric oxyntic mucosa is low and the serotonin cell density in the antrum of the stomach is high in IBS-C patients, while in IBS-D patients the ghrelin cell density in the gastric oxyntic mucosa is high and the densities of cholecystokinin (CCK) and secretin cells are reduced in the duodenum[132]. Ghrelin is a peptide hormone that was first isolated from the stomach, and originates mostly from endocrine cells in the oxyntic mucosa of the stomach, although small amounts are expressed in the small intestine, large intestine, and the arcuate nucleus of the hypothalamus[198-200]. Ghrelin has several functions, and plays a role in regulating the release of growth hormone from the pituitary gland, which increases appetite and feeding, and also plays a major role in energy metabolism[201-204]. Furthermore, ghrelin has been found to accelerate gastric and small- and large-intestine motility[83,110,205-214]. Serotonin acts on 5-HT$_{1p}$ receptors, which are located on a subset of inhibitory motor neurons of the myenteric plexus, relaxing the stomach *via*

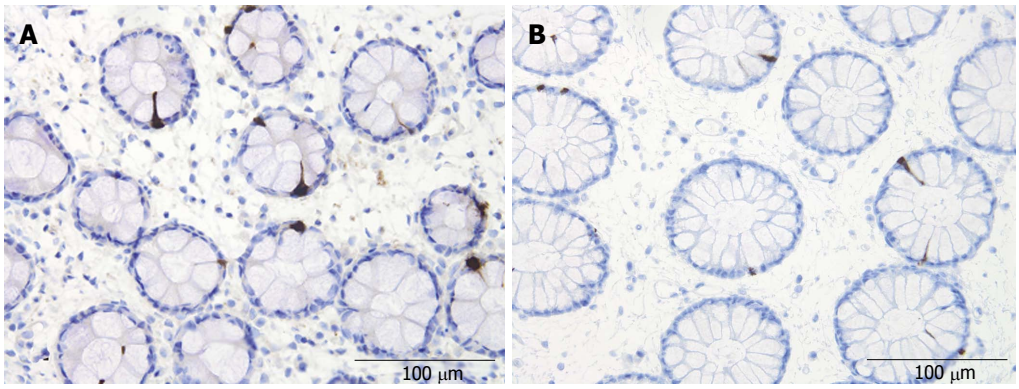

Figure 4 Peptide YY-immunoreactive cells. A: In the colon of a healthy subject; B: In a patient with irritable bowel syndrome (IBS). The density of peptide YY cells in the colon is lower in IBS patients than in healthy controls.

a nitrergic pathway and delaying gastric emptying[98,215-217]. CCK relaxes the proximal stomach in order to increase its reservoir capacity, and inhibits gastric emptying[218-220]. Secretin also inhibits gastric emptying[76,221]. It is therefore conceivable that low gastric ghrelin and high serotonin contribute to the slow gastric emptying in IBS-C, while the high gastric ghrelin and low intestinal CCK and secretin contribute to the rapid gastric emptying in IBS-D.

Several studies have found small-bowel transit to be delayed in IBS-C and accelerated in IBS-D[195,222-226]. However, a study from the Mayo clinic found no overall association between these IBS subgroups[161]. Studies on the motor patterns of the small bowel in IBS yielded contradictory results, which is probably due to marked inter- and intraindividual variations of small-intestine motor patterns[194,227-243]. As mentioned above, ghrelin cell density is low in the gastric oxyntic mucosa and PYY cell density is high in the ileum of IBS-C patients. Since ghrelin stimulates small-intestine motility and PYY stimulates the absorption of water and electrolytes, and is a major regulator of the ileal brake[244-249]. Moreover, it inhibits prostaglandin E2 and vasoactive intestinal polypeptide (which stimulate intestinal fluid secretion)[250-252], it is possible that the abnormalities in gastric ghrelin and ileal PYY contribute to the slow small-intestine transit in IBS-C. Secretin inhibits the contractile activity of the small intestine, and so the high ghrelin cell density and low duodenal secretin cell density may play a role in the rapid small-intestine transit in IBS-D.

It has been reported by some that colorectal transit is delayed in IBS-C and accelerated in IBS-D[153,222,223,253-258]. However, others have found that the colorectal transit time does not differ between IBS patients and controls[225,253,254]. The myoelectric and motor patterns of the large intestine of IBS patients have been investigated by several studies, which have yielded contradictory results[196,254-279]. In IBS-C, ghrelin cell density in the gastric oxyntic mucosa is low and ileal PYY cell density is high. Given that ghrelin stimulates intestinal motility and PYY stimulates the ileal-break, these abnormalities in ghrelin and PYY may promote the delayed colorectal transit observed in some IBS-C patients. CCK and secretin inhibit intestinal motility, and the cell densities of both are low in the duodenum of IBS-D patients. These factors together with a high gastric ghrelin cell density may contribute to the development of the accelerated colorectal transit seen in IBS-D patients.

In PI-IBS, the serotonin cell densities are high both in the small and large intestines, and CCK cell density is high in the small intestine. Serotonin primarily targets the mucosal projections of the intrinsic primary afferent neurons, which initiate peristaltic and secretory reflexes[156-161,175,280-289]. As mentioned above, CCK stimulates intestinal motility; thus, high serotonin and CCK levels could be responsible for the diarrhea seen in PI-IBS.

Abnormal secretion

Few studies have investigated gastrointestinal secretion in IBS patients. Enhanced intestinal secretion in response to bile acid perfusion in the ileum has been reported in these patients[290]. Increased reactivity of the secretory component of the migrating motor complex has been observed in the small intestine of IBS patients, and especially in those with IBS-D[291]. Among the abnormalities in the gut endocrine cells in IBS patients listed in Table 2, the low duodenal CCK and secretin observed in IBS-D, and the high ileal PYY cell density observed in IBS-C are particularly interesting with respect to gut secretions. CCK stimulates the secretion of digestive enzymes from pancreatic exocrine glands, and secretin stimulates pancreatic bicarbonate and fluid secretions[218,219]. The secretion of pancreatic bicarbonate increases the pH of the gut contents, which are highly acidic after leaving the stomach, and PYY stimulates the absorption of water and electrolytes[76]. This change in pH is essential for lipid digestion, since pancreatic lipase is irreversibly inactivated below pH 4.0[218]. It is tempting to speculate that IBS-D patients could suffer from fat maldigestion and a functional pancreatic insufficiency. Indeed, pancreatic enzyme substitution and a low-fat diet have been applied in clinical practice for these patients, with some success[292]. Moreover, an increase in PYY in the ileum of IBS-C patients may result in increased absorption of water from the feces, resulting in hard feces that worsen their to obstipation (Figure 4).

In conclusion, there are sufficient grounds to suspect

that the abnormalities in the gastrointestinal endocrine cells play a role in the development of visceral hypersensitivity, gastrointestinal dysmotility, and abnormal gastrointestinal secretion.

IS IBS AN ORGANIC DISORDER?

It has long been considered that IBS is caused by psychological stress and/or brain dysfunction, and it is overrepresented in patients with psychiatric illness/and or sexually and/or physically abused individuals. During the past decade there has been rapid progress in our understanding of IBS, and there is accumulating evidence of a biological etiology for this condition. Research to establish effective treatments for IBS have been intensified, and societal attitudes toward IBS patients are slowly changing.

This review presents evidence for an anatomic defect in IBS patients, namely the gastrointestinal endocrine cells. However, the data presented on the gastrointestinal endocrine cells in sporadic IBS were obtained by only two research groups. Further studies performed by other researchers involving different patient cohorts are needed before these observations can be confirmed. Conversely, while the data for PI-IBS were reported by several research groups from different countries and related to different patient cohorts, studies on PI-IBS have focused mainly on serotonin and are mostly restricted to the rectum. Further studies of other endocrine cells in different segments of the gastrointestinal tract are needed in PI-IBS. It should be noted that the gastrointestinal endocrine cells interact in an integrated manner with each other and the ENS, and together constitute the so-called neuroendocrine regulatory system of the gut[76,293-295]. It is thus possible that IBS patients have an abnormality in the ENS, in addition to those in the endocrine cells. However, investigating the ENS is very difficult since it would require whole-wall biopsy sampling under laparoscopic control, which represents a risk for both patients and controls. Regardless of the ethical issues this raises, it is unlikely that either patients or healthy subjects would voluntarily submit to laparoscopy and whole-wall biopsy sampling.

The abnormalities in the gut endocrine cells differ between sporadic IBS and PI-IBS/IBD-IBS, and their etiologies also appear to be different. Familial aggregation, twin, and genetic studies provide evidence for a genetic predisposition in sporadic IBS[296-306], and these patients describe their symptoms as commencing in childhood, suggesting the presence of genetically defective gastrointestinal endocrine cells. However, gastrointestinal mucosal cells - including the endocrine cells - have a rapid turnover, and it is also possible that factors related to luminal content such as diet or bacterial flora can provoke an increase or decrease in the endocrine cell population.

The etiology of the gastrointestinal endocrine cell abnormalities in PI-IBS and IBD-IBS appears to differ from that of sporadic IBS. Patients who develop PI-IBS and IBD-IBS likely have a genetic predisposition (host related) as well as other factors, such as infecting-organism-associated risk factors[307-315]. Following infection, these patients develop a low-grade inflammation that manifests as an increased intraepithelial and mucosal infiltration of lymphocytes and mast cells[143-149,316]. There is some evidence that inflammation and immune cells affect the gastrointestinal endocrine cells[317]. The secretion of serotonin by enterochromaffin (EC) cells can be enhanced or attenuated by the secretory products of immune cells, such as $CD4^+$ T, and also modulates the immune response[126,317]. The EC cells are in contact with or very close to $CD3^+$ and $CD20^+$ lymphocytes, and several serotonergic receptors have been identified in lymphocytes, monocytes, macrophages, and dendritic cells[318]. Therefore, it is conceivable that the abnormalities in the gastrointestinal endocrine cells in PI-IBS and IBD-IBS are caused by endocrine/immune interactions (*i.e.*, the endocrine/immune axis), which are in turn caused by low-grade inflammation in predisposed individuals[319,320].

CONCLUSION

The gut NES comprises the gastrointestinal endocrine cells and the ENS[76]. This regulatory system controls all gastrointestinal functions independently from the CNS[76,156]. However, the gut NES and the CNS are connected, and the CNS modulates the gastrointestinal functions through this connection[152]. Thus, a defect in the gut NES should be suspected in patients with IBS[76,294]. The gastrointestinal endocrine cells serve as chemical and mechanical transducers for afferent projections to the ENS, and subsequently to the CNS[294,321]. The present review describes evidence in the literature of an anatomic defect in the NES in IBS, namely defective gastrointestinal endocrine cells. Therefore, in line with some other gastroenterologists, we consider it highly likely that IBS is an organic disorder[107].

The endocrine cells interact in an integrated manner with each other. It is possible that the abnormality in many endocrine cells of the gut seen in IBS is caused by a defect in one or more endocrine cell types, which in turn results in changes in the other endocrine cell types.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Then and now: The progress in hepatitis B treatment over the past 20 years

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Abstract

The ultimate goals of treating chronic hepatitis B (CHB) is prevention of hepatocellular carcinoma (HCC) and hepatic decompensation. Since the advent of effective antiviral drugs that appeared during the past two decades, considerable advances have been made not only in controlling hepatitis B virus (HBV) infection, but also in preventing and reducing the incidence of liver cirrhosis and HCC. Furthermore, several recent studies have suggested the possibility of reducing the incidence of recurrent or new HCC in patients even after they have developed HCC. Currently, six medications are available for HBV treatment including, interferon and five nucleoside/nucleotide analogues. In this review, we will examine the antiviral drugs and the progresses that have been made with antiviral treatments in the field of CHB.

Key words: Chronic hepatitis B; Treatment of hepatitis B; Hepatocellular carcinoma; Nucleoside analogues; Nucleotide analogues

Core tip: Chronic hepatitis B virus (HBV) infection is one of the leading causes of death across the world due to its worldwide distribution and potential sequelae. Advances in knowledge in combination with the development of potent and effective antiviral therapy for chronic hepatitis B have led to decreased complications from the virus. Timely use of nucleotide/nucleosides may improve liver function and increase survival in patients with hepatic decompensation. Maintained suppression of HBV replication with antiviral therapy halts the progression of liver disease, may reverse liver fibrosis, and can reduce the development of cirrhosis and hepatocellular carcinoma.

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INTRODUCTION

In the decades after World War Ⅱ, clinical and epidemiological studies began to differentiate among various types of hepatitis[1]. However, it was the discovery of an antigen by Blumberg and his colleagues, now known as hepatitis B surface antigen (HBsAg), in the serum of an Australian Aborigine that reacted with the antibody (now known to be anti-HBs) in serum of a hemophiliac patient that provided the first clue[2]. Subsequent development of acute hepatitis in a technician in his laboratory provided the essential link to the illness. For his discovery and subsequent work on the disease progression related to hepatitis B virus (HBV), Blumberg received the Nobel

Prize in Medicine 1976[3,4]. In 1970, Dane *et al*[5], identified the whole virus particle (Dane particle) using electron microscopy. In 1972, hepatitis B e antigen (HBeAg) was identified by Magnius *et al*[6]. By the early 1980's the genome of the virus had been sequenced and the first vaccine (initiated by Millman *et al* and developed by Hilleman *et al*) were tested[7,8]. This plasma vaccine became available in 1983 and was rightly designated "The First Cancer Vaccine" by World Health Organization. The close link between HBV and hepatocellular carcinoma (HCC) was lucidly documented by Beasley *et al*[9] in their historical prospective study of 22707 men in Taiwan (Figure 1).

Since the discovery of the virus, our understanding and knowledge about the complexities of HBV have grown tremendously. Chronic HBV infection is one of the leading causes of death across the world due to its worldwide distribution and potential sequelae. People infected with the virus are at risk of developing hepatic decompensation, liver cirrhosis and HCC with 15% to 40% of individuals developing serious sequelae in their lifetime[9]. Despite the implementation of effective universal vaccination programs, over 300 million people are still chronically infected with HBV worldwide with 75% of infected individuals residing in the Asia-Pacific region[10,11].

Increased knowledge of the natural history of chronic hepatitis B (CHB) and clinical data demonstrating improved outcomes with medical interventions have led to publication of various treatment guidelines aimed at providing direction regarding initiation/on-treatment management of antiviral therapy and monitoring of outcome measures. These advances in knowledge in combination with the development of potent and effective antiviral therapy for CHB have led to decreased complications from the virus[12]. In this review, we will discuss the advances in the understanding of the natural history of CHB and the progress of anti-HBV treatment over the past two decades.

HEPATITIS B VIRUS

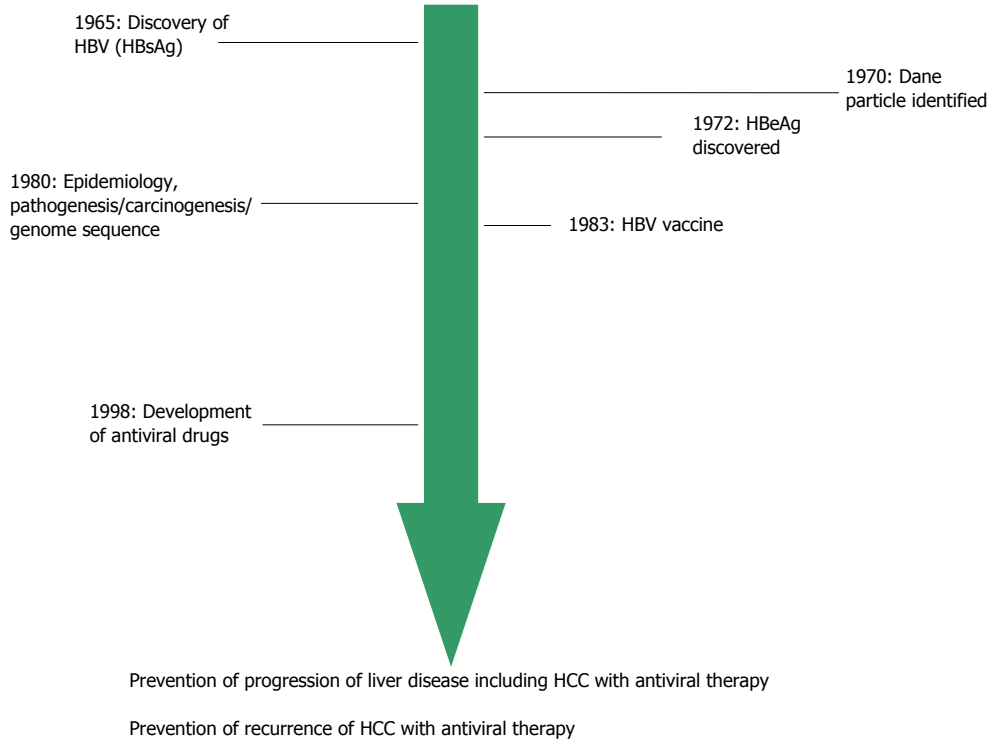
HBV belongs to a group of closely related DNA viruses termed Hepadnaviruses[13-15]. This family of viruses has a strong preference for infecting liver cells and has a similar life cycle in their hosts. The virus consists of a nucleocapsid and an outer envelope composed mainly of three HBsAgs, which play a central role in the diagnosis of HBV infection. The nucleocapsid contains hepatitis B core antigen (HBcAg), a DNA polymerase-reverse transcriptase, and the viral genome[16]. The genome consists of a partially double-stranded circular DNA molecule of about 3200 base pairs in length. The pre-surface 1/pre-surface 2 and surface genes code for the various HBsAgs. The protein encoded by the pre-core/core gene undergoes post-translational modification to yield HBeAg, which is a seromarker for high viral replication[16]. The viral DNA polymerase-reverse transcriptase is encoded by the polymerase gene and is of central importance for viral replication. Different from all known mammalian DNA viruses, hepadnaviruses replicate using a reverse transcription of an RNA intermediate[17-19]. Based on this unique replication cycle of HBV, antiviral therapeutic strategies have been mainly aimed at the reverse transcription of HBV RNA with nucleotide/nucleoside analogues[20].

The presence of HBV DNA in serum is the best indication of active viral replication. Antibody to HBsAg is produced in exposure to the envelope antigen and confers protective immunity. Antibody to HBcAg is detectable in all patients who have ever been exposed to HBV. However, unlike antibody to HBsAg, this antibody is not protective, but can be helpful in distinguishing acute from chronic infection if IgM antibody (anti-HBc IgM) is present. Antibody to HBeAg appears when the antigen has been cleared and the virus is no longer replicating or has reduced replication[12].

PATHOGENESIS AND CARCINOGENESIS OF HEPATITIS B

Liver injury in CHB is the result of the host's immune responses against HBV; an HLA-class I antigen-restricted, cytotoxic T lymphocyte-mediated response against HBV antigens expressed on hepatocytes would result in apoptosis and necrosis of the hepatocytes[21]. Accordingly, CHB is a dynamic state of interactions among HBV, the patient's hepatocytes and the immune system. Based on these interactions, the natural course of CHB can be divided into different changing phases, although not all patients go through all of the phases (Figure 2)[22].

The first phase is the "immune tolerant phase" which consists of HBeAg seropositivity, high viral loads, but with a normal serum alanine aminotransferase (ALT) and near-normal liver histology. Adult-acquired chronic HBV infection usually has a very short "immune tolerant phase". In contrast, the perinatally or early childhood-acquired chronic HBV infection has a long "immune tolerant phase"[22-24]. The "immune clearance phase" usually develops during adolescence or adulthood. This phase is characterized by positive HBeAg, high serum HBV levels and increased ALT levels, sometimes complicated by hepatic decompensation[21]. These events may lead to progression to fibrosis or development of cirrhosis in some patients during the HBeAg-positive phase, but may also result in a declining serum HBV DNA level and may eventually lead to HBV DNA seroclearance and HBeAg seroconversion to its antibody (anti-HBe) in most patients. A 3-year clinical study in patients with chronic hepatitis B, or patients in the "immune clearance phase", showed that cirrhosis developed at an estimated annual incidence of 2.1%, being higher in those seropositive for HBeAg at entry (2.4%/year)[25]. The estimated annual incidence of spontaneous HBeAg seroconversion was reported to be 2%-15%, depending on factors such as age, ALT level and HBV genotype[26,27]. Following HBeAg seroconversion, most of the patients enter an "inactive



Dr. Baruch S Blumberg discovered an Antigen in the blood of Australian Aborigine and named Australia Antigen which was found to be HBsAg. Made the first HBV vaccine (plasma vaccine Heptavax B). Received Nobel Prize in Medicine in 1976.

Dr. R Palmer Beasley conducted a study of over 22000 government workers found that risk of liver cancer is 60 times higher in chronic HBV infected persons.

Figure 1 Progress in the field of hepatitis B virus. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HCC: Hepatocellular carcinoma.

phase" with sustained normal serum ALT, low serum HBV DNA and no or minimal necro-inflammatory histological changes, although some of them may have already developed advanced fibrosis or cirrhosis[27-29]. Spontaneous HBsAg seroclearance may occur several years after HBeAg seroconversion at an incidence of 0.7%-2.4% per year depending on age at time of infection[29].

As early as the 1970's, chronic infection with the HBV was associated with the development of HCC. A powerful substantiation of the association between HBV infection and HCC was the results of a prospective cohort study reported by Beasley *et al*[9] in 1981. These investigators followed more than 22000 male municipal workers in Taiwan and found that those who were seropositive for HBsAg had rates of HCC that were significantly greater than were the rates in uninfected controls. They calculated the relative risk for HCC among those who were HBV-infected to be 63 compared to uninfected controls. More recent cohort studies have confirmed the high risk of HCC in HBsAg-positive individuals as originally identified in the Beasley study. An example is the Haimen City cohort that included about 11000 HBsAg-positive subjects followed over a mean period of 8 years[30]. The mechanism by which HBV infection causes HCC is not completely understood. Evolution to HCC may be the direct effect of the virus itself, or it may be an indirect effect, through the process of the inflammation, regeneration and fibrosis associated with cirrhosis due to the HBV infection[31,32]. HBV DNA has been shown to become integrated within the chromosomes

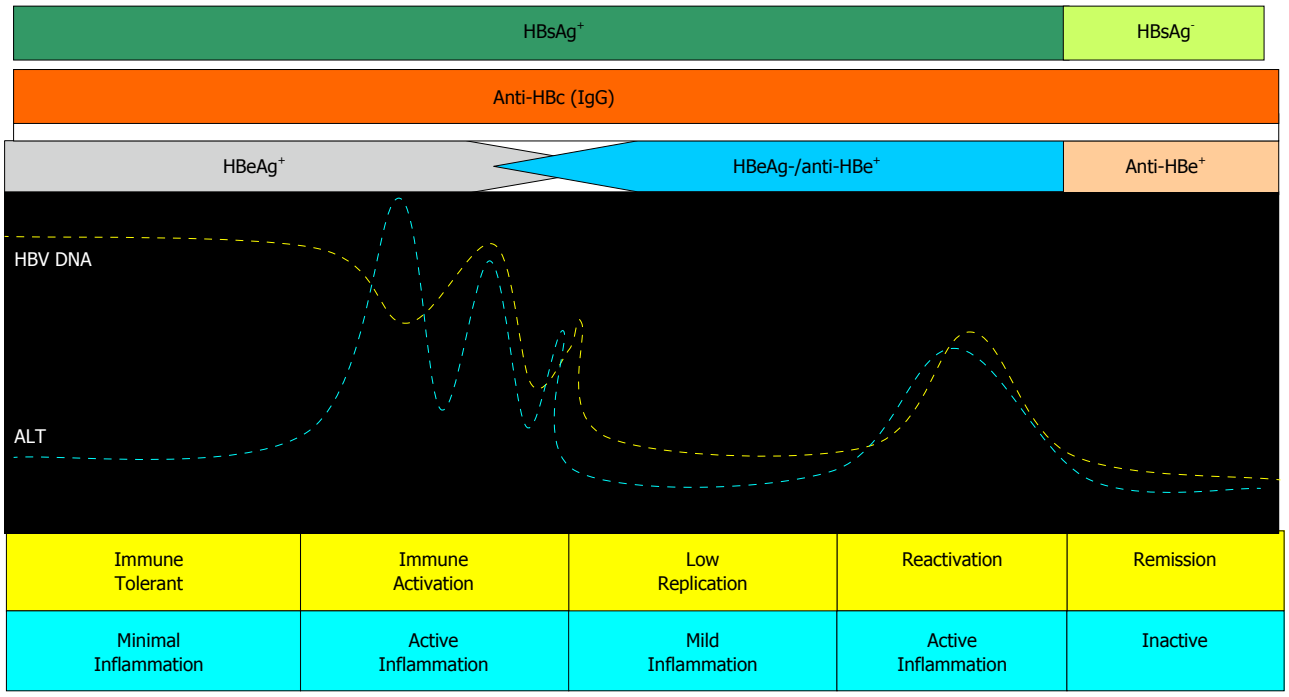


Figure 2 Five phases of chronic hepatitis B. Adapted from Tong *et al*[37]. ALT: Alanine aminotransferase; Anti-HBc: Hepatitis B core antibody; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; Anti-HBe: Hepatitis B e antibody.

of infected hepatocytes, the integration of viral genetic material occurring in a critical location within the cellular genome. The hepatitis B x gene (*HBx*) product has been implicated in causing HCC because it is a transcriptional activator of various cellular genes associated with growth control[32,33]. The HBx gene expression is also associated with activation of the Ras-Raf-MAP kinase pathway, an important cellular pathway that has been implicated in hepatocarcinogenesis.

HBsAg seroclearance usually confers protection against HCC but may still carry a risk for HCC although at a very low rate and usually in patients in whom cirrhosis or superinfection with other viruses had already developed before HBsAg seroclearance[34,35]. Studies further indicate that serum HBV DNA level is associated with cirrhosis and HCC development in a dose-dependent manner starting from serum HBV DNA level[34-38]. These findings suggest that HBV replication, with subsequent immune-mediated liver injuries, is the primary driving force for liver disease progression. It has also been identified that patients of Asian background are at higher risk for HCC because they are more likely to have been infected early in life and carcinogenic processes could have taken place earlier[37]. This may explain why some patients even with well suppressed viral replication still develop HCC.

EPIDEMIOLOGY OF HEPATITIS B INFECTION

As stated earlier, HBV infection is common and clinically consequential worldwide. In endemic countries, an estimated 50 million new cases are diagnosed annually. In Asia, HBV is the leading cause of chronic hepatitis, cirrhosis, and HCC[37]. The HBV carrier rates in Asia have been reported to be as high as 20% in the male population of Guangxi Province, China[38]. A recent study, conducted in China, showed that HBV carrier rates have fallen to 7.2% in regions where hepatitis B vaccination programs had been implemented[39]. In South Korea, the HBV carrier rates ranged from 5.0% to 8.6% in the 1970s and 1980s and have subsequently declined to 3.7%-5.7% as a result of national vaccination programs[40,41]. In other parts of Asia, the HBV infection rates remain high, particularly in countries in which vaccine programs have not yet been implemented. Notably, the HBsAg rates among Asians residing in the United States are similar to rates reported in their countries of origin, especially in first-generation immigrants to the United States[42-45]. Therefore, the disease burden from HBV, including mortality from liver disease progression and development of HCC, remains a major health problem among Asian Americans with CHB.

PROGRESSION OF HEPATITIS B TREATMENT

Currently, six treatments are approved for hepatitis B, including interferon (IFN) (two formulations: IFN and PEG-IFN) and five nucleotide/nucleoside analogues (lamivudine, adefovir, entecavir, telbivudine and tenofovir) (Figures 3 and 4)[36,46]. The aim of hepatitis B treatment is to achieve sustained viral suppression of HBV replication. With viral suppression, the ultimate goal would be prevention of cirrhosis and HCC. Response to treatment

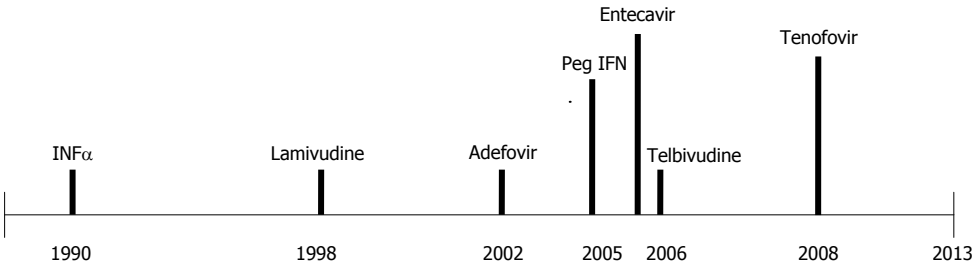


Figure 3 Timeline of approved therapies for chronic hepatitis B. IFN: Interferon.

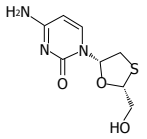
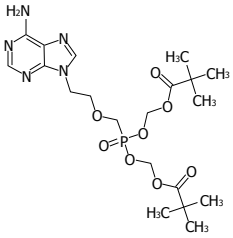
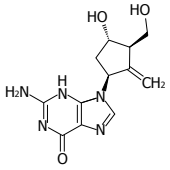
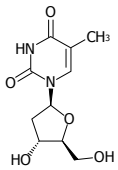
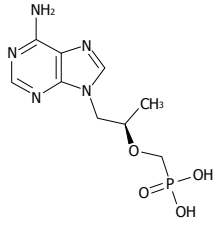
Name	Trade name	Strong points	Weak points	Approved	Chemical structure
Interferon alpha-2b and pegylated interferon 2a	Intron A Pegasys	Finite duration of treatment Durable response post-treatment No known resistance	Needle injection High cost 65%-70% fail to respond Significant side effects	1991 2005	Human leukocyte clone
Lamivudine	Epivir (Zeffix)	Oral Safe with negligible side effects Effective and safe in pregnancy Least expensive	Long term treatment is necessary High incidence of resistance	1998	
Adefovir dipivoxil	Hepsera	Oral Low resistance	Long term treatment is necessary Long term treatment renal toxicity Less potent than other treatments	2002	
Entecavir	Baraclude	Oral Potent viral suppression Safe with negligible side effects Low resistance	Long term treatment is necessary High cost	2005	
Telbivudine	Tyzeka	Oral Potent viral suppression Effective and safe in pregnancy	Long term treatment is necessary High incidence of resistance	2006	
Tenofovir	Viread	Oral Potent viral suppression Safe with negligible side effects No known resistance for 6 years' study Effective and safe in pregnancy	Associated with osteopenia Long term treatment is necessary	2008	

Figure 4 Characteristics of approved drugs for treatment of hepatitis B.

is judged based on decrease in serum HBV DNA level, loss of HBeAg with or without seroconversion to anti-HBe, loss of hepatitis B surface antigen (HBsAg) with or without seroconversion to HBs antibody, normalization of serum ALT levels, and a decrease in hepatic inflammation on liver biopsy[46,47].

The importance of monitoring individuals with low-serum HBV DNA and normal ALT levels regardless of their HBeAg status has also become recognized in recent years. Serial ALT and HBV DNA monitoring every 3 mo for 1 year after the initial diagnosis and 6-12 mo thereafter is usually recommended to detect intermittent flares of hepatitis B[37,48]. This regimen is also useful to differentiate chronic active HBeAg negative hepatitis from inactive carriers in recently diagnosed HBV carriers. Treatment should be initiated regardless of the level

of viremia if active inflammation is also detected on liver biopsy[37,47,49]. In the case of HBeAg negative CHB, treatment is continued indefinitely until HBsAg becomes undetectable. Nonetheless, close follow-up is important, and prompt retreatment is necessary if elevation of HBV DNA and ALT levels are observed[37]. Therefore, achieving maximum viral suppression without the development of antiviral drug resistance, reducing progression to cirrhosis and decreasing the risk of developing HCC are the primary treatment endpoints.

Interferon

In 1991, conventional IFNα-2a was the first successful treatment approved for CHB with widespread use. Its major mechanism of action is immune modulation, although there is also a weak anti-viral effect[50]. Peg IFNα-2a replaced standard IFN in 2005 due to improved pharmacokinetic properties and a less demanding injection schedule with comparable efficacy. Long-term follow-up of patients treated with conventional IFN therapy showed that responders had a decreased incidence of hepatic decompensation and HCC, and improved overall survival compared with non responders[51-53]. Forty-eight weeks of therapy with Peg IFN results in a 27% rate of HBeAg seroconversion and 25% rate of loss of HBV DNA. Six months after discontinuation of therapy, the HBeAg seroconversion rates increased to 32%. Loss of HBsAg with the appearance of anti-HBs occurred in 4%-6% of patients after 1 year of treatment and 6 mo of post treatment follow-up[51,54,55]. Even after discontinuation of IFN therapy, 12%-65% of patients lost HBsAg within 5 years of HBeAg loss. This results in the highest rate of off-treatment sustained response after 1 year of therapy[53,54]. Achieving early virological response, defined as > 2 $\log_{10}$ drop in serum HBV DNA or suppression to levels below 10^5 copies/mL in the first 2 wk of therapy, is associated with induction of long-term remission after stopping therapy[56,57].

Despite the fixed duration of Peg IFN therapy and the lack of antiviral drug resistance compared with oral agents, the use of Peg IFN only accounts for no more than 10% of all prescriptions for the treatment of CHB in the United States[54]. This low rate can be explained by the drug's substantial side effect profile and the need for administration by injection.

Lamivudine

Lamivudine (LAM) was the first nucleoside analogue reverse transcriptase inhibitor that was approved for use by the United States Food and Drug Administration (FDA) in 1998. Although it is not as used as commonly today due to the presence of better oral agents with higher genetic barrier to resistance; it played a major role in the transition CHB treatment and allowed reduction in cirrhosis and risk of HCC to be achieved with some success.

One-year therapy with lamivudine is associated with 16%-18% rate of HBeAg seroconversion; the HBeAg seroconversion rate increases to 50% with 5 years of therapy[58-60]. LAM therapy also results in 60%-70% HBV DNA suppression in HBeAg-negative CHB after 1 year of therapy[56]. The durability of response is lower than the interferon therapy regardless of the HBeAg status and has been reported to range between 50% and 80% for HBeAg-positive CHB and 20%-25% for HBeAg-negative CHB patients.

Treatment of HBV with LAM has been shown to slow the rate of development of fibrosis, as well as decrease of HCC incidence[60-62]. Use of LAM is associated with a significant risk of development of resistance with prolonged use. Five years of therapy can lead to 65%-70% rate of resistance[59]. However, thorough review of studies investigating the LAM resistance during CHB treatment revealed a diverse range of methodologies to assess resistance[63]. Studies that use purely genotypic methods report resistance rates at 1 year ranging from 14% to 32%. However, these may overestimate clinically relevant resistance. Studies that use virologic resistance report lower one year resistance rates, ranging from 6.4% to 15.4% and may provide more relevant measure of resistance. When comparing resistance rates with antiviral drugs in CHB, it is important to consider the methodology and definition of resistance used[63]. In addition, baseline HBV DNA was closely related to the resistance rate[64] and HBV DNA level at 6 mo on LAM therapy was an important predictor for LAM resistance at one year and later[65].

The largest and most compelling study suggesting that antiviral treatment might decrease the risk of HCC was a randomized, controlled trial of LAM *vs* placebo in patients with advanced chronic hepatitis B and high serum levels of HBV DNA[60]. The primary outcome of the study was progression of liver disease, including an increase in Child-Pugh score, bleeding from esophageal varices and the development of HCC. The study was halted early because of a distinct benefit for the group on LAM treatment compared to the placebo group. Instead of continuing the study for intended 5 years, all received LAM at the end of 3 years on trial. In this study, at year 3, the rate of HCC was 3.9% among LAM recipients *vs* 7.4% among placebo recipients ($P = 0.047$). Other retrospective studies observed similar results[61,66].

Adefovir dipivoxil

Adefovir dipivoxil (ADV) was the first nucleotide analogue approved in United States in 2002 for the treatment of CHB. The arrival of this agent provided new insights into the treatment of CHB. ADV did not only have increased antiviral potency but also had an intrinsic stereoscopic structure which was an important factor against the emergence of viral resistance.

One year of therapy with ADV leads to a 12% rate of HBeAg seroconversion and 53% rate of histological improvement in HBeAg-positive patients[67]. Once HBeAg seroconversion occurs, it is sustained in 91% of patients[68]. Like LAM, HBeAg-negative patients require

therapy indefinitely with ADV, and resistance is also a problem with prolonged ADV use. Persistence of viremia after 48 wk of therapy is linked to development of resistance[69]. Resistance rates of 0%, 3%, 18% and 29% have been reported at 1, 2, 4 and 5 years of therapy[70]. These high resistance rates and its potential renal toxicity have lead to declining use of ADV in light of newer therapeutic agents. Furthermore, ADV was highly effective for LAM resistant HBV[71,72].

Entecavir

During the period between 1998 and 2004, LAM and ADV for treatment naive CHB and ADV for LAM-resistant CHB were the main treatment strategies available for CHB. In 2005, entecavir (ETV), a nucleoside analogue, entered the arena when it was approved in United States. It is a potent inhibitor of HBV polymerase at a dose of 0.5 mg daily resulting in 6.98 $\log_{10}$ copies/mL decrease in HBV DNA levels compared to a 5.4 $\log_{10}$ copies/mL reduction with LAM[73]. In clinical studies, patients who received ETV for 52 wk achieved superior virological response, with HBV DNA < 400 copies/mL (67% *vs* 36%), histological improvement (72% *vs* 62%) and normalization of ALT (78% *vs* 70%) compared with those who received LAM[73]. However, there was no difference between ETV and LAM in achieving HBeAg seroconversion (21% *vs* 18%). ETV is also superior to LAM in HBeAg-negative patients, but requires indefinite treatment to maintain viral suppression to prevent relapse[74-76]. ETV demonstrates better virological suppression (91% *vs* 73%) and improved histology (70% *vs* 61%). In addition, analysis of two studies of patients who received continuous ETV for up to 5 years revealed that 94% of patients continue to have HBV DNA < 300 copies/mL at 5 years[76].

In long term studies, up to 96% of patients (mainly HBeAg-positive CHB) had histological improvement and 88% of the patients had improvement in fibrosis score after 6 years of ETV therapy; this holds true even in patients with cirrhosis[77]. ETV has also been shown to be superior to ADV in achieving rapid viral suppression within 2 wk of therapy. Even though a 48-wk therapy with ETV compared with ADV was associated with a higher rate of HBV clearance (58% *vs* 19%) and ALT normalization (76% *vs* 63%), there was no difference in the rate of HBeAg loss (18% *vs* 22%) and HBeAg seroconversion (15% *vs* 22%)[78].

One of the most important differences from LAM and ADV is that, ETV has a high genetic barrier with a very low incidence of resistance. The cumulative incidence rate of resistance after 6 years of therapy in nucleoside-naïve patients remains low at 1.2%[79,80]. The low resistance rate is related to both profound viral suppression and the requirement of at least 3 sites of genetic mutations to confer resistance. However, the chance for ETV resistance is much higher in patients who already developed LAM resistance[80]. ETV therapy has also been associated with HBsAg loss, improvement of liver histology, decreased risk of HCC and very low to undetectable HBV DNA levels[81,82]. The ability to decrease the incidence of HCC in patients with CHB has been the most exciting attributes of antiviral therapy. Recent report from Hosaka *et al*[83] compared the incidence of HCC in 472 ETV-treated patients and 1143 non-treated HBV patients. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). The treatment effect was found to be greater in patients at higher risk of HCC.

Telbivudine

While newer treatments for LAM-resistant disease were still under investigation, telbivudine (TLV), another nucleoside analogue was approved by the FDA in 2006 for treatment of chronic CHB. In HBeAg-positive CHB patients, the rate of HBeAg seroconversion with TLV therapy was 22% and 30% at 1 and 2 years respectively. Viral suppression was limited to HBV DNA levels of < 300 copies/mL after 1 and 2 years of therapy in 60% and 56% of HBeAg-positive patients[84]. In HBeAg-negative CHB patients, HBV suppression was noted in 88% and 82% of patients at 1 and 2 years of therapy, respectively[84,85]. Resistance to TLV has been reported to be 21.6% in HBeAg-positive patients and 8.6% in HBeAg-negative patients after 1 and 2 years of therapy respectively. Although TLV barrier to antiviral resistance is higher then LAM, is not recommended as a first-line agent[37,48,51]. Although this treatment is not used often due to its high rate of resistence it is effective and safe for the prevention of mother-to-child transmission of HBV from chronically infected mothers with a high degree of infectivity late in pregnancy.

Analysis of the baseline characteristics of the patients enrolled in the GLOBE trial revealed important predictive factors of response to therapy. The strongest predictors of achieving a good response to TLV therapy in HBeAg-positive patients were serum HBV DNA < 9 $\log_{10}$ copies/mL, or ALT ⩾ 2 times the ULN at baseline with undetectable serum HBV DNA at week 24 of therapy[86]. Extensive review of TLV for treatment of hepatitis B indicated that there was a specific group of patients who are likely to achieve good therapeutic response with TLV. Patients with low baseline HBV DNA who could achieve negative HBV DNA at week 24 had the best outcome with TLV[87]. With this data, LAM and TLV can be used in countries where cost is a major concern by selecting patients with favorable baseline HBV DNA and ALT levels. Another important aspect of TLV is its renoprotective effect as recently reported by Gane *et al*[88]. In approximately 2500 patients treated with TLV, there was a trend towards in increased GFR in both compensated and decompensated CHB. The mechanism of this renoprotective effect by TLV is unknown.

Tenofovir

Rescue therapy for patients with viral resistance to the nucleoside analogues was the usage of adefovir until 2008 when, tenofovir disoproxil fumarate (TDF), the

second nucleotide analogue was approved for the treatment of CHB. It is structurally related, but more potent than ADV. Forty-eight weeks of TDF compared with ADV therapy in HBeAg-positive CHB resulted in more patients achieving viral suppression defined as < 400 copies/mL (76% *vs* 13%), normalization of ALT (68% *vs* 54%), histological improvement (67% *vs* 12%) and HBsAg loss (3.2% *vs* 0%)[89]. Data from the TDF trials revealed an excellent durability of response, with a viral suppression (HBV DNA < 400 copies/mL) of 99% and 100% in HBeAg-negative and HBeAg-positive CHB respectively after 4 years of therapy[90,91]. Sub-analysis of the Asian subset of 145 patients revealed similar efficacy (97%) in achieving viral suppression defined as HBV PCR < 400 copies/mL[92]. Four years of TDF therapy has led to HBeAg loss in 41% of patients and HBeAg seroconversion in 29%[91]. TDF is also superior to ADV in achieving increased viral suppression (93% *vs* 63%), an improved inflammatory score and viral suppression (71% *vs* 49%) in a phase Ⅲ study of HBeAg-negative patients[89]. However, besides profound viral suppression, the most impressive characteristic of TDF is that no resistance has been detected to date with 5 years of follow up[92,93]. Due to these excellent features, TDF is recommended as first line agent for treatment-naïve CHB patients. Furthermore, treatment with TDF for 5 years showed regression of cirrhosis in 74% of those who showed cirrhosis at baseline[94].

Emtricitabine

Emtricitabine is a nucleoside analogue structurally similar to LAM. Emtricitabine was approved by the FDA since 2003 for treatment of HIV infection and is not approved by the FDA for CHB. It is currently being studied as an add-on to TDF therapy in the form of Truvada (tenofovir 300 mg/emtricitabine 200 mg). Like lamivudine, its use as monotherapy for treatment of CHB is limited by its intermediate genetic barrier to resistance. Two years of emtricitabine therapy is associated with 13% risk of development of resistance[95].

A randomized trial in ADV-experienced patients showed equal efficacy in viral suppression to < 400 copies/mL between tenofovir and Truvada at 24 wk of therapy[96]. After 24 wk in the randomized arm, patients were switched to open label Truvda if they had detectable HBV DNA defined as > 400 copies/mL. Eighty one per cent of patients in each treatment arm achieved serum HBV DNA < 400 copies/mL at the end of week 48 according to intention-to-treat analysis[96]. The presence of baseline ADV resistance or LAM resistance did not impact the efficacy of TDF nor Truvada. Both TDF and Truvada were equivalent through week 168 of therapy in achieving viral suppression at a rate of 82%, independent of pre-existing ADV or LAM-resistant mutations[97].

ROLE OF ANTIVIRALS IN PREVENTION OF HCC RECURRENCE

With the advent of antiviral therapy, it is now possible to reduce inflammation, regress cirrhosis and reduce the incidence of HCC in patients with CHB. The incidence of HCC recurrence after resection of HBV-related HCC is high. Newer data has shown that there is a role for antiviral therapy for those who have already developed HCC. Since 2005, there have been retrospective studies, small and large in numbers that showed improvement of survival in patients who received concomitant antiviral therapy after curative liver resection and local tumor ablation[98-102]. Treatment with nucleoside/nucleotide analogues may prevent *de novo* primary tumors and further progression of liver disease, thereby decreasing recurrent HCC. Recent large cohort studies further confirmed the benefit of antiviral therapy in this group of patients with decrease in mortality with the antiviral treatment[103,104]. The longest survivors of those who benefited from antiviral therapy following the existing tumor ablation have reached over 12 years (Hann *et al*, personal communication). This novel treatment strategy may offer a significant alternative to liver transplantation to relieve the current graft shortage.

VIROLOGIC BREAKTHROUGH, COMPLIANCE AND SAFETY OF TREATMENT

As noted above, the development of anti-viral resistance is a barrier in achieving successful therapy in CHB. In large retrospective review of 11000 CHB patients on nucleoside/nucleotide therapy, mean adherence rate to therapy was 87.8% with 1-year persistence of 81%. Although adherence to CHB therapy is high, new and younger age patients tend to be less compliant[105]. In a study of 148 CHB patients on nucleoside/nucleotide therapy with mean follow-up of 3 years, 39 patients had at least one virologic rebound with 38% having no genotypic resistance and 10 patients with further HBV DNA decline while continued on current re-treatment[106]. Medication non-adherence is a common cause of intermittent virologic rebound and should be addressed before changing therapy. In a study of 84 patients treated with LAM, ADV, or ETV who stopped therapy after reaching defined endpoints, 42% of HBeAg-positive and 47% HBeAg negative patients had virologic relapse with HBV DNA more than 1000 copies/mL at a mean of 4.3 mo[107].

Concerns about the possible lose of bone mineral density (BMD) and has been raised from the results of clinical studies on CHB treatment[108-111]. BMD loss has been reported in chronic liver disease. However, accelerated BMD loss has been reported in patients specifically on TDF[108]. This BMD loss has raised concerns regarding the long term safety of TDF. BMD should be monitored in patients on TDF with bone density scans and factors that also contribute to bone loss should be given consideration when selecting a treatment option for CHB[108,109]. Like BMD, renal function is frequently impaired in patients with compensated CHB. These oral antiviral agents

are all primarily eliminated unchanged through renal route. Therefore, inpatients with renal insufficiency, dose reduction and/or increased dose intervals are recommended. Renal impairment is frequent after long-term treatment with adefovir[110]. Similarly, a decrease of eGFR has been observed in retrospective cohorts of CHB patients during long-term tenofovir or entecavir-treated[111].

CONCLUSION

Although a vaccine has been available for hepatitis B since 1982, this chronic infection is still far from eradicated across the world. Timely use of nucleotide/nucleosides may improve liver function and increase survival in patients with hepatic decompensation. Maintained suppression of HBV replication with antiviral therapy halt the progression of liver disease, may reverse liver fibrosis, and can reduce the development of cirrhosis and HCC. Due to the availability of effective and potent treatment options for HBV, there has been a decrease in the proportion of annual liver transplants performed for this indication[112]. However, one must remember that this can only achieved with an excellent compliance on the part of patients, early detection of drug resistance and correct choice of medications. Nonetheless, current therapies may not always prevent all adverse sequela. HCC must be monitored using ultrasound and α-fetoprotein assays to improve outcomes by increasing early detection and the chance of curative treatment. Developing safe and affordable agents as well as management strategies to improve sustained HBV suppression should be the ultimate goal in the treatment of chronic HBV infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Outcomes and management of viral hepatitis and human immunodeficiency virus co-infection in liver transplantation

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Abstract

Liver transplantation for human immunodeficiency virus (HIV) positive patients with viral hepatitis co-infection is increasingly offered in many North American and European liver transplant centers. Prior studies have demonstrated acceptable post-transplant outcomes and no increased risk of HIV complications in patients co-infected with hepatitis B virus (HBV). However, liver transplantation in HIV positive patients with hepatitis C virus (HCV) has poorer outcomes overall, requiring careful selection of candidates. This review aims to summarize the published literature on outcomes after transplant in HIV patients with HBV or HCV related end-stage liver disease and recommendations for management. In particular the pre-transplant factors impacting outcomes in HCV/HIV co-infected candidates and importance of multidisciplinary management will be discussed.

Key words: Hepatitis B virus; Human immunodeficiency virus co-infection; Hepatitis C virus; Co-infection; Liver transplantation

Core tip: Liver transplantation is not contraindicated in viral hepatitis patients co-infected with human immunodeficiency virus. Patients should meet standard listing criteria for liver transplantation. Management of these patients should be done through a multidisciplinary management approach including pharmacists and infectious diseases physicians.

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INTRODUCTION

Liver transplantation offers the promise of improved quantity and quality of life for patients with end stage liver disease with 1-year survival approaching 90%[1]. Historically, HIV infection was considered to be a contraindication to transplantation[2] with early outcomes in the pre-highly active anti-retroviral (HAART) era being abysmal[3]. With the improvement of HAART, patients with HIV have comparable life expectancy to the general population[4,5]; similar to those with other chronic medical conditions, such as diabetes. As such, liver transplant is now considered a potential treatment option to the over 1.1 million infected with HIV in the United States[6] and 34 million worldwide[7].

Patients living with HIV have a significant burden of liver disease; one large series suggests that liver disease is related to over 14% of all cause mortality[8] with

three-quarters of this being attributable to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. The increased burden of co-infection with HBV or HCV in HIV patients relates to similar mechanisms of transmission of the viruses such as sexual or vertical transmission, blood transfusion or intravenous (*iv*) drug use. In areas of low HBV endemicity, such as North America and Western Europe, HIV and HBV co-infections occur primarily in immigrant populations and in adult populations due to shared sexual and percutaneous modes of transmission[9]. The prevalence of HBV co-infection in Western countries has been reported as between 6%-14%[10] with rates of co-infection in endemic areas such as Africa and southeast Asia nearing 30%[11]. For HCV, the rates of co-infection also reflect the shared risk factors for transmission with approximately 10% acquired through high risk sexual exposures and the vast majority via blood-borne contact[12]. The global burden of HIV co-infection is significant with approximately 7 million persons co-infected with HCV and 4 million with HBV worldwide[13,14].

IMPACT OF VIRAL HEPATITIS/HIV CO-INFECTION

Patients co-infected with either HBV or HCV have more aggressive liver disease than those with mono-infection. In a large epidemiological study of 23441 patients infected with HIV, HIV-HBV co-infected patients were reported to have a 3-fold higher risk of liver related mortality compared to HBV mono-infection[8]. Differences in survival were demonstrated in the early HAART era; a Taiwan study showed a 5-year survival of 75% in patients with HIV and chronic hepatitis B [positive serum hepatitis B surface antigen (HBsAg)] versus approximately 90% survival in HBsAg-negative, HIV infected patients[15]. A subsequent meta-analysis of the co-infected population was concordant with this finding, although HAART did reduce the risk of death from 1.6 (95%CI: 1.07-2.39) to 1.28 (95%CI: 1.03-1.60)[16]. Accordingly, HIV co-infected patients without HAART seem to have more aggressive HBV-related liver disease and progression to cirrhosis[17]. As well, HBV co-infection has also been demonstrated to adversely impact HIV patient outcomes either with or without HAART[18,19].

In HCV/HIV co-infection, the HCV disease course is negatively impacted with an increased HCV viral load as compared to HCV mono-infected patients[20] as well as accelerated fibrosis progression[21-24]. Insulin resistance in HCV mono-infected patients has been associated with increased fibrosis and impaired response to treatment[25,26] although in the co-infected population the impact of insulin resistance is less clear[27-30]. HCV co-infected patients have increased healthcare resource utilization[31] and increased mortality[32] versus those living with HCV alone. Treatment of HCV is often more complex, due to the interaction between HCV and HIV medications[33]. Further complicating matters is that co-infected patients have inferior responses to interferon and ribavirin based therapy; pooled analysis showed sustained virological response rates of 38% overall with genotypes 1 and 4 being 25% and genotype 2 and 3 being 60%[34] although the addition of protease inhibitors may lead to similar responses in genotype 1 infections[35]. The evidence that HIV disease activity is aggravated by HCV co-infection is controversial[36]. Nonetheless, achieving adequate control of the HIV with the use of HAART is important as it may reduce mortality as compared to no treatment[37] and reduce the rate of fibrosis to that of a HCV mono-infected patient[38] albeit may not completely achieve fibrosis regression[39].

OUTCOMES AFTER LIVER TRANSPLANTATION IN HCV/HIV CO-INFECTION

The published outcomes of HCV/HIV co-infected patients with regards to survival and HCV recurrence were analyzed in a recent meta-analysis[40]. This analysis compared HIV/HCV co-infected patients to those infected with HCV alone. There was no difference between groups with regards to the rate of acute cellular rejection (OR = 0.88; 95%CI: 0.44-1.76) or with regards to HCV recurrence rates (OR = 0.66; 95%CI: 0.27-1.59) although the evidence quality is described as being low. A significant reduction in survival was seen in co-infected patients compared to the HCV mono-infected population (HR = 2.81; 95%CI: 1.47-5.37) although this again was based on weaker evidence overall. More recently, there have been two large prospective multicenter cohort studies examining outcomes of HCV-HIV co-infection published. In Spain, a series of 86 consecutive HCV-HIV co-infected patients were compared to a matched series of 252 HCV mono-infected patients[41]. Patients with HIV were eligible if they met Spanish consensus guidelines[42] including $CD4^+$ T cell counts > 100 cells/L (> 200 cells/L with history of opportunistic infection), suppressed HIV viral load and no AIDS defining events other than *Pneumocystis* pneumonia, esophageal candidiasis or tuberculosis.

In this cohort, 55% of the population had genotype 1 HCV infection and 15% were co-infected with HBV; the median model for end-stage liver disease (MELD) score at the time of transplant was 16 and the waitlist time was 4 mo. Notable differences between the HCV comparison group and the HCV/HIV co-infected group were lower rates of genotype 1 in the HCV mono-infected group, lower rates of acute rejection and lower rates of significant fibrosis (> Stage 2) with post-transplant recurrence of the HCV. Survival in the HCV/HIV co-infected group was similar in the first year (88% *vs* 90%) but diverged at 3 years (62% *vs* 76%) and 5 years post transplant (54% *vs* 71%). Similar rates and trends were seen for graft survival. Factors predicting poor survival on multivariate analysis included HCV genotype 1 and an increased donor risk index; having a low HCV RNA level had a significant protective effect. Low center experience

Table 1 Summary of outcomes post orthotopic liver transplant in hepatitis C virus/human immunodeficiency virus co-infection

Ref.[1]	Study period	Country	Patients	Median follow-up (mo)	Survival	Graft survival
Terrault *et al*[2]	2003-2010	United States	89	32	76% 1 yr 60% 3 yr	72% 1 yr 53% 3 yr
Miro *et al*[41]	2002-2006	Spain	84	44	88% 1 yr 62% 3 yr 54% 5 yr	NR
Duclos-Vallée *et al*[43]	1999-2005	France	35	44	82% 1 yr 73% 2 yr 51% 5 yr	NR
De Vera *et al*[44]	1997-2005	United States	27	27	67% 1 yr 56% 3 yr 33% 5 yr	63% 1 yr 52% 3 yr 31% 5 yr
Ragni *et al*[94]	1997-2001	United States	15	17	80% 1 yr 57% 3 yr 36% 5 yr	NR
Vennarecci *et al*[95]	2002-2006	Italy	11	26	83% 1 yr 58% 3 yr[2]	NR
Anadol *et al*[96]	1997-2011	Germany	19	61[3]	58% 5 yr	NR

[1]Studies with ⩾ 10 patients; [2]Survival reported for cohort of 12, including one hepatitis B virus/human immunodeficiency virus co-infected patient; [3]For all patients. NR: Not reported.

was also independently associated with an increased risk of death.

The main North American experience published to date is based on data from the National Institutes of Health-sponsored Solid Organ Transplantation in HIV study. This multicenter United States trial compared a group of 86 HIV/HCV co-infected patients to HCV mono-infected patients and to all transplants in patients over the age of 65 years using the United Network for Organ Sharing (UNOS) database[2]. Eligible patients had similar entry criteria to the Spanish study including a $CD4^+$ T cell count > 100 cells/L for 6 mo and being on a stable HAART regimen for at least 3 mo with undetectable viral loads; patients intolerant to HAART were allowed entry if they were predicted to have suppression of HIV post transplant based on past medication exposure and anti-HIV drug resistance testing. There was a more liberal policy regarding opportunistic infections in that after April 2002, patients with treated opportunistic infections (excluding lymphoma, visceral Kaposi's sarcoma, chronic cryptosporidiosis and progressive multifocal leukoencephalopathy) were eligible for enrolment in the trial.

In this study, the HCV/HIV group was younger, had a lower body mass index (BMI), longer warm graft ischemic time and were more likely to have a deceased donor transplant than the HCV mono-infected group. More patients in the co-infected group received anti-HCV treatment. The co-infected group had significantly poorer survival compared to the HCV mono-infected population with 1-year survivals of 76% *vs* 92% and 3 year survivals of 60% *vs* 79%. The graft survival was also worse in HCV/HIV coinfected patients with 1-year graft survival rates of 72% *vs* 88% and 3 year graft survival of 53% *vs* 74%. The only factor identified as a risk factor for patient survival in this group was HIV infection. Risk factors for losing graft function included having a combined liver-kidney transplant (HR = 3.8), BMI < 21 kg/m^2 (HR = 3.2), HCV+ donor (HR = 2.5) and an older donor (HR = 1.3/decade). Table 1 summarizes the data from the larger HCV trials published to date.

Based on the literature, several major risk factors for poor transplant survival in the HCV/HIV co-infected population have been reported. These include high MELD scores[43,44], HCV genotype 1[41], African descent[44], as well as viral load[41,44]. Few studies have looked at factors impacting graft survival; Terrault *et al*[2] identified that receiving either a HCV+ graft or an older organ, undergoing a simultaneous liver-kidney transplant or being underweight decreased graft survival. Fibrosing cholestatic hepatitis (FCH), an often fatal complication of hepatitis C post transplant is relatively uncommon in the mono-infected population with an incidence of about 5%-8%[41,45,46]. The incidence is increased in the co-infected population by two to three fold with the largest series suggesting a prevalence of 19%[41,44,47]. Commonly associated factors for FCH in the mono-infected population including acute rejection and older donor age[48] were not identified as predictors in the co-infected population[47].

OUTCOMES AFTER LIVER TRANSPLANTATION IN HBV AND HIV CO-INFECTION

In general, the results for HBV/HIV co-infected patients are very good and are similar to that of HBV mono-infected patients. One of the largest published series of HBV/HIV co-infected patients undergoing transplant consists of 22 patients predominantly from the Solid Organ Transplantation in HIV Multi-Site Study[49]. Patients were required to have either undetectable HIV viral loads or be predictably suppressible, $CD4^+$ T cell count > 100 cells/L, absence of prior opportunistic infections and no history of visceral Kaposi's sarcoma. Post transplantation, patients received a combination of hepatitis

Table 2 Summary of outcomes post orthotopic liver transplant in hepatitis B virus/human immunodeficiency virus co-infection

Ref.	Study period	Country	*n*	Median follow-up (mo)	Survival	Graft survival	Comments
Coffin *et al*[49]	2001-2007	United States	22	42	85% 1 yr 85% 3 yr	85% 1 yr 85% 3 yr	About 50% had detectable HBV pre transplant
Tateo *et al*[97]	1999-2007	France	13	32	100%	100%	1 co-infected with HDV, 2 with HCV, 4 with HCV and HDV
Anadol *et al*[96]	1997-2011	Germany	10	61[1]	90% 1 yr 80% 5 yr	NR	
Schreibman *et al*[98]	1999-2006	United States	8	NR	75% 1 yr 75% 3 yr	NR	2 co-infected with HCV, 1 fulminant hepatic failure
Norris *et al*[99]	1995-2003	United Kingdom	4	22	100% 1 yr	NR	

[1]For entire series. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NR: Not reported; HDV: Hepatitis D virus.

B immune globulin (HBIG) and antiviral therapy with indefinite use of HBIG targeting anti-HBs titers of > 100 IU/L; HBV antiviral treatment was based on their treatment prior to transplant. Co-infected patients were compared to patients with HBV undergoing transplantation at the University of California, San Francisco. Overall, the co-infected group was younger (median age 47 years *vs* 58 years), included more males (100% *vs* 65%) and fewer were transplanted for HCC as a primary indication (9% *vs* 25%). Both groups had similar donor characteristics other than the co-infected group receiving younger donors (39 years *vs* 51 years); immunosuppression regimens in the co-infected population were more likely to use cyclosporine and less likely to be receiving mycophenolate mofetil. The three-year survival of 85% was not significantly different to the HBV mono-infected population; there was no evidence of HBV disease activity in either group. Similar positive outcomes have been seen in other small trials (Table 2).

ECONOMIC STUDIES OF CO-INFECTED LIVER TRANPLANTATION

Liver transplant has significant cost to payers with estimated costs of transplant in the HCV mono-infected population of $169000 USD initially and subsequent annual costs of $38000[50]. Very limited study has been done on the economic impact of HIV on the cost of transplantation. One study estimated that the presence of HIV adds an additional cost of approximately $38000 to the cost of liver transplantation[40]. Further economic analyses to assess cost-effectiveness are needed.

SELECTION OF HIV POSITIVE PATIENTS FOR LIVER TRANSPLANTATION

Patients with HIV should meet the generally accepted indications for liver transplant as patients without HIV including advanced liver disease with a MELD of at least 15 or having an indication for an appealed MELD score (*i.e.*, MELD exception points) such as hepatocellular carcinoma, hepatopulmonary syndrome or portopulmonary syndrome[51]. They must be able to tolerate the surgery from a cardiovascular and respiratory standpoint and have a satisfactory psychosocial assessment. Patients with a history of substance abuse, including alcohol, must be abstinent and have completed a treatment program. Most programs, including ours, require a minimum of 6 mo of abstinence even though this interval is arbitrary[52].

Patients should be on a stable treatment regimen for their HIV with documented adherence and viral loads being undetectable for at least a 6-mo interval. Preferred antiretroviral agents as part of the HAART regimen include raltegravir, efavirenz, maraviroc and rilpivirine. Zidovudine and protease inhibitors are avoided when possible due to significant anemia and drug interactions respectively. Most nucleoside reverse transcriptase inhibitors can be used. Didanosine and stavudine are the only agents truly contraindicated given the risk of potentially fatal lactic acidosis. Antiretroviral therapy is now recommended in all HIV-infected patients, regardless of CD4 count, assuming they understand the risks and benefits of therapy and are committed to adherence[53]. In the context of potential transplantation and subsequent immunosuppression as well as the numerous options for therapy currently, we would recommend all patients be on HAART prior to transplant.

Current guidelines from the American Society of Transplantation (AST), based on the National Institutes of Health studies, suggest that patients with no AIDS defining conditions should have a $CD4^+$ T cell count > 100 cells/L and for patients with AIDS defining disease, a $CD4^+$ T cell count > 200 cells/L for at least 3 mo before transplant[54]. In practice, there may be significant variation between centers. In our center, we require a CD4 count $\geqslant$ 150 cells/L for at least 6 mo or, if the CD4 count is between 100-150, as is not uncommon in those with portal hypertension and hypersplenism, the CD4% should be $\geqslant$ 20% for at least 6 mo. Patients treated with interferon based anti-HCV treatment experience a drop in their absolute CD4 counts[55]. Therefore, to accurately reflect their immune status in the context of transplantation, they should have their CD4 count performed prior to beginning interferon treatment; a reasonable interval would be 4-6 mo. We consider a history of multi-drug resistant HIV a relative contraindication to transplantation given the potential challenges of controlling HIV post transplant; however as newer options for therapy continue to be developed, this needs to be reviewed on a

Table 3 Contraindications to liver transplantation in human immunodeficiency virus positive patient

Condition	Comment
Progressive multifocal leukoencephalopathy	
Cryptosporidiosis	Chronic intestinal > 1 mo duration
Lymphoma	Primary CNS
Visceral Kaposi's sarcoma	Cutaneous KS considered if remission with immune reconstitution and no active/vascular residual cutaneous lesions on physical exam and chest CT scan
Encephalopathy, HIV-related	Unless diagnosed prior to HAART and resolved on HAART with marked improvement in mental status and increased $CD4^+$ T-cell count and no evidence of progression of CNS disease and are otherwise considered eligible from a functional standpoint

HIV: Human immunodeficiency virus; CNS: Central nervous system; CT: Computerized tomography; KS: Kaposi's sarcoma; HAART: Highly active anti retroviral therapy.

case by case basis by the infectious diseases consultant to assess the likelihood of HIV viral breakthrough and the potential for future options.

In general, most opportunistic infections (OI) are not absolute contraindications to transplantation; absolute contraindications are listed in Table 3, which are congruent with the AST guidelines[54]. Patients should have been successfully treated for the OI with a reasonable interval of time elapsing following the completion of therapy to allow for immune reconstitution with HAART; we suggest at least 12 mo since the infection. Consultation with an infectious diseases specialist with regards to the risk of recurrence post transplant as well as the need for any additional prophylactic therapies post transplant is also recommended.

For the HIV/HCV co-infected patient, simultaneous liver-kidney transplantation is not recommended given poor outcomes[2] as compared to the mono-infected HCV patient[56]. Similarly, HCV positive grafts should be avoided in HIV co-infected patients given inferior survival[2]. Patients should ideally have a BMI > 21 kg/m^2; in otherwise acceptable candidates with BMIs < 21 kg/m^2, nutritional supplementation and reassessment once the BMI exceeds 21 is reasonable; this is in keeping with the 2013 AST guidelines[54]. We would suggest that patients with natural MELD scores > 25 be reviewed on a case by case given the worse outcomes seen in this population. Additionally, it is recognized that co-infected patients have more rapid deterioration of liver disease and a higher risk of death on the list than mono-infected patients[1,57], thus a detailed discussion of potential benefit of live donor liver transplantation should occur. At this time, we do not recommend re-transplantation of patients with HIV/HCV co-infection outside of study protocols given the poor 42% 3 year survival seen in a small series of 14 patients[58].

SUMMARY OF RECOMMENDATIONS FOR THE PRE-TRANSPLANT MANAGEMENT OF THE PATIENT WITH HIV AND VIRAL HEPATITIS CO-INFECTION

In co-infected HCV patients, successful treatment of HCV will likely offer significant improvement in transplant outcomes given the adverse effects of recurrent HCV in the co-infected[44] and mono-infected HCV populations[59] as well as increased mortality on the wait-list[60]. However, treatment can be challenging due to the interaction of novel directly acting anti-HCV agents (*i.e.*, viral protease and polymerase inhibitors) with HAART[33] especially in the context of advanced liver disease and risk of decompensation. If treatment is not feasible, complications of liver disease should be managed similar to the non-HIV infected population.

For patients with HIV-HBV co-infection, the ideal antiretroviral regimen should contain tenofovir, with appropriate dose adjustment for renal function. If tenofovir is poorly tolerated, entecavir is suggested in conjunction with a fully suppressive HIV treatment regimen. If patients have any history of lamivudine exposure, given high rates of lamivudine resistant HBV in the co-infected population, it is recommended that if entecavir is used, it be at a 1 mg dose daily.

Both HBV/HIV and HCV/HIV co-infected patients with HCC seem to have a higher risk of tumor progression outside of most transplant centers' criteria including the current UNOS standard of the Milan criteria[61], and total tumor volume[62]. Given this, we would obtain imaging every 3 mo to monitor tumor development. As well, given that an α-fetoprotein (AFP) rise of > 15 μg/L per month is associated with a poor prognosis in this population[63], it is suggested that AFP be done monthly as a marker of tumor progression.

SUMMARY OF RECOMMENDATIONS FOR THE POST TRANSPLANT MANAGEMENT OF THE HIV/VIRAL HEPATITIS CO-INFECTED PATIENT

Immunosuppression

Overall, immunosuppression in the HIV co-infected population is similar in principle to that of the mono-infected patient. The use of an induction agent is controversial; however, given high rejection rates seen[2], we feel that the use of the interleukin 2 inhibitor basiliximab as a steroid sparing agent is reasonable. Thymoglobu-

Table 4 Drug-drug interactions: Antiretrovirals and immunosuppressants[54,102]

	Steroids	Calcineurin inhibitors (cyclosporine/tacrolimus)	mTOR inhibitors (sirolimus, everolimus)	Antimetabolites (mycofenylate mofitl)
PI	Significant increase	Significant increase in immunosuppression levels in general. Calcineurin inhibitor levels may increase or decrease with exposure to either amprenavir or fosamprenavir	Significant increase in immunosuppression levels	Generally no effect; levels may decrease with nelfinavir, lopinavir/ritonavir
NNRTI	Mild decrease in level	Mild decrease in level	Mild decrease in level	No effect on immunosuppressant levels. May decrease nevirapine levels
NRTI	No effect	No effect	No effect	May be increased with zidovudine
Integrase inhibitors	No effect	Increased with elvitegravir	Increased with elvitegravir	Increased with elvitegravir
CCR5-agonists			No effect	
Fusion inhibitors			No effect	

PI: Protease inhibitor; mTOR: Mammalian target of rapamycin; NNRTI: Non-nucleoside reverse transcriptase inhibitors; NRTI: Nucleoside reverse transcriptase inhibitors; CCR5: Chemokine receptor type.

lin would not be recommended given the high rate of graft loss seen in the HIV renal transplant group due to increased HCV replication[64]. The maintenance immunosuppression regimen in HIV-positive recipients is not well defined, and even less is known in HCV/HIV in co-infected patients[54]. Most programs have used calcineurin inhibitors as the backbone of the maintenance protocol with cyclosporine potentially having some in vivo suppression of HIV[65] although cyclosporine may put patients at higher risk of rejection as compared to tacrolimus[64] and may lead to poorer outcomes in the HCV population[66]. Mycophenolate mofetil as an adjunct agent may have anti-HCV[67] and anti HIV effects[68,69]. Sirolimus may be considered as a calcineurin inhibitor-sparing agent in the context of renal insufficiency. As well, sirolimus may reduce HIV replication through blocking of the HIV entry receptor, CCR5[70], has antitumor properties in HCC[71] and demonstrated improved outcomes in a small series of HIV/HCV coinfected patients[72]. However, analysis of the Scientific Registry of Transplant Recipients database showed worse graft survival and overall HCV patient survival[73] as well as an increased risk of hepatic artery thrombosis[74]. Thus the risk-benefit ratio of sirolimus will need to be carefully considered.

Medication management in HIV positive transplant recipients is challenging due to bidirectional drug interactions between immunosuppressants and antiretrovirals which may lead to altered drug exposure, toxicity, rejection or poorly controlled HIV[75]. Close communication between pharmacists managing the HIV antivirals and immunosuppression will be critical. Therefore, both the HIV team and the transplant teams should be informed about any medication changes. Antiretroviral therapy should be given up to the time of transplant and then restarted as soon as possible post transplant once oral therapy is tolerated and ideally no longer than a week post operatively; generally in well suppressed patients before transplant, the HIV viral loads will not rebound within that time-frame. If there is poor oral absorption, HAART levels may be sub-therapeutic leading to increased risk of resistance and viral breakthrough. The key drug-drug interactions are summarized in Table 4.

Prophylaxis

In HBV patients post transplant, most patients initially receive HBIG as prophylaxis for recurrent infection[76]. The use of a potent HBV medication such as tenofovir or entecavir is also recommended. Likely, with suppression of HBV with a newer nucleos(t)ide analogue, HBIG may be able to be stopped in many patients[77-79] or may not even be required[80]. In the context of a HBV-HIV co-infected patient, until further data exists with regards to HBIG sparing therapy, we would use HBIG in combination with tenofovir or entecavir with consideration of indefinite HBIG use due to a high incidence of occult HBV post liver transplant as well as to account for possible anti-HBV drug interruptions.

Given the immunodeficiency from the HIV as well as the antirejection medication, infection prophylaxis is critical (Table 5). From an infection standpoint, perioperative prophylaxis to cover gastrointestinal pathogens, including *Enterococcus*, as well as *Candida* in those at high risk, in addition to cytomegalovirus prophylaxis with valganciclovir is identical to patients without HIV. All co-infected patients should remain on lifelong prophylaxis for *Pneumocystis jirovecii* pneumonia with one tablet of single strength trimethoprim/sulfamethoxazole daily. For those intolerant, alternatives include monthly inhaled pentamidine, oral dapsone or atovaquone.

Summary of post transplant management issues in HIV/viral hepatitis co-infection

Rejection: Patients with HIV are at increased risks of acute cellular rejection[2,41]; careful monitoring is required. Given the increased risk, protocol biopsies are recommended peritransplant at baseline, 6 mo, 12 mo and then annually subsequently. When rejection is clinically suspected, a biopsy should be obtained. The preferred method to treat rejection is to increase calcineurin inhibitor levels or sirolimus levels depending on the agent being

Table 5 Post transplant prophylaxis

Post transplant prophylaxis	Comment
PJP prophylaxis	Trimethoprim/sulfamethoxazole SS one tablet daily life long Alternatives: Dapsone 100 mg daily, pentamidine 300 mg inhaled or *iv* monthly or atovaquone 1500 mg daily[54]
CMV	Valganciclovir 900 mg daily[1]; oral (1 g *tid*) or *iv* (5 mg/kg daily) ganciclovir for 3 mo in D+/R-; prophylaxis or pre-emptive monitoring and therapy in R+
Fungal	High risk patients[2] should receive Fluconazole 400 mg *po* daily × 14 d minimum[100]
HBV (in HBV co-infected patients)	Life long HBIG targeting 100 IU/L plus either tenofovir or entecavir

[1]Valganciclovir is not FDA approved for use in liver transplantation; many centers use this agent off label[101]; [2]High risk features include repeat or prolonged surgery, high transfusion requirements, renal failure, colonization with Candida or Choledochojejunostomy[100]. PJP: *Pneumocystis jeroveci* pneumonia; SS: Single strength; CMV: Cytomegalovirus; HBV: Hepatitis B virus; HBIG: Hepatitis B immunoglobulin; FDA: United States Food and Drug Administration.

used, as well as increasing mycophenolate mofetil levels. Use of anti-lymphocyte depleting agents, such as thymoglobulin, is to be avoided if possible due to prolonged immunosuppression of T cells with thymoglobulin[81]. High dose steroids in the context of HCV co-infection leads to more aggressive disease activity and rapid progression in fibrosis[82].

Occult HBV: In patients who have occult HBV (HBsAg negative, HB core antibody positive), we would suggest annual monitoring of HBsAg levels. Given most HAART regimens includes medication that has activity for HIV and HBV, such as tenofovir, emtricitabine or lamivudine, reactivation is low risk, especially with the use of tenofovir.

Recurrent HCV: Treatment of the hepatitis C should be offered post-transplantation when there is histologic evidence of recurrent disease. Earlier therapy in disease may be associated with improved outcomes in the mono-infected population[83] and thus consideration could be given to starting therapy in all HIV/HCV co-infected liver transplantation recipients once stable and on low dose maintenance immunosuppression. HIV/transplant pharmacists should be consulted with regards to drug selection given the complex three-way interactions between HCV protease inhibitors, antiretroviral agents and immunosuppressants.

Treatment of hepatitis C is critical with the presentation of fibrosing cholestatic hepatitis (FCH) given its marked effect on survival. Currently, the typical treatment is the use of pegylated interferon and ribavirin with a median survival of 22 mo; this is about 20% the survival of co-infected HCV-HIV patients without fibrosing cholestatic hepatitis[47]. Recently, there has been two reports of the use of a protease inhibitor (telaprevir and boceprevir[84]) including in a co-infected patient[85] with good effect, although careful monitoring of the calcineurin inhibitors is needed with both telaprevir and boceprevir[86]. One case report exists of the combination of sofosbuvir and daclatasvir being successfully used to treat FCH[87]; these drugs are not yet commercially available. Thus, consideration may be currently given to triple therapy for managing FCH given its abysmal prognosis with likely better-tolerated and more efficacious therapy to come.

CONCLUSION

Significant advances have been made with regards to transplanting patients with HIV including those with viral hepatitis co-infection. Transplanted HBV co-infected patients obtain similar outcomes to the HBV mono-infected patient with outcomes less successful in the HCV co-infected population. Treatment of HCV offers the best chance of improved outcomes. Many new treatment strategies for HCV are in advanced stages of development including pan-genotypic interferon free regimens and it is likely these regimens will allow for easier treatment of HCV and improved survival post transplant.

Historically, in the United States, organs could not be knowingly transplanted from donors with HIV by federal law[88] with similar policies existing in Canada[89,90] Given the current armamentarium of antiretroviral medications and the ability to suppress HIV, there is potentially a pool of organs that could be used to benefit patients with HIV; one model suggests that over 500-600 patients with HIV could benefit from receiving organs from donors also infected with HIV[91]. A series of 14 patients with HIV receiving HIV positive kidneys has showed good 1 year outcomes suggesting that this may be a viable option[92]. Recent passage of the HIV Organ Policy and Equity Act changes previous federal policy and enables HIV positive organs to be used in HIV positive patients[93]. With this development, further research can then be conducted with regards to outcomes and determining the appropriate population these organs can be used in. In the future, further delineation of the optimal HCV/HIV co-infected candidate will be identified so as to bring outcomes closer to that of the HCV mono-infected patient.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Core promoter: A critical region where the hepatitis B virus makes decisions

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Abstract

The core promoter (CP) of the viral genome plays an important role for hepatitis B virus (HBV) replication as it directs initiation of transcription for the synthesis of both the precore and pregenomic (pg) RNAs. The CP consists of the upper regulatory region and the basal core promoter (BCP). The CP overlaps with the 3'-end of the X open reading frames and the 5'-end of the precore region, and contains *cis*-acting elements that can independently direct transcription of the precore mRNA and pgRNA. Its transcription regulation is under strict control of viral and cellular factors. Even though this regulatory region exhibits high sequence conservation, when variations appear, they may contribute to the persistence of HBV within the host, leading to chronic infection and cirrhosis, and eventually, hepatocellular carcinoma. Among CP sequence variations, those occurring at BCP may dysregulate viral gene expression with emphasis in the hepatitis B e antigen, and contribute to disease progression. In this review these molecular aspects and pathologic topics of core promoter are deeply evaluated.

Key words: Hepatitis B virus; Core promoter; Variants; Basal core promoter; Transcription regulation

Core tip: This review summarized the progress in our understanding of the core promoter of hepatitis B virus. This critical genomic region is involved in regulating hepatitis B virus (HBV) gene expression and viral replication, involving both host and virus-derived factors on its regulation. Such pivotal functions appear modified when genomic variations are detected and clinical implications are characterized. This review emphasizes several aspects of the HBV core promoter molecular biology and highlights its role on HBV life cycle. Finally, the most frequent genomic variations with their consequent clinical correlations are described.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. Nearly 350 million are chronically infected carriers and are at high risk of complications from cirrhosis and primary liver cancer. Among these HBV carriers, 7%-30% are believed to be infected with HBV variants that express little or no hepatitis B e antigen (HBeAg)[1].

The HBV is an enveloped virus, containing a partially double-stranded DNA genome that is replicated *via* an RNA intermediate using its own encoded reverse transcriptase (RT). The HBV RT does not have proofreading or editing activity, therefore, together with this enormous

daily virion production, errors inevitably occur during replication. The rate of HBV virion production (up to 10^{11} virions per day) is considerably higher than for human immunodeficiency virus (HIV) and hepatitis C virus, and approximately 10-fold higher than for other DNA viruses. The half-life of HBV in plasma ranges from 1 to 3 d whilst the half-life of an infected hepatocyte is 10-100 d. The error rate of the HBV pol has been calculated as 10^{-7} per nucleotide per day. Therefore, on a daily base, approximately 10^{14} nucleotides are replicated with potentially 10^{7} base pairing errors[2].

The major viral translational products include the precore and core, polymerase, large, medium and small envelope proteins and the X-protein as transcriptional regulator are encoded by four overlapping genes. The lifecycle of HBV includes viral attachment and entry, viral uncoating in the cytoplasm and entry of the partially double-stranded genome into the nucleus. In the nucleus the covalently closed circular DNA (cccDNA) is synthesized in the form of a viral minichromosome, creating a stable intermediate responsible for the persistence of the virus and rebound viraemia after withdrawal of antiviral therapy in some patients. The lack of cccDNA in artificial host cells (*e.g.*, hepatocytes of HBV transgenic mice) suggests that host specific factors may regulate cccDNA formation. However, in a model of hepatocyte nuclear factor 1α (HNF1α)-null HBV transgenic mice Raney *et al*[3] have reported that HBV cccDNA in the nucleus as well as its potential precursor protein-free relaxed circular HBV DNA in the cytoplasm were present in the hepatocytes, suggesting that cycling of viral replication intermediates into the nucleus may occur in this *in vivo* model system.

The cccDNA is the template for transcription of pregenomic (pg) RNA as well as subgenomic mRNAs, which are translated into the viral proteins in the cytoplasm. Encapsidation follows the binding of the polymerase and core to the pgRNA in the cytoplasm. Synthesis of the minus-strand DNA by reverse transcription and partial synthesis of the plus-strand is accomplished by the HBV polymerase within the nucleocapsid. At this point the nucleocapsids are enveloped by budding into the endoplasmic reticulum followed by secretion from the cell or return to the nucleus to amplify the cccDNA reservoir in the nucleus[4].

The typical course of hepatitis B infection involves an HBeAg-positive phase with high serum HBV DNA levels. Subsequently, patients undergo a process of seroconversion in which HBeAg is lost and antibodies to HBeAg (anti-HBe) appear. Generally this signals the decline of HBV DNA to levels that are not detectable by unamplified assays and a return of aminotransferase to normal values. Among some patients, for reasons that are not yet clear, the immune pressure associated with seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop anti-HBe, active HBV DNA replication continues with associated liver damage[1,5].

The core promoter region regulates transcription of the pre-core region. Therefore certain mutations in this region can affect HBeAg synthesis without adversely affecting the ability of the HBV to replicate[6-9].

The primary aim of this literature review is to describe the molecular biology, function and variants of the hepatitis B virus core promoter.

HBV GENOMIC STRUCTURE AND ORGANIZATION

The HBV genome is a partly double stranded DNA composed of a minus (-) and a plus (+) strand that has evolved structurally to a compact genetic organization (Figure 1). At the 5'-end of the two strands, exhibits short cohesive end regions that contain two direct repeats (DRs) termed DR1 and DR2 that allow to maintain the circular configuration and are essential for viral replication[10]. The whole genome is carrying in the full-length coding (-) strand DNA (3.2 kb), while the incomplete non-coding (+) strand DNA region extends to around two-thirds of the genome length with a variable 3'-end. The viral polymerase is covalently linked to the (-) strand DNA. This strand also contains on both, 5'- and 3'-ends, an 8-9-nucleotide-long terminal redundancy, termed "r" that is critical for the formation of relaxed circular (rc) DNA synthesis. Once the viral (-) strand DNA synthesis is completed, a residual short RNA oligomer derived from the 5'-end pgRNA remains covalently bond to the 5'-end of the (-) strand DNA to serve as a template for (+) strand DNA synthesis. A small quantity (5%-10%) of double-stranded linear DNA can also be found packaged into the nucleocapsid instead of rcDNA. During infection, both forms of viral DNA (circular and linear) can be transformed to cccDNA in the hepatocyte nucleus.

The HBV genome contains four overlapping open reading frames (ORFs) (P, preC/C, preS/S and X) encoded by the (-) strand with six start codons, four promoters (preS1, preS2, core and X), and two enhancer elements (EN Ⅰ and EN Ⅱ) located upstream of the core promoter. The viral genome also contains a common 3' polyadenylation signal in the core gene and a number of *cis* -acting signals essential for DNA replication.

Seven viral proteins (HBe; core; large, medium and small envelopes; polymerase; and HBx) are produced by translation of the polyadenylated and capped viral RNA transcripts (3.5, 2.4, 2.1 and 0.7 kb). The open reading frame (ORF) P comprised four distinguishable domains that encode viral DNA polymerase (Pol, 90 kDa) exhibiting multiple functions and involving the terminal protein, the spacer -that exhibits no enzymatic function-, the polymerase and reverse transcriptase activity -that catalyzes viral genomic synthesis-, and the fourth domain that encodes ribonuclease H[11]. The ORF preC/C partially overlaps with ORF P and encodes the precore-core protein (HBeAg), the core protein (HBc), and the pgRNA. The pgRNA is initiated five nucleotides downstream from the precore initiation codon; it serves as a template for

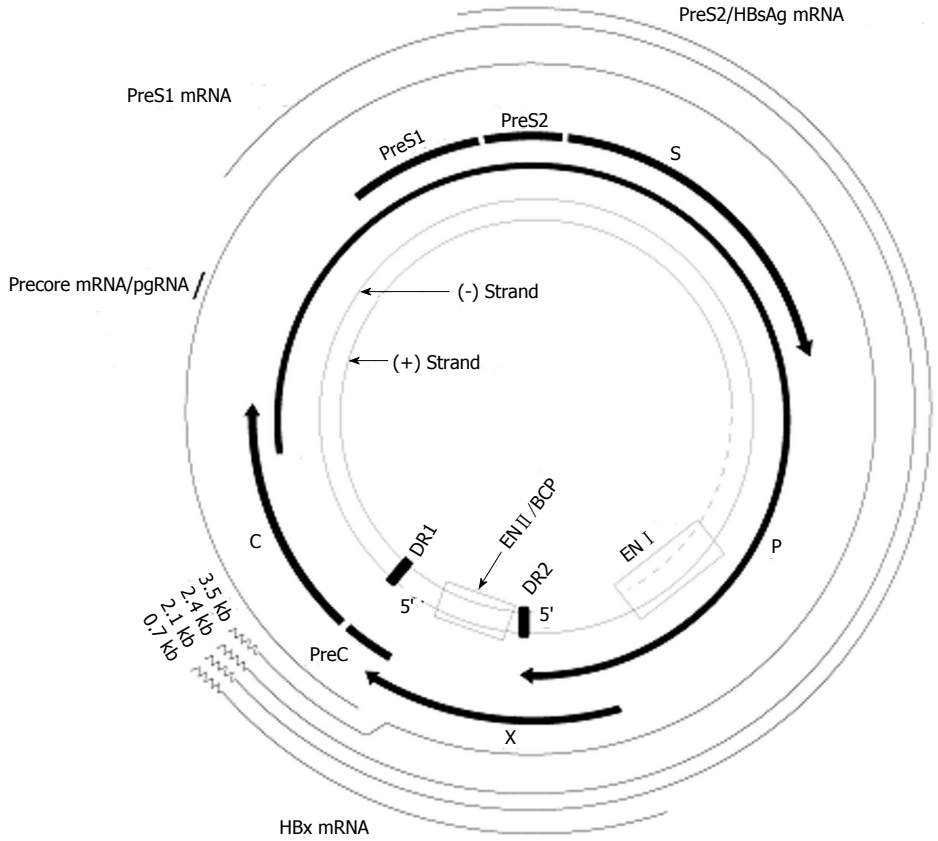

Figure 1 Genomic organization of hepatitis B virus. The inner circle depicts the rcDNA including the complete minus-strand DNA and the incomplete plus-strand DNA. The direct repeats, DR1 and DR2, as well as the two enhancers, EN Ⅰ and EN Ⅱ, are shown. The outer circle depicts the four viral RNAs, the core (C) or pgRNA, the pre-S (L) mRNA, the S mRNA, and the X mRNA. The common 3'-ends (poly-A) of the three mRNAs are indicated by the curve lines. The four protein-coding regions are shown including the precore (PC) and core genes, the polymerase gene, and the *X* gene. The envelope genes *pre-S1* (*L*), *pre-S2* (*M*), and surface (*S*) overlap with the polymerase open reading frame.

the translation of core protein (p21) and the viral DNA synthesis by reverse transcription within the nascent nucleocapsids. The precore polypeptide (p25) is generated from the preC transcript, it is not packaged into viral nucleocapsids instead it is proteolytic cleaved in both N and C termini when it moves to the secretory pathway to generate the mature secreted soluble HBeAg (p17)[11] (Figure 2). The synthesis of three integral envelope glycoproteins (large L, medium M and small S) is directed by the ORF preS/S, located within ORF P (between the spacer and the RT domains). The three glycoproteins contain HBsAg and they have distinct N-terminal domains because they use three in-frame AUG start codons[12]. The smallest ORF X overlaps with ORF P. It encodes for the small protein HBx (17 kDa) which shares no homology with any known gene. This ORF is only found in all mammalian hepadnaviruses and plays a central role in the pathogenesis of HBV-induced hepatocellular carcinoma[13,14].

REGULATION OF HBV DNA TRANSCRIPTION

HBV promoters and enhancers

The HBV genome contains four promoters (core, preS1, preS2 and X) and two enhancers I and Ⅱ (EN I and EN Ⅱ) as *cis*-acting elements to control HBV transcription of the four 3.5, 2.4, 2.1 and 0.7 kb mRNAs, which have heterogeneous 5'-ends, with the exception of preS1, which lacks a TATA box and produces transcripts with a defined 5'-end[15].

The transcription of HBV mRNA can be modulated by both EN I and EN Ⅱ[16]. EN I (approximately 200 bp long) is the main stimulating element and up-regulates significantly preC and HBx mRNAs but has little effect on surface protein mRNAs. Its location partially overlaps the X promoter and exhibits binding sites for ubiquitous (NF1, AP1 and NFκB) and liver-specific [LRH-1/hB1F, HNF1, HNF3b, HNF4 and CCAAT/enhancer binding protein (C/EBP)] transcription factors[17,18]. EN I contains three distinct domains: a 5' modulator element, a central core domain, and a 3'domain that overlaps with the HBx ORF. The central core domain contains the enhancer activity and binding sites for HNF3, RFX1, EFC, NF1, and a retinoic acid response element (RARE), which interacts with HNF4, the peroxisome proliferator-activated receptor α-retinoid X receptor α (PPAR/RXR α) heterodimers and Chicken ovalbumin upstream promoter transcription factors (COUP-TF). Interestingly, signal transducer and activator of transcription-3 (STAT-3) factor may bind to NF1- and HNF3-binding sites and in-

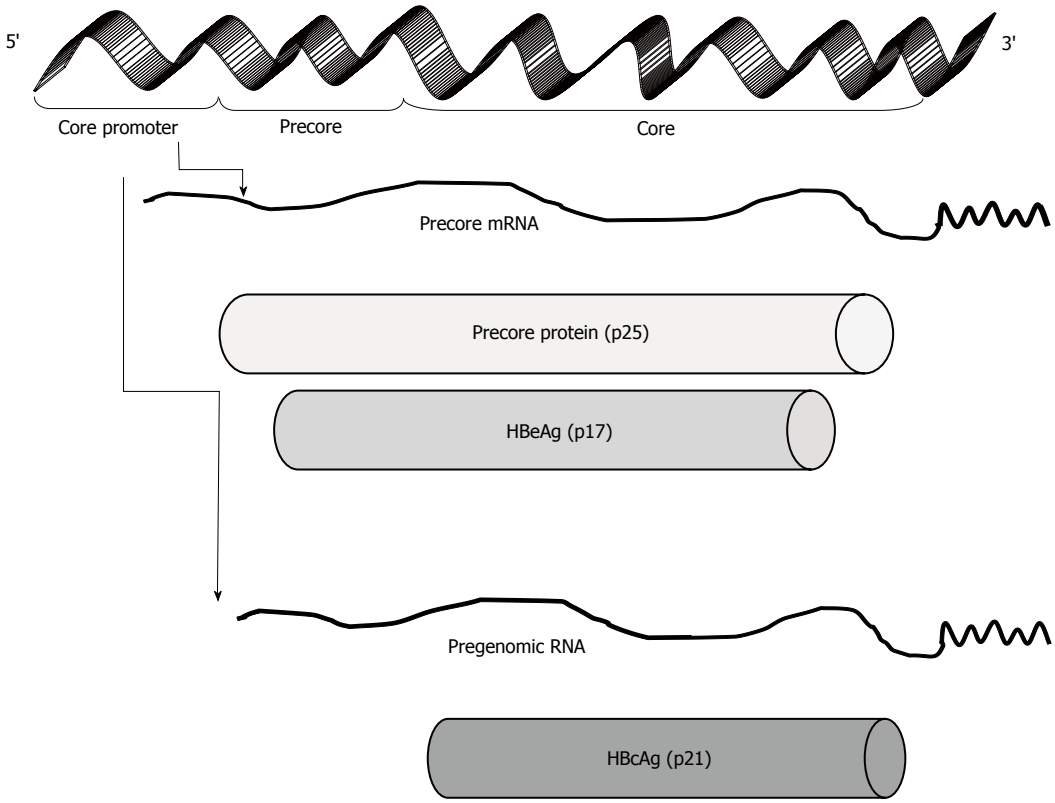

Figure 2 Transcription and translation of hepatitis B virus precore. The core promoter region regulates the transcription of precore mRNA and pregenomic RNA from the same open reading frame. The precore mRNA is translated into precore protein, which is processed at the N-terminal and C-terminal ends to HBeAg, a secretory protein. Pregenomic RNA is reverse transcribed into HBV DNA and also translated into core protein (HBcAg), which overlaps with HBeAg. HBcAg: Hepatitis B core protein antigen; HBeAg: Hepatitis B e antigen.

crease EN I function. Despite the 5' modulator element and the 3' domain have no enhancer activity, the former can interact with C/EBP and HNF1 transcription factors and increase the central core domain activity. EN Ⅱ is located upstream of the core promoter and increases preferentially the transcription of the preS1, preS2, and X promoters as well as the pg/pc mRNAs. EN Ⅱ contains two regions, Ⅱ A and Ⅱ B, that act concomitantly as potent enhancer elements that bind various transcription factors such as C/EBP, RXR, PPAR, HNF4, HNF3, FTF, hepatic leukemia factor and Sp1[19,20].

The preS1 and preS2 promoters control the envelope protein expression. The first one controls the transcription of the L mRNA (2.4 kb) while the preS2 promoter controls the transcription of S and M mRNAs (2.1 kb), and it seems to be stronger than the preS1 promoter and leads to the production of more S and M surface proteins, which is necessary for virus secretion[21].

The transcription of HBx is under the control of its own promoter X to generate an approximately 0.7 kb-long RNA. The X promoter has no TATA box, but its sequence is located upstream of the transcription initiation site and overlaps with the ENI 3'-end[13,22].

MOLECULAR BIOLOGY OF THE HBV CORE PROMOTER REGION

The 3.5 kb RNA synthesis is regulated by two partially overlapping but genetically distinct core promoters that overlap the 3' end of the X ORF and the 5' end of the pre-C/C ORF and direct the transcription of both preC and pregenomic RNAs[23].

The core promoter (CP, nt1575-1849) of the viral genome has a pivotal role in the replication and morphogenesis of the virus[24] (Figure 3). It directs initiation of transcription for the synthesis of both the precore mRNA and pgRNA. The CP consists of the BCP (nt 1743-1849) and the upper regulatory region (URR, nt 1613-1742), the latter containing both positive and negative regulatory elements that modulate promoter activity[25]. The BCP is sufficient for accurate initiation of both pre-C mRNA and pgRNA transcription *in vivo*. BCP contains major nuclear binding sites which is recognized by a variety of the nuclear receptors super family, including HNF4 and PPARα-RXRα heterodimer and a series of transcription factors such as C/EBP to regulate the transcription both the pre-core RNA and the core RNA. Different biological functions of pre-core and pregenomic RNA, as well as differential regulation of BCP by these transcriptional factors may have profound effects on HBV replication and pathogenesis.

The BCP minimal essential sequence is a 108-bp fragment (nts 1742-1849) that contains direct repeat 1 (DR1) which is required for reverse transcription[26]. It overlaps with the 3'-end of the X ORF and the 5'-end of the precore region, and contains *cis*-acting elements that can

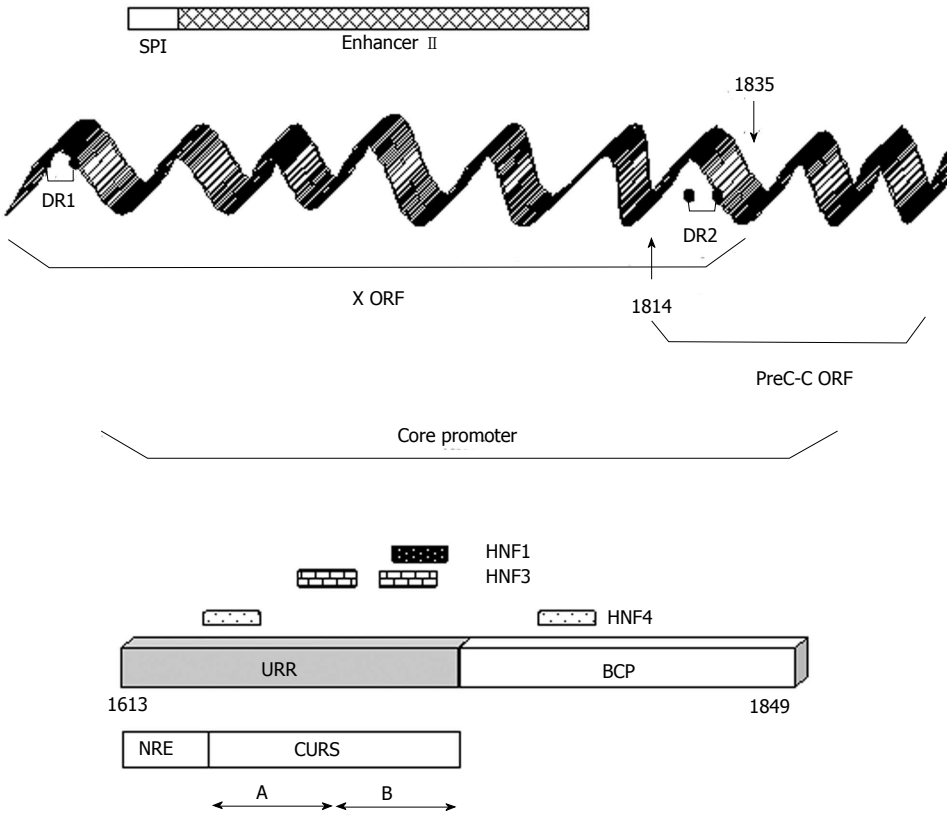

Figure 3 Molecular biology of the hepatitis B virus core promoter. The core promoter (CP) overlaps the 3'-end of the X open reading frame (ORF) and the 5'-end of the pre-C/C ORF. The CP comprises the upper regulatory region (URR) and the basic core promoter (BCP). The former involves a negative regulatory element (NRE) and a core upstream regulatory sequence (CURS) which can be further subdivided into two domains: CURS-A and CURS-B. The sequence of enhancer II and binding sites hepatocyte nuclear factor 1, 3 and 4 (HNF1, HNF3, HNF4) are shown. SPI: S promoter; DR: direct repeat.

independently direct transcription of the precore mRNA and pgRNA, both of which are about 3.5 kb in length[27]. The URR comprises the negative regulatory element (NRE, nt 1613-1636) and the core upstream regulatory sequence (CURS, nt 1636-1742)[28]. The NRE, upstream of EN Ⅱ, can repress both EN Ⅱ and CURS in differentiated liver cells. It exhibits a minor inhibitory effect on its own but is strongly repressive in the presence of EN Ⅱ. At least three different subregions are distinguished: NRE-α, NRE-β and NRE-γ which suppress strongly the CP activity. NRE-γ also suppresses EN Ⅱ activity. The activity of the NRE is cell-type dependent which could be ascribed to a differential regulation by cell type-dependent protein factors[23,29].

The CURS acts as a regulatory region neither as a promoter nor with EN Ⅱ participation. It can be subdivided into two domains: CURS-A (nts 1636-1703) and CURS-B (nts 1704-1743). The former can exert a positive regulatory effect on the BCP, although to a lesser degree than does the whole CURS. It can be subdivided in several sequence motifs, namely, α (nts 1646-1668) which is able to bind different transcription factors, γ (nts 1671-1686) and δ (nts 1687-1703), which positively regulate BCP activity; in contrast, β (nts 1704-1714) regulates negatively[26].

The EN Ⅱ element regulates the activity of the CP and partially overlaps the BCP and URR. Sequences located directly at 5' of the BCP can positively regulate this promoter in a liver-specific manner through *cis*-acting elements in the CURS overlapping EN Ⅱ. Further upstream there are sequences that can negatively modulate the promoter through the NRE.

Sequences within the EN Ⅰ (nts 1074-1234) can also activate the BCP which is required for augmenting the CP extremely weak promoter activity. This whole region contains nucleotide motifs that constitute transcription factor binding sites, imparting at the same time liver cell specificity for optimal function of these elements. This interaction between the *cis*-acting elements with ubiquitous and liver-specific transcription factors is absolutely necessary for liver-specific expression from the CP. Moreover, the interaction between these trans-acting factors and the *cis*-acting elements allows the virus to coordinately or differentially regulate the transcription of the two mRNAs[27]. Moreover, the stimulation of the BCP by EN Ⅱ and CURS can be repressed by the effect of the upstream NRE[30].

The liver tropism of HBV may involve different factors including the BCP and the enhancers as well as the interaction of viral *cis*-acting elements with transacting factors present in various cell types and during different stages of liver cell differentiation[18]. The cooperative interaction of different liver enriched and ubiquitous factors is necessary for liver-specific expression from the CP.

Several transcriptional factors bind to regulatory sequence elements of the CP such as C/EBP, HNF1, and HNF3. Multiple members of the nuclear receptor superfamily of transcription factors, including HNF4, RXR, PPAR, COUP-TF1 and apoA I regulatory protein (ARP1), bind this regulatory element and differentially regulate synthesis of pre-C mRNA and pgRNA[19]. Their effects depend on their interactions: for example, binding of HNF4 and TR2 may repress preC RNA synthesis, whereas PPAR α and RXR α can mediate its activation, and COUP-TF1 can inhibit both preC and pgRNA synthesis. Human Sp1 transcription factor increases the synthesis of pgRNA by binding to three binding sites, but has no effect on the synthesis of pre-C mRNA[21]. By binding to the AT-rich regions of the CP the ubiquitous TBP plays an important role in the regulation of transcription. Thus, the synthesis of pre-C mRNA and pgRNA occurs from separate promoters which are in turn, differentially regulated[28].

Selection of the transcription initiation sites of pre-C mRNA and pgRNA is not liver-cell specific and the URR is not required for the precise initiation of either RNA type[21,31]. The 5'-ends of pre-C mRNAs are more varied than are those of pgRNA. The latter has been mapped to a single nt region: 1815 ± 5 whereas the 5'-ends of pre-C mRNA map to nts 1785-1786 and 1791-1797. Such discrepancy could be ascribed to the presence of three TATA-like boxes (AT-rich regions) within the BCP that are not canonical TATA box (TATAAA) and a fourth TATA-like box that controls the transcription of pgRNA. The three AT-rich sequences: TA1, 5'-AGATTA-3' (nts 1750-1755); TA2, 5'-TTAAA-3' (nts 1758-1762); and TA3, 5'-TATTA-3' (nts 1771-1775); are located 20-35 bp 5' to the pre-C mRNA start sites and can bind recombinant TATA-binding protein (TBP) as well as are required for the optimal transcription of pre-C mRNA. TA4, 5'-CATAAATT-3' (nts 1788-1795), functions as both a TBP-binding site for initiation of transcription 25-30 bp downstream at the start site of pgRNA, and as the initiator (Inr) sequence for some of the pre-C mRNAs. The Inr is a minimal promoter element that overlaps the transcription start site. The 5' boundary of the pgRNA promoter is at nt 1788, the first nt of its TATA box-like sequence. The pre-C Inr consists of 5'-CATA-3' (nts 1788-1791), overlaps the pre-C mRNA initiation sites and resembles the optimal Inr sequence: 5'-CA(T/G)T-3'. Using mutational studies it was shown that the 3' boundary of this Inr element is at nt 1792. On the other hand, the pgRNA Inr element is the sequence 5'-CAACT-3' (nt 1817-1821) that overlaps the transcription initiation sites of pgRNA and two of its bases match the two most important bases of the optimal Inr sequence. Therefore, the 15-nt sequence (1788-1802) is sufficient to direct the initiation of both pre-C and pgRNA because it contains the TATA-like element of the pregenome promoter and the Inr of the pre-C promoter. The transcription of pre-C mRNA and pgRNA is regulated in a coordinate manner although the transcription of the two mRNA species can be separated. Sequences in the URR can stimulate the expression of both messages to a similar extent[28].

The pgRNA starts 3' to the precore AUG and is translated into the core and polymerase proteins. As well, it serves as the template for the synthesis of the negative DNA strand of the virus by reverse transcription after encapsidation within the neosynthesized core particle[10]. The precore mRNA, which is slightly longer than the pgRNA and is initiated upstream of the precore start codon, is the template for the translation of the precore/core protein that is post-translationally processed by proteolysis to produce HBeAg, as previously described.

BASAL CORE PROMOTER VARIANTS

Considering its pivotal role in viral replication, sequence variations within the CP in natural isolates is restricted. Nevertheless, several studies have focused on mutations within this region and, in particular, BCP because it may dysregulate viral gene expression and contribute to disease progression. In the CP, two highly conserved regions involved in the regulation of transcription have been identified: nts 1770-1808 and nts 1813-1849. When mutations in these regions occur, they are present in viral population with coexistence of wild-type variants that must have a compensatory effect, overcoming the potentially lethal effect of the mutation and allowing viral replication to proceed (Table 1). The pre-C mRNA initiation sites map within the first region[28,32] as does TA4 that controls pgRNA synthesis. Mutations within TA4 severely reduce the synthesis of pgRNA, leaving synthesis of the pre-C mRNAs unaffected[27,31]. In addition, this region has been shown to be essential for the trans-activating function of X protein[33]. The second conserved region contains the initiation site of pgRNA transcription[28,32] as well as coding for the C-terminal of the X protein. The overlap of this region with the 5'-end of the pre-C ORF containing DR1 is a further reason for the low sequence divergence. Mutations in the CP region may have repercussions on viral gene expression and/or replication, with a concurrent impact on viral pathogenesis.

The double mutation A1762T and G1764A in the BCP has been described in various disease states or settings of HBV infection[34-37]. The presence of the double mutation is clearly associated with downregulation of HBeAg production, as demonstrated by transfection studies[7,9]. There is evidence to suggest that the double BCP mutation results in decreased levels of the precore mRNA and therefore diminished production of HBeAg. Taking into consideration that HBV BCP contains a binding site for nuclear receptors, the double mutation selectively abolishes the binding of several nuclear receptors without affecting that of HNF4. It could stimulate the expression of the precore RNA and the core RNA from the core promoter of both the wild-type (WT) HBV and the double mutant, although its effect on the former was more prominent. The 1762/1764 double mutation also creates a binding site for the transcription factor HNF1 and changes two amino acids in the overlapping X protein sequence. HNF1, which did not affect the wild type BCP, suppressed the precore RNA expres-

Table 1 More frequent basal core promoter mutations and their clinical relevance

Nucleotide position	Clinical relevance
1762T + 1764A	Chronic HBV
(BCP double mutant)	Fulminant hepatitis
	Decrease in HBeAg production and increase viral replication
	Diminished binding of a liver-specific transcription factor, resulting in a decrease in HBeAg, but unchanged amounts of HBV pregenomic RNA
	Enhance viral replication through the combined effects of *X* gene mutations and the appearance of a HNF-1 transcription factor binding site
	Elevated ALT (diminishing circulating HBeAg levels → augment the host immune response to HBV-infected hepatocytes → increasing hepatocyte apoptosis)
	More often in patients with HBV genotypes that have 1858C (*i.e.*, genotype C)
1762T	HBeAg seroconversion and histological inflammation
1764A	No suppression on HBV RNA transcription and only slightly decreases the efficiency of virus replication
1653T	Usually together with the 1762T + 1764A in patients with fulminant hepatitis and hepatocellular carcinoma
1753-1757	Together with the 1762T + 1764A mutation, have been detected in patients with fulminant hepatitis and in patients with HCC
	ALT levels and histological changes
1764A/T + 1766A/G	Found in active and inactive disease in conjunction with 1810T + 1811T double mutation
	1762A1766A mutation, together with 1762T, was found in fulminant hepatitis and HCC patients
	The 1764T + 1766G mutation was found in a patient with fulminant recurrent hepatitis after liver transplantation, but was absent in patients with fulminant hepatitis.
1766T + 1768A	Fulminant hepatitis
	Together with 1762T + 1764A, in a patient with recurrent hepatitis following liver transplantation
	With 1764A in a HBeAg-negative asymptomatic carrier
	Exacerbation of HBV infection and created two overlapping low-affinity HNF1 sites

BCP: Basal core promoter; HBV: Hepatitis B virus; HNF-1: Hepatocyte nuclear factor 1; ALT: Alanine transaminase; HCC: Hepatocellular carcinoma.

sion of the double mutant. Furthermore, the X protein did not affect the HNF4 activity on the core promoter and affected the HNF1 activity on the core promoter of only the double mutant[38] (Table 1).

During *in vivo* infection its increased presence could be temporally correlated with HBe seroconversion often preceding HBeAg clearance by many years[39] but still there are discrepant results on its relationships with the HBeAg negativity considering that some of the studies were performed at different stages of chronic liver disease. More recently its presence was also related with higher viral load levels[40] in opposite with other previous findings[7,41]. These discrepancies could be associated with the upregulation of pgRNA production, promoting encapsidation and core protein production[42] whereas others, however, reported decreased precore mRNA levels, but wild-type levels of replication and gene expression[42]. Such differences observed in transcript levels when BCP mutations are detected could be explained by the alteration of a nuclear receptor binding site for HNF1 that appears to be essential for this activity. By using the woodchuck animal model and the woodchuck hepatitis virus (WHV), such variations in the HNF1 binding site diminished significantly the synthesis of viral pgRNA[43].

The BCP double mutation is correlated with the L130M and V131I mutations in the overlapping X ORF gene product. The removal of the nuclear receptor binding site had no effect on the transcription of HBV mRNAs as was shown by transfection studies in Huh7 hepatoma cells, the two codon change in the X protein suppressed both transcripts, and the creation of the HNF1 site restored the pgRNA level[44]. In addition, between both, the T1762 change is decisive for the mutant phenotype as was revealed by analysis of revertants with either one or the other of the BCP mutations[7]. Studies from chronically infected patients have provided further information regarding the double A1762T/G1764A mutation in relation to genotype[45,46]. Compared with genotype A and B cases, patients with genotypes C and D have lower rates and usually delayed onset of spontaneous HBeAg seroconversion. HBV-genotype C has a higher frequency of A1762T/G1764A mutation and preS deletion, and a higher viral load than genotype B. Similarly, genotype D has a higher prevalence of BCP A1762T/G1764A mutation than genotype A[47]. The A1762T/G1764A BCP mutations in genotype C isolates correlated with increased replication capacity, while the A1752G/T mutation found in genotype B isolates correlated with low replication capacity[48]. Interestingly, genotype C isolates with wild-type BCP sequences replicated less efficiently than genotype B isolates, due to less efficient transcription of the pgRNA, but more efficient virion release was observed with the former[49]. Core promoter changes are significantly more common in patients infected with HBV genotypes exhibiting C at nucleotide 1858, while precore stop codon changes appears exclusively in those genotypes that have T at nucleotide 1858. Nevertheless, the double BCP and the precore stop codon mutations are far from being mutually exclusive[50]. Among HBV genotype C infected patients from China, those acutely infected with BCP/precore mutant viruses had higher viral load than chronic patients with the mutant virus but both showed a lower prevalence of A1762T/G1764A, G1896A, and G1899A, but higher prevalence of T1758C mutations. The T1758C and A1762T/G1764A mutations appeared as mutually exclusive[51].

The double mutation has been detected with increased frequency in patients with fulminant hepatitis, including children, those with HBeAg- and anti-HBe-positive chronic hepatitis, and HCC patients, but less frequently in asymptomatic chronic carriers. In addition, it has been detected in immunosuppressed, liver transplant, and seronegative patients. Interestingly, it was recently reported that BCP T1762/A1764 mutant is an independent risk factor for progression to cirrhosis rather than hepatocellular carcinoma (HCC) in chronic HBV infection[52]. There are differences in the prevalence of such variants between studies in various settings, and this may relate to differences between prevalent genotypes, the importance of which is not always recognized and therefore not clearly reported[53-56]. Very recently Yang *et al*[57] have postulated that a quantitative analysis of the BCP double mutation (and G1896A) can predict interferon-induced HBeAg seroconversion.

The BCP mutations, as mentioned here, have been found in patients regardless of HBeAg status. However, in anti-HBe-positive patients, the double mutation was often accompanied by T1753C/G mutation. In addition, other point mutations upstream and downstream of T1762/A1764 have been described, occurring either alone or in combination with the double mutation, and in different settings, including chronic hepatitis, fulminant hepatitis B, HCC, and liver transplantation[46,58,59]. More recent studies have identified additional mutations in the BCP region that are prevalent in HCC patients, and particularly novel individual and combination patterns of mutations in the X/precore region of HBV genotype D1 as predictors of HCC[46]. These include T1673/G1679, G1727, C1741, C1761, A1757/T1764/G1766, T1773, T1773/G1775 and C1909 (Table 1).

The mutations G1613A (NRE) and C1653T, both of which tended to also occur simultaneously in HCC patients may suppress HBeAg production, enhanced viral DNA synthesis, and bound RFX1 with higher affinity. Since the 1762/1764 mutations overlap with the X ORF and lead to amino acid changes in the X protein (L130M and V131I, respectively), this double substitution on its own or together with G1386A (V5M) was encountered at a significant rate in HCC patients by activating NF-κB activity[60]. Synergistic effects of A1896, C1653T and T1762/A1764 mutations in genotype C2 on development of hepatocellular carcinoma has been reported[61]. Thus, to summarize, T1653, V1753, T1762, A1764, T1766, and A1768 have been found to occur more frequently in HCC patients[62].

The double BCP mutation was found to be associated with increased risk of HCC independent of genotype, while the precore A1896 mutation was associated with decreased risk. According the the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus study, the infection by genotype B strains exhibiting the BCP double mutation over a 10-year period increases 23% the risk of developing HCC. Likewise, the presence of family history of HCC multiplies the risk of HCC at each stage of HBV infection[63-64].

OTHER CORE PROMOTER VARIANTS

Core promoter mutations other than those at positions 1762 and 1764 can have major impact on viral DNA replication and HBeAg expression[34]. *In vitro* studies reveal that CP mutants with additional mutations (T1753C and/or C1766T) replicated at high level (Table 1). When mutational complexes were assayed, the 1762/1764/1766 and 1753/1762/1764/1766 showed higher replication rates and lower HBeAg expression than 1762/1764 mutations alone, whereas the 1753/1762/1764 variant was not much different from the double BCP mutant. Single mutations including T1753C/A/G (V), 1762/1764, A1846T, G1896A, 1899 and A/G1913, as well as triple mutations 1753/1762/1764 and 1762/1764/1766 or T1768A, were more frequently detected in patients with acute-on-chronic liver failure than in patients with chronic hepatitis, and mostly associated with genotype B- than C-infected patients[65].

Deletions within the CP region -mostly at BCP- and varying in length from 1 to 21 bp have been reported once again in different settings. These include fulminant hepatitis, chronic hepatitis, asymptomatic infection, serologically silent infection, HCC, renal dialysis with atypical HBsAg-negative infections characterized by either the presence of viral antibodies alone or no serological markers, and liver and renal transplantation, HIV coinfection[66]; they are also found in patients who have survived hematological malignancies or solid tumors[28,67]. Such deletion variants are often characterized by low viremia levels, and may need help from the wild-type virus for survival[68]. Likewise, reduced levels of transcription and progeny virus production were obtained by transfection with clones having an 8 bp deletion in the BCP (nt 1768-1775)[69]. It appears, therefore, that the strain genetic background in which the BCP and G1896A mutations arise, in relation to additional ones, determine replication rate, expression of HBeAg, and pathogenicity[23]. A limited number of reports have shown the presence of deletions in the URR[70,71] or in the region of the CP overlapping the pre-C ORF[72].

Insertions within the BCP have been described in patients with CH[73] and in those undergoing orthotopic liver transplantation[74]. One patient who had a fulminant exacerbation of CH after heart transplantation was reported to be infected with a high-replicating HBV variant carrying an 11-bp insertion in the CP[75]. This insertion occurred shortly before, or during, FH and created a binding site for HNF1. Thus, the emergence or presence of a novel HNF1, or putative HNF3, site may be related to fulminant exacerbating hepatitis in immunosuppressed patients. This concurs with the presence of such strains in renal transplant patients with severe liver disease but not in those with mild disease[28,71]. Simultaneous detection of deletion and insertion at BCP was recently reported in a single patient with HCC from South Africa with concomitant presence of other variations that could

accelerate the HCC development[76].

The G1862T substitution affecting codon 17 of the precore protein of the virus was only observed in patients infected with genotype B. This variant was five times more common in patients with fulminant hepatitis than in chronic carriers. The G1862T mutation leads to substitution of valine for phenylalanine, affecting the - 3 position in the signal peptidase recognition motif, impairing the processing of the precore/core protein into HBeAg[77]. A similar behavior was described for viral variants carrying the C1856T mutation. It leads to P15S substitution at precore. This amino acid change affects the signal peptide also, and may therefore affect production of HBeAg from the precursor precore/core, but there are no experimental data supporting this proposition at the moment[23]. This variant was initially found by chance while sequencing the complete HBV genome of an HIV positive patient with a strange serological profile[78], and then in Hong Kong Chinese patients with chronic hepatitis[79].

HBV possesses the smallest genome of any virus known to infect man. Therefore, it is not surprising that HBV utilizes its genetic material economically. The core promoter is an emblematic model of this strategy whose DNA harbors several pivotal functions. Its sequence involves three critical elements (EN Ⅱ, BCP, and the overlapping X ORF) which are able to execute critical viral functions (stimulate the S, X, and CP; regulate the transcription of both pre-C mRNA and pgRNA; encode the X protein with trans-activating functions, respectively). Its integrity appears essential to maintain viral replication, and variation may contribute to the persistence of HBV within the host, leading to chronic infection and, eventually, cellular transformation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Is hepatitis B-virucidal validation of biocides possible with the use of surrogates?

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Abstract

The hepatitis B virus (HBV) is considered to be a major public health problem worldwide, and a significant number of reports on nosocomial outbreaks of HBV infections have been reported. Prevention of indirect HBV transmission by contaminated objects is only possible through the use of infection-control principles, including the use of chemical biocides, which are proven to render the virus non-infectious. The virucidal activity of biocides against HBV cannot be predicted; therefore, validation of the virucidal action of disinfectants against HBV is essential. However, feasible HBV infectivity assays have not yet been established. Thus, surrogate models have been proposed for testing the efficacy of biocides against HBV. Most of these assays do not correlate with HBV infectivity. Currently, the most promising and feasible assay is the use of the taxonomically related duck hepatitis B virus (DHBV), which belongs to the same *Hepadnaviridae* virus family. This paper reviews the application of DHBV, which can be propagated *in vitro* in primary duck embryonic hepatocytes, for the testing of biocides and describes why this model can be used as reliable method to evaluate disinfectants for efficacy against HBV. The susceptibility levels of important biocides, which are often used as ingredients for commercially available disinfectants, are also described.

Key words: Hepatitis B virus; Surrogate model; Duck hepatitis B virus; Disinfectants; Testing virucidal efficacy

Core tip: There is a need for disinfectants with proven virucidal activity against the hepatitis B virus (HBV). Feasible HBV infectivity assays are not available; therefore, the establishment of surrogate models for HBV infection is of high importance. This paper reviews the application of the most promising and feasible assay, the use of the duck hepatitis B virus, which can be propagated *in vitro* in primary duck embryonic hepatocytes. The paper also describes how and why this model can be used to evaluate the efficacy of disinfectants against HBV.

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WHY EVALUATE BIOCIDES FOR THEIR EFFICACY AGAINST HEPATITIS B VIRUS?

Approximately 350 million people, 5% of the total population, are chronically infected with the hepatitis B virus (HBV)[1]. Thus, the hepatitis B is considered to be a major public health problem worldwide. Furthermore,

nosocomial infections resulting from HBV, in patients during hospitalization and interventional procedures, as well as in health care workers, have been described[2,3]. Although infections attributed to transfusion of contaminated blood or blood products and transmission from infected health care workers have been reduced over the past decades by prophylactic measures, such as HBV screening of blood or vaccination of health care workers, there is still a significant number of reports about nosocomial outbreaks of HBV infections[4-7]. Common transmission pathways include the use of multi-dose vials[8], dental or biopsy equipment[9], dialysis units[10], contaminated finger-stick devices[11,12], contaminated acupuncture needles[13], reuse of syringes[14], endoscopes[15], or unsafe surgical and injection procedures[16-19]. Prevention of indirect HBV transmission by contaminated objects is only possible through the use of fundamental infection-control principles, including the use of chemical biocides[15,20], which are proven to destroy the viral infectivity. Thus, HBV must be inactivated as a result of the disinfection of instruments, surfaces, and biological materials. The use of biocides with proven microbiocidal activity against the pathogens most likely to contaminate a patients' environment has been recommended by the United States Healthcare Infection Control Practices Advisory Committee as part of their guidelines to prevent the transmission of infectious agents in health care settings[21].

In general, HBV can be inactivated by chemical biocides, such as formaldehyde, glutaraldehyde or peracetic acid, which possess broad-spectrum virucidal activity according to the norm EN 14476:2007[22]. However, like all enveloped viruses[23], HBV is thought to be relatively sensitive to biocides. A German guideline for testing the virucidal activity of chemical disinfectants in the medical field characterizes disinfectants effective against enveloped viruses as biocides with limited virucidal activity. In contrast, disinfectants effective against non-enveloped and enveloped viruses are defined as biocides with virucidal activity[24,25]. Thus, broad-spectrum biocides, of which there are few, are not required for the inactivation of HBV like other blood-borne viruses. However, validation of the virucidal action of disinfectants against HBV is essential because the virucidal activity of biocides against HBV cannot be predicted[26]. In addition, human blood plasma protects the virus from inactivation. In the literature, HBV has been described as an enveloped virus that may be difficult to inactivate[27]. The virus possesses a relatively high thermal and dry resistance. At 25 ℃ and a relative air humidity of 42%, the HBV can be infectious for more than 1 wk[28]. Therefore, the virucidal efficacy of biocides against HBV must be validated using reliable and robust laboratory methods.

METHODS FOR TESTING THE EFFICACY OF BIOCIDES AGAINST HBV

The most common methods for the evaluation of virucidal activity of biocides are infectivity assays, which measure the infectivity of viruses in cell culture systems after the virus has been exposed to the biocide in suspension[24,25]. Recently, more practice-relevant methods have been developed, testing viral infectivity after exposure to viruses dried on non-porous surfaces[29]. These procedures mimic the conditions found in actual practice. A crucial component of these assays is that the tested viruses can easily be propagated in cell cultures and the infectivity can be determined reliably by the evaluation of virus-induced cytopathic changes or other methods detecting viral antigens, which are produced during the viral replication cycle. However, the *in vitro* propagation of non-cytopathogenic HBV is difficult, especially in obtaining human liver cells. Historically, the virucidal efficacy of biocides has been stringently determined *in vivo* through the use of chimpanzee infection assays, albeit with decreased sensitivity[30-34]. Currently, animal protection and economic reasons prohibit the use of higher primates for routine tests of commercial products[35]. For *in vitro* infectivity testing, the use of the hepatoma cell line HepG2[36], which has been described in the literature[27,37], has been debated[38]. In comparison, re-differentiated HepaRG cells[39] are well accepted and reproducible as an HBV infectivity system[38]. The specificity of this HBV infection model has been determined by both the neutralization capacity of HBV envelope protein-specific antibodies and the competition with an envelope-derived peptide. However, this infectivity system has not been applied in the past for testing the hepatitis B-virucidal activity of biocides. The reasons for this are the following: HepaRG cells are very expensive, can only be used in highly HBV-specialized laboratories and require highly concentrated HBV suspensions or human sera with a high viral load. Thus far, the most promising HBV infectivity assay seems to be the use of primary hepatocyte cultures derived from Tupaias, small-squirrel-like animals living in Southeast Asia[38,40]. However, the availability of Tupaia hepatocytes is limited, thus the model is too costly for routine use. Furthermore, purified virus must contain approximately 10^9 particles/mL to demonstrate an inactivation factor of at least 10^4[38]. The virus can be obtained from human serum by sedimentation in a density gradient[41].

Thus, surrogate models have often been reported for testing the efficacy of biocides against HBV. To measure the virucidal activity of disinfectants against HBV, Hilfenhaus *et al*[42] and Thraenhart *et al*[35] validated the integrity of viral DNA using a polymerase chain reaction (PCR) technique. Several groups[43,44] have also examined HBV inactivation by measuring the enzymatic activity of the viral DNA polymerase. In addition, the destruction of HBV antigenicity and the decrease in the immunochemical reactivity of different HBV antigens, such as HBsAg, HBcAg and HBeAg, was outlined to verify the virucidal efficacy of alcohol antiseptic, formaldehyde and peracetic acid-containing disinfectants[26,45,46]. Finally, the irreversible morphological alterations of HBV particles were determined to be an indicator of HBV inactivation by

chemical biocides[43,47,48]. However, this test is subjective, and there is a qualitative but not a quantitative measurement. In conclusion, all of the abovementioned studies have shown that the results do not correlate with HBV infectivity.

DUCK HEPATITIS B VIRUS AS A SURROGATE VIRUS FOR HBV

The most promising and feasible assay for the evaluation of hepatitis B-virucidal efficacy of biocides is the use of the taxonomically related duck hepatitis B virus (DHBV) belonging to the same family-*Hepadnaviridae*-and within the genus *Avihepadnavirus*, while HBV is in the genus *Orthohepadnavirus*[49]. DHBV shares many physical properties with the closely related HBV but a sequence comparison of the two viruses indicated that there is a low nucleotide identity[49,50]. Furthermore, there are differences in the genome size (3.2 kb for *Orthohepadnaviruses* and 3.0 kb for *Avihepadnaviriruses*), and the host range of the viruses is restricted to mammals (*Orthohepadnaviruses*) or birds (*Avihepadnaviruses*). In addition, the *Avihepadnaviriuses* have larger core proteins and lack M surface protein[49]. In contrast to the *Orthohepadnaviruses*, some envelope proteins of the *Avihepadnaviruses* are not glycosylated but are phosphorylated. In addition, the proteins of the envelope are not connected by disulfide bridges and instead contain lysine side chains[51]. The model infection of DHBV in Pekin ducks has been used extensively for studying aspects of HBV infection in humans[50,52]. It has been concluded that DHBV and HBV differ primarily between the hosts they infect and the nature of the disease they produce. This has no bearing on the ability of disinfectants to abolish infectivity of the viruses[53]. Furthermore, the DHBV model has similar disinfectant inactivation kinetics to those observed in the limited studies of HBV transmission in chimpanzees[31,54]. Thus, DHBV infectivity tests have been used for testing the virucidal activity of chemical biocides against HBV in the United States and Australia[54-57] and have been proposed in Europe[58].

It is of great value that the DHBV is maintained in domestic duck flocks through vertical transmission from viremic ducks. The virus infects the developing liver *in ovo* and is not sufficiently recognized by the host immune system to produce hepatitis and liver disease or to eliminate the virus[49,59]. Thus, DHBV can be propagated *in vivo* in ducklings or *in vitro* in primary duck embryonic hepatocytes to assess viral infectivity[56,60-62]. Several authors have reported using *in vivo* DHBV assays[54,55,63-65]. To estimate DHBV infectivity, the diluted viral suspensions exposed to the biocides are injected intraperitoneally or intravenously into naïve ducklings not infected with DHBV. The ducklings are euthanized 2 wk later, and their livers are removed to be analyzed for DHBV DNA using PCR[66]. However, these *in vivo* tests conflict with ethical and legal aspects of animal protection. Therefore, the preferred method for testing the efficacy of disinfectants against DHBV is the *in vitro* assay. This protocol is in accordance with the recommendations of the United States Environmental Protection Agency[67].

Viral propagation of DHBV in duck embryonic hepatocytes is not trivial because DHBV is a non-cytopathogenic virus. This approach requires that additional tests, such as immunofluorescence[68], PCR[69] or Southern blotting[70], be used to verify the growth of the virus. Additionally, viral propagation requires a source of DHBV-free Pekin ducks, appropriate eggs for the preparation of embryonic hepatocytes, *in vitro* cultures of hepatocytes, and congenitally infected Pekin ducks as source of the virus. It is advantageous that experimental investigations on embryonated hen and duck eggs are, in general, regarded experimentally as in between *in vivo* and *in vitro* systems and do not conflict with ethical and legal aspects of animal protection[71].

IN VITRO DUCK HEPATITIS B VIRUS MODEL FOR TESTING VIRUCIDAL EFFICACY OF BIOCIDES

In Germany, an assay protocol for testing DHB-virucidal efficacy of biocides by DHBV infection of primary duck embryonic hepatocytes has been established and successfully evaluated for virucidal testing in several studies[68,72,73]. The primary duck embryonic hepatocytes were obtained from fertilized Pekin eggs and incubated for 21 d[68]; the liver tissue was harvested from several embryos[74]. A crucial step was to ensure the absence of DHBV in the source tissue using a qualitative PCR technique[68,75]. To digest the liver tissue, a solution comprised of trypsin, ethyl diamine tetraacetate solution, phosphate-buffered saline and glucose was effective. Digestion of the liver could be inhibited by the addition of fetal calf serum[69,76,77]. DHBV-negative cells were seeded in 24-well culture plates not containing collagen 1 such as CellBIND™ (Corning, Acton, United States)[74]. This step is necessary to ensure stable attachment of hepatocytes to the surface of culture vessels for successful DHBV propagation (Figure 1). The optimal growth medium can be modified according to previous reports[69,78,79]. This medium supports the maintenance of differentiating hepatocytes, which is important for the susceptibility of cells to the virus and for DHBV replication[60,80-82]. A suitable microenvironment can be achieved by coating the growth surface with Matrigel or other substrates containing extracellular matrix molecules[83,84]. Alternatively, the use of co-culture systems of hepatocytes with non-parenchymal liver cells has been described as a suitable method to maintain hepatocyte differentiation *in vitro*[85,86].

The use of hepatocytes cryopreserved by the suspension method is also suitable when freshly isolated cells from the liver of duck embryos are not available due to seasonal differences[74]. Growth medium[74] was supplemented with 10% fetal calf serum and suitable

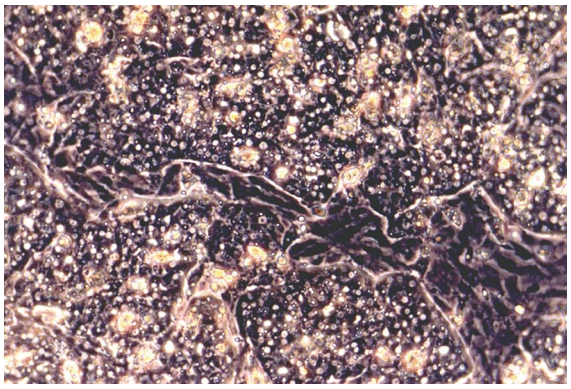

Figure 1 Primary duck embryonic hepatocytes grown in CeLLBIND™ plates at day 3 of cultivation. Monolayers of hepatocytes, which show typical polygonal morphology, are interrupted by areas of non-parenchymal cells (light microscopy, phase contrast, x 200).

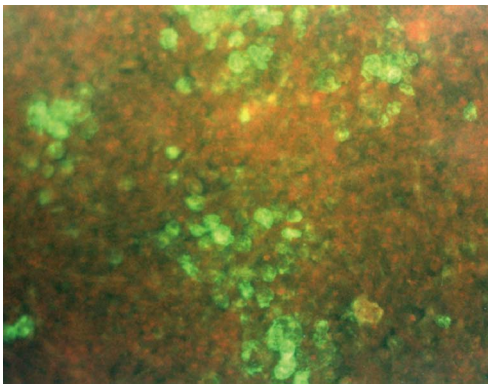

Figure 2 Detection of duck hepatitis B virus-specific surface antigen six days after inoculation of primary duck embryonic hepatocytes by indirect immunofluorescence. Polyvalent rabbit anti-DHBs (kindly provided by Dr. D. Glebe, Institute of Medical Virology, National Reference Centre for Hepatitis B and D, Justus Liebig University, Giessen, Germany) and goat anti-rabbit IgG Alexa Fluor® 488 (Life Technologies, Darmstadt, Germany) were used as antibodies (fluorescence microscopy, x 125).

cryoprotective agents, such as 10% DMSO or cryosafe-1. The freeze-thaw process does not significantly reduce the susceptibility of primary duck embryonic hepatocytes to DHBV infection, suggesting no loss of viral receptors on the cell surface.

As virus pool, DHBV-containing serum from congenitally infected ducks must be used[72,73]. Sera should contain between $10^{6.0}$ and $10^{8.0}$ tissue culture infective doses 50% of DHBV per mL, corresponding to $10^{9.0}$ and $10^{11.0}$ DHBV genomic copies. To avoid reduction of viral titers, the uninterrupted storage of aliquots at -80 ℃ is strongly recommended. One should, however, be aware that the Pekin duck is an unreliable source of the test virus, which causes difficulties for standardization. Another disadvantage is that the titers of infectious virus are often too low to detect sufficient reduction of viral titers especially when cytotoxic biocides are tested[73]. On the other hand, the DHBV prepared from the DHBV DNA-transfected hepatoma cell line D2[87] is not suitable for testing virucidal efficacy of biocides either because this virus is more sensitive than the wild type DHBV naturally occurring in the serum of Pekin ducks[72].

Virucidal tests are recommended to be carried out in accordance with national guidelines for testing the virucidal efficacy of chemical disinfectants in human medical areas[25]. At the end of the chosen exposure time, the test compounds must be immediately removed from the mixture of virus and test formulation by rapid dilution of samples or the use of sephadex-based methods[88], particularly when cytotoxic residues must be removed. However, previous experience has shown that sephadex columns can withhold the infectious virus, thus leading to inaccurate results. It is recommended that the primary duck embryonic hepatocytes are infected at day 4 of cultivation. Due to the *in vitro* method of preparing of hepatocytes, the type I interferon system is stimulated, thereby inhibiting DHBV replication during the first 2-3 d after primary cell plating[89,90]. Thus, 4 d post-infection, a high number of DHBV-infected hepatocytes are infected[68,69]. On the other hand, DHBV-negative hepatocytes lose their susceptibility to DHBV infection after the day 5 of cultivation[60,80]. This results from the dedifferentiation of hepatocytes and/or the loss of the cellular receptor for virus attachment. The presence of DMSO in the cell culture medium is also critical for this process because DMSO not only allows maintenance of viral replication but also prolongs the susceptibility of cultured hepatocytes to DHBV infection[80]. Following viral infection, the cells should be incubated for at least 6 d to achieve infection rates of approximately 40% as shown by specific fluorescence, a surrogate marker of productive viral infection[68].

Indirect immunofluorescent antigen staining has been recommended for detection of DHBV surface(s) antigen in primary duck embryonic hepatocytes to verify DHBV infection[68,72]. To this end, a polyvalent rabbit anti-DHBs antiserum that is not commercially available must be used. As shown in Figure 2, the infected hepatocytes can be easily identified because they appear in clusters[79]. A 4-$\log_{10}$ reduction of infectivity (inactivation $\geqslant$ 99.99%) is regarded as evidence of sufficient virucidal activity[25]. As the guidelines of the United States Environmental Protection Agency[56] state, an *in vitro* assay requires a demonstration of at least 3-$\log_{10}$ reduction in viral titers beyond any disinfectant dilutions that exhibit cell culture cytotoxicity. Although fluorescent analysis has a subjective output and requires experience for the analysis of results, indirect immunofluorescence can be employed for routine testing and has been applied to detect a variety of human and animal viruses[91]. An advantage of this method is that the efficacy of biocides against DHBV infection can be rigorously evaluated because production of DHBV surface proteins in hepatocytes is a late step in the viral life cycle and correlates well with the production of mature virus particles[92]. In contrast, PCR-based methods identify the presence of viral DNA, but this may not necessarily correlate with the number of infectious virus particles[93].

Table 1 Studies published in the literature to evaluate the efficacy of biocides against duck hepatitis B virus

Year	Country	Ref.	Evaluated biocides or inactivation procedures
1991	Australia[1]	Murray *et al*[54]	Glutaraldehyde; mix of glutaraldehyde, non-ionic alcohol derivate, quaternary compound and tri-ethyleneglycol surfactant
1993	United Kingdom[1]	Tsiquaye *et al*[63]	Sodium hypochlorite; sodium dichloroisocyanurate
1996	Australia[1]	Deva *et al*[94]	Glutaraldehyde
1998	United States[2]	Eble *et al*[70]	Photochemical inactivation by 8-methoxypsoralen
1999	Australia[1]	Chaufour *et al*[55]	Glutaraldehyde; ethylene oxide
1999	Australia[1]	Vickery *et al*[64]	Hydrogen peroxide
2000	Australia[1]	Vickery *et al*[95]	Glutaraldehyde
2001	United States[2]	Wagner *et al*[96]	Photoinactivation by dimethylmethylene blue
2002	United States[2]	Wang *et al*[69]	*N*-alkyl dimethyl benzyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride
2004	United States[2]	Moore *et al*[97]	Ethylene oxide
2005	Australia[2]	Druce *et al*[65]	Ethylene oxide
2006	Germany[2]	Sauerbrei *et al*[72]	Peracetic acid; povidone-iodine; formaldehyde
2008	United States[2]	Roberts *et al*[98]	*Ortho*-phthalaldehyde
2012	Germany[2]	Sauerbrei *et al*[73]	Ethanol; isopropanol; peracetic acid; glutaraldehyde; formaldehyde

[1]DHBV *in vivo* test system; [2]DHBV *in vitro* assay. DHBV: Duck hepatitis B virus.

EVALUATION OF BIOCIDES USING DUCK HBV

Several study groups in Australia, the United States, the United Kingdom and Germany have used the DHBV *in vivo* test system and the DHBV *in vitro* assay to evaluate the DHB-virucidal efficacy of chemical biocides or photochemical inactivation procedures. Table 1 gives an overview of the evaluated biocides and procedures of each study group. The majority of groups waived the *in vivo* test system. Since the year 2000, DHBV *in vitro* assays have been used almost exclusively. A recent study determined the DHB-virucidal activity of the following five different chemical biocides: ethanol, isopropanol, peracetic acid, glutaraldehyde and formaldehyde, which are often ingredients present in commercially available disinfectants[73]. Testing was carried out as modified quantitative suspension test[25] in the presence of a protein load of 10% fetal calf serum. Table 2 lists the minimal concentrations and contact times to reach virucidal efficacy. This means that $\geqslant$ 40% ethanol or isopropanol, $\geqslant$ 0.05% peracetic acid and $\geqslant$ 0.1% glutardiaaldehyde within $\geqslant$ 1 min significantly inactivate infectious DHBV corresponding to a 4-$\log_{10}$ reduction in viral titers. For a 0.7% formaldehyde solution, which resulted in high hepatocytotoxicity, a longer contact of $\geqslant$ 30 min is needed. These results show that the DHBV, as an enveloped virus, is considered to be relatively sensitive to inactivation by virucides. Limited, unpublished data with HBV and Tupaia hepatocytes corroborate these findings (personal communication: D. Glebe, Institute of Medical Virology, National Reference Centre for Hepatitis B and D, Justus Liebig University, Giessen, Germany). This is also in agreement with the susceptibility of levels of HBV detected by direct chimpanzee inoculation[30]. Thus, the results presented for DHBV are likely also valid for HBV. However, it must be considered for the interpretation of the *in vitro* data obtained by the quantitative suspension test that recommendations for the application of the agents in practice can be concluded only to a limited extent. Such favourable conditions as during the homogeneous suspension are seldom to be found in practice. Thus, results of the suspension test should not be regarded as practical application in every case but they allow conclusion of the efficacy of single disinfectants and, therefore, they also allow to compare the efficacy of different disinfectants[25]. For comparison, information on stability of HBV published by the World Health Organization[99] is summarized in Table 3. These biocides or measures, including concentrations, temperatures and contact times, are recommended for clinical practice to destroy infectious HBV. In contrast, Table 2 lists the minimal concentrations and contact times for the duck hepatitis B-virucidal activity of several biocides in the quantitative suspension test in which a protein load of 10% fetal calf serum was used. When selecting the most effective method for destroying infectious HBV, it should be taken into account that the amounts of serum HBV varies considerably among HBV-infected patients[100]. Thus, there can be differences in methods according to the level of viremia in patients.

Table 2 Minimal concentrations and contact times for the duck hepatitis B virus-virucidal activity of ethanol, isopropanol, peracetic acid, glutaraldehyde and formaldehyde against duck hepatitis B virus in the presence of a protein load of 10% fetal calf serum

Biocide	Concentration (%)	Contact time (min)
Ethanol	40	1
Isopropanol	40	1
Peracetic acid	0.01	2
	0.05	1
Glutaraldehyde	0.05	2
	0.1	0.5
Formaldehyde	0.7	30

Results of quantitative suspension tests are shown[73].

Additionally, the study by Sauerbrei *et al*[73] has shown that biocides tested against DHBV are efficacious against the vaccinia virus strain Lister or the modified vaccinia Ankara strain[101], which are used in guidelines for the declaration of limited virucidal activity of biocides[25]. The testing of these viruses does not present any difficulties; therefore, it can be expected that in the absence of more direct tests, the results of DHBV, and even of

Table 3 Information on the stability of hepatitis B virus published by the World Health Organization[99]

Biocide/ measure	Concentration/ temperature	Contact time	Remarks
Sodium hypochlorite	0.25%	3 min	Antigenicity of hepatitis B surface antigen is destroyed, infectivity is probably destroyed
Sodium hypochlorite	5%	10 min	Inactivation of virus
Glutaraldehyde	2% (room temperature)	5 min	Inactivation of virus
Glutaraldehyde	2% (98 ℃)	2 min	Inactivation of virus
Formaldehyde	5%	2 min	Inactivation of virus
Isopropanol	70%	2 min	Inactivation of virus
Ethanol	80% (11 ℃)	2 min	Inactivation of virus
Autoclaving	121 ℃	20 min	Lost of infectivity
Heat sterilization	160 ℃	1 h	Lost of infectivity

the vaccinia virus or its modified Ankara strain, may be extrapolated to HBV. Therefore, the surrogate DHBV model can provide highly valuable data for the susceptibility of HBV to disinfectants.

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TOPIC HIGHLIGHT

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Non-invasive diagnosis of hepatitis B virus-related cirrhosis

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Abstract

Chronic hepatitis B (CHB) infection is a major public health problem associated with significant morbidity and mortality worldwide. Twenty-three percent of patients with CHB progress naturally to liver cirrhosis, which was earlier thought to be irreversible. However, it is now known that cirrhosis can in fact be reversed by treatment with oral anti-nucleotide drugs. Thus, early and accurate diagnosis of cirrhosis is important to allow an appropriate treatment strategy to be chosen and to predict the prognosis of patients with CHB. Liver biopsy is the reference standard for assessment of liver fibrosis. However, the method is invasive, and is associated with pain and complications that can be fatal. In addition, intra- and inter-observer variability compromises the accuracy of liver biopsy data. Only small tissue samples are obtained and fibrosis is heterogeneous in such samples. This confounds the two types of observer variability mentioned above. Such limitations have encouraged development of non-invasive methods for assessment of fibrosis. These include measurements of serum biomarkers of fibrosis; and assessment of liver stiffness *via* transient elastography, acoustic radiation force impulse imaging, real-time elastography, or magnetic resonance elastography. Although significant advances have been made, most work to date has addressed the diagnostic utility of these techniques in the context of cirrhosis caused by chronic hepatitis C infection. In the present review, we examine the advantages afforded by use of non-invasive methods to diagnose cirrhosis in patients with CHB infections and the utility of such methods in clinical practice.

Key words: Chronic liver disease; Chronic hepatitis B; Hepatitis B virus; Cirrhosis; Liver stiffness measurement; Transient elastography; Acoustic radiation force impulse imaging; Real-time elastography; Magnetic resonance elastography; FibroTest; Aspartate aminotransferase to platelet ratio index

Core tip: Chronic hepatitis B (CHB) infection is associated with significant morbidity and mortality because it can progress to cirrhosis or hepatocellular carcinoma. Early diagnosis of liver cirrhosis in CHB patients is important to prevent the disease progression. Non-invasive diagnosis has been developed remarkably and showed high diagnostic accuracy for cirrhosis. New methods and the combination of non-invasive diagnosis tools may contribute to the improvement to diagnose the cirrhosis with CHB.

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INTRODUCTION

Serologically, hundreds of millions of people are chronic

hepatitis B (CHB) virus surface antigen (HBsAg) carriers and it is estimated that over 200000 CHB patients die worldwide each year from cirrhosis[1,2]. The prognosis is worse in those infected by hepatitis B virus (HBV) in areas in which the virus is endemic. The annual rate of progression from chronic hepatitis to cirrhosis is 2%-5% in HBV e antigen-positive and 3%-10% in e antigen-negative patients[3].

Early diagnosis of liver cirrhosis in CHB patients is important because cirrhosis per se is an independent predictor of mortality. The 1-year mortality rates vary from 1% in patients with early-stage cirrhosis to 57% in those with decompensated disease[4-6]. In addition, the 5-year cumulative risk that a cirrhotic patient will develop hepatocellular carcinoma is 10%-17%[3]. Diagnosis of cirrhosis in CHB patients may trigger early initiation of antiviral treatment, which improves clinical outcomes. Also, development of complications in such patients is monitored regularly[7,8].

Traditionally, cirrhosis has been diagnosed with the aid of clinical information, including laboratory and imaging data. However, such methods have their limitations, and a liver biopsy has usually been required to confirm a diagnosis of liver cirrhosis.

Cirrhosis occurs in response to chronic liver injury and constitutes the final stage of progressive hepatic fibrosis characterized by distortion of hepatic architecture and formation of regenerative nodules[9]. If a liver biopsy sample is to be adequately diagnostic, the sample should be at least 2-3 cm in length and contain more than 11 complete portal tracts[10]. However, it is usually difficult to fulfill these requirements. Also, the evaluation of simultaneous biopsies taken from the left and right lobes yielded different fibrosis scores in 33% of patients[11]. Intra- and inter-observer variation contribute to test variability[12]. Biopsy is associated with a bleeding risk of 1%[13] and a mortality rate of approximately 0.01%[14].

Therefore, it is necessary to investigate the new diagnostic methods for improving the limitations and complications of liver biopsy. Non-invasive modalities, including transient elastography (TE), acoustic radiation force impulse imaging (ARFI), real-time elastography (RTE), and magnetic resonance elastography (MRE), have been developed to overcome the problems discussed above. Also, markers of cirrhosis have been sought in blood. Such non-invasive modalities have attracted much attention[15-22]. In the present review, we examine the roles played by non-invasive diagnostic modalities in terms of assessment of cirrhosis and prediction of the prognosis of CHB patients.

CLASSICAL DIAGNOSIS OF LIVER CIRRHOSIS USING CLINICAL INFORMATION

Clinicians usually take a clinical history, conduct a physical examination, and review laboratory and imaging data to diagnose liver cirrhosis in CHB patients.

First, history-taking is a basic clinical approach. For example, a family or past history of HBV infection is an important clue to decide whether cirrhosis may or may not be caused by HBV. The histories of blood transfusion, needle injury, and sexual activity are important factors for the route of infection.

Second, physical examination is both simple and essential. Spider angioma, splenomegaly, hepatomegaly, a distended abdomen, the presence of shifting fluid, and pitting edema suggestive of portal hypertension, all aid in the diagnosis of liver cirrhosis in CHB patients. Blood samples should be evaluated for the presence of HBV surface antigen, platelet count, albumin level, prothrombin time, and total bilirubin concentration. Simple blood test data do not afford great sensitivity in diagnosis of cirrhosis, but one meta-analysis found that the presence of ascites [likelihood ratio (LR) = 7.2], a platelet count < 160000/mm^3 (LR = 6.3), and spider nevi (LR = 4.3) were all usefully predictive of cirrhosis[23]. These simple blood tests, and data from physical examination, are necessary to evaluate the severity of cirrhosis, being parameters used to derive Child-Pugh or Model for End-Stage Liver Disease (MELD) scores for cirrhotic patients.

Third, imaging modalities including ultrasound, abdominal computed tomography (CT), and magnetic resonance imaging (MRI), can aid in detection of cirrhosis or hepatocellular carcinoma in HBV patients. Liver cirrhosis is typically associated with splenomegaly, hepatomegaly, a coarse echo pattern of the liver parenchyma, nodularity of the liver surface, a blunt angle, ascites, and thrombus of the portal vein[24-27]. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of ultrasound used to predict the presence of HBV-related cirrhosis were 77.8%, 92.5%, 87.5%, 86.0%, and 86.6%, respectively, and were higher in CHB patients than in those with chronic hepatitis C (CHC)[27]. Such radiological findings are also important when abdominal CT or MRI is used to diagnose liver cirrhosis. Additionally, other radiological findings including the velocity of portal flow, the shape of the hepatic vein waveform (as revealed by Doppler testing), and the caudate lobe/right lobe ratio with use of the main or right portal vein to set the lateral boundary (as revealed on CT or MRI), are useful[24,28-30]. Of these parameters, a caudate lobe/right lobe ratio over 0.65 is suggestive of the presence of liver cirrhosis. The sensitivity, specificity, and accuracy of this measure were 84%, 100%, and 94%, respectively[30]. Liver surface nodularity, a platelet count less than 100000/mm^3, an albumin level less than 3.5 g/dL, and an international normalized prothrombin time ratio of 1.3 or more, are also associated with liver cirrhosis. Each of these findings is associated with a specificity and sensitivity of 90% and 61%, respectively, in terms of cirrhosis diagnosis[31].

An overview of clinical history, physical examination, and review of laboratory and imaging test data, combine to aid in the diagnosis of HBV-caused cirrhosis. The use

of clinical strategies that combine elements of these diagnostic modalities is crucial to diagnose cirrhosis in CHB patients and to plan suitable treatment.

TRANSIENT ELASTOGRAPHY

Measurement of liver stiffness

TE is a representative noninvasive method used to diagnose cirrhosis or assess the extent of fibrosis using ultrasound elastography. The propagation velocity of transient shear waves of low amplitude and frequency (50 Hz) is affected by the elasticity of the liver parenchyma. If the waves propagate rapidly, the fibrotic burden is large[32]. The outcome is expressed as a pressure (thus in kilopascals; kPa) and ranges from 2.5 to 75 kPa (normal, 5 kPa)[33-35]. The reliability of the technique has been reported to be over 60% and the median interquartile range less than 30%[36-38]. The generator is placed over an interspace of the ribcage of the right upper quadrant. The test usually takes 5-10 min. Typically, 10 or more measurements are taken but three valid measurements suffice to reliably diagnose liver fibrosis in CHB patients[39].

Diagnostic performance

Most studies have found that TE performs well when used to diagnose and measure the extent of liver fibrosis in patients with CHC infection or other liver conditions[40-42]. TE also afforded acceptable diagnostic accuracy in CHB patients[18-20]. Marcellin *et al*[20] reported that TE used to diagnose cirrhosis in CHB patients had a diagnostic accuracy of 94%, a sensitivity of 57%, a specificity of 97%, a PPV of 67%, and an NPV of 96%. When maximal sensitivity and specificity are required, the cutoff value falls to 11.0 kPa (sensitivity 93%, specificity 87%, PPV 38%, and NPV 99%). Chan *et al*[19] found that TE yielded a good diagnostic performance in clinical practice. The maximal diagnostic accuracy was associated with a cutoff of 13.4 kPa but this value was 12.0 kPa when the sum of sensitivity and specificity was maximized. A liver stiffness measurement (LSM) cutoff value can easily be determined for each particular clinical requirement. A highly sensitive cutoff is useful when screening for early-stage cirrhosis whereas a highly specific cutoff would aid in detection of significant fibrosis or cirrhosis. A cutoff affording a high diagnostic accuracy would be valuable to ensure correct diagnosis. Interestingly, the cutoffs obtained by maximizing the sum of sensitivity and specificity, the area under the receiver operating characteristic (AUROC) curve, and diagnostic accuracy, were slightly lower for CHB than CHC patients. This is because the extent of fibrosis is slightly less in the former patients[43]. Cirrhosis in CHB patients is more macronodular in nature than in patients with CHC or alcoholic liver disease.

Kim *et al*[44] found that LSMs and biopsy data were not in agreement with measures of necro-inflammatory activity in CHB patients. LSMs were significantly higher in cirrhotic patients in whom such activity was maximal (grades 3-4; grade 4 is termed F4) than when the activity was less (grades 1-2) (median of 19.2 kPa in the former patients *vs* 11.9 kPa in the latter; $P = 0.009$). The results of liver biopsy and LSM differed in CHB patients with cirrhosis, principally when necro-inflammatory activity of grades 3-4 significantly increased the LSMs.

In addition, serum levels of aminotransferases must be considered when interpreting TE data from hepatitis B patients because alanine aminotransferase (ALT) flares (which are frequent in CHB patients) cause TE values to be artificially inflated[45]. Thus, patients with higher ALT levels tend to have greater LSMs than do those with lower ALT levels, even when the stage of liver fibrosis is identical. Thus, TE values of CHB patients should be interpreted cautiously because it is possible that patients with low-grade fibrosis may score as false-negatives whereas those with high ALT levels may yield false-positive values. In patients with normal ALT levels, the optimal cutoff values for reliable detection of cirrhosis ranged from 8.4 to 12.0 kPa. However, in patients with elevated ALT levels [> 1-5-fold the upper limit of normal (ULN)], the optimal cutoff values ranged from 8.4 to 13.4 kPa[19]. Kim *et al*[46] suggested that an appropriate LSM cutoff value in CHB patients with ALT ≤ ULN was 10.1 kPa, but 15.5 in those with ALT > ULN and ALT < 2 × ULN. When only patients with ALT < ULN were studied, the cutoff values allowing discrimination of each stage of fibrosis were reduced, and the sum of LSM sensitivity and specificity increased.

Both the total bilirubin level and the time of performance of LSM have been explored in terms of the ability to predict development of cirrhosis in CHB patients. The total bilirubin level was significantly associated with changes in LSMs. However, the LSM was not reduced when measured soon after the levels of ALT and total bilirubin normalized[47]. This suggests that normalization of the LSM may occur only after laboratory measurements on serum return to normal. Therefore, biochemical findings should be well-stabilized before LSMs can be considered reliable.

LSM data did not differ when the detector was placed in the fifth, sixth, and seventh intercostal spaces[48]. All LSMs accurately predicted development of cirrhosis. It was not necessary that the site of LSM should correspond to the liver biopsy site in cirrhotic HBV patients.

A meta-analysis of 50 studies found that the mean AUROC for reliable diagnosis of significant fibrosis and cirrhosis was 0.94, with an optimal cutoff LSM of 13.01 kPa[49]. TE diagnosed severe fibrosis or cirrhosis more reliably than less advanced fibrotic stages. The data suggest that TE is an excellent tool for use in clinical practice to confirm the presence of cirrhosis when other clinical data are not definitive.

Another meta-analysis of the data of nine studies showed that use of TE to diagnose cirrhosis was associated with 87% sensitivity and 91% specificity[50]. TE affords good diagnostic accuracy when used to quantify the extent of liver fibrosis in patients with either CHB or CHC. Chon *et al*[51] performed a meta-analysis of 18 stud-

ies with 2772 patients and found that the cutoff value for detection of cirrhosis in Asian CHB patients was 11.7 kPa, with a sensitivity and specificity of 84.6% and 81.5%, respectively. The mean AUROC for diagnosis of cirrhosis was 0.929.

Advantages and disadvantages of TE

TE data are reproducible and the results have been validated in detail. The procedure time is less than 5 min. It is easy to evaluate patients either at the bedside or in the outpatient clinic. TE is useful when employed to follow-up disease progression and to predict hepatic events preceding cirrhosis[52].

However, it is difficult to obtain TE data from obese patients [those with a body mass index (BMI) above 28 kg/m^2] and patients with narrow intercostal spaces, ascites, space-occupying tissue abnormalities, extrahepatic cholestasis, or congestion[15,53,54]. The difficulty of reliably measuring liver stiffness in patients with ascites is a major disadvantage, because ascites is a typical sign of cirrhosis.

Recently, an XL TE probe (as distinct from the standard M probe) has been used to gather data from obese patients. The XL probe afforded the best diagnostic performance of all probes tested and a relatively low level of measurement failure[55]. Compared to the M probe, the XL probe operates at a lower frequency (3.5 MHz *vs* 5 MHz), is longer (ultrasound transducer focal length of 50 mm *vs* 35 mm), yields vibration of greater amplitude (peak-to-peak 3 mm *vs* 2 mm), and has a greater depth of measurement (35-75 mm *vs* 25-65 mm). Failure of LSM using the M probe occurred in 29.1% of patients with BMIs of 30 kg/m^2 or more, but the failure rate was only 6.8% when the XL probe was employed.

Interestingly, eating prior to performance of TE can compromise the accuracy of LSMs in both CHC patients and those with other conditions[56,57]. The impact of ingestion on liver stiffness values is proportional to the stage of fibrosis, being maximal in patients with cirrhosis. This is because liver stiffness increases as portal blood flow and the hepatic vein pressure gradient rise after a meal[58]. Therefore, it is necessary for a patient to fast for at least 2 h prior to performance of TE.

ACOUSTIC RADIATION FORCE IMPULSE IMAGING

Measurement of liver stiffness

ARFI, which employs conventional B-mode sonography, is an alternative to TE. ARFI mechanically excites tissue for a brief period *via* delivery of a high-intensity acoustic pulse to a region of interest (ROI). Shear waves propagate and generate localized tissue displacement[59]. The shear-wave velocity (SWV) is recorded in m/s and quantified in a region smaller than that examined when TE is employed (10 mm long and 6 mm wide). The ROI can be chosen by the operator. The SWV increases with stiffness. Thus, the SWV is an intrinsic and reproducible property of tissue[59-61].

The probe is usually placed between the ribs on the right side (*e.g.*, in segment 8) to reduce the effect of cardiac motion on measurement. Thus, the device is located approximately where a liver biopsy is usually performed 1 cm under the capsule. Minimal scanning pressure is applied and the patient is asked to stop breathing for a moment, to minimize motion[62-64]. Data can also be obtained from the left lobe, which always appears to be stiffer than the right lobe, although data from both lobes correlate well with the stage of fibrosis[65]. The difference in measurement may be explained by variation in the pressure with which the ultrasonographic probe is placed. The probe is not directly applied to the right lobe because the chest wall intervenes to prevent direct probe contact with the liver.

Diagnostic performance

ARFI SWV diagnostic cutoff values of 1.75-2.00 m/s have been reported in patients with liver cirrhosis[62-67]. Chen *et al*[68] explored the association between spleen stiffness and advanced liver fibrosis. The optimal cutoff value was 3.32 m/s for detection of cirrhosis (80.0% sensitivity, 88.4% specificity, 55.5% PPV, 96.0% NPV). However, these data should be interpreted with caution because the proportion of HBV patients in the tested population was too small to allow the diagnostic performance of ARFI in cirrhotic HBV patients to be evaluated. ARFI has been used to examine patients with a variety of liver diseases, principally hepatitis C infection.

Friedrich-Rust *et al*[69] were the first to use ARFI to assess the severity of liver fibrosis in HBV patients. In this prospective European multicenter study, the median SWV was 0.76-2.96 m/s in CHB patients. In terms of Metavir fibrosis scores, the median velocities were 1.10 m/s for F0 patients, 1.14 m/s for F1 patients, 1.23 m/s for F2 patients, 1.60 m/s for F3 patients, and 1.75 m/s for F4 patients. The accuracy of cirrhosis diagnosis was 0.97. The cutoff for diagnosis of significant fibrosis (F grade $\geqslant$ 2) was 1.39 m/s with a sensitivity of 50%, a specificity of 90%, a PPV of 67%, an NPV of 73%, a positive likelihood ratio of 5.125, and a negative likelihood ratio of 0.554. However, no optimal cutoff for diagnosis of cirrhosis in CHB patients was derived, because the number of patients with F3/F4 fibrosis was too small. The diagnostic accuracy of ARFI in HBV patients with different degrees of fibrosis was lower than reported in previous studies on CHC patients who had fibrosis ranging from mild to significant, or cirrhosis. The accuracy of ARFI when used to detect mild fibrosis was 66% in HBV patients compared to 73% in studies on patients with CHC and other liver diseases. The accuracy values were 73% and 82%-90% in patients with significant fibrosis, 94% and 90%-99% in those with severe fibrosis, and similar (97% and 87%-99%) in those with cirrhosis[62-72]. Further randomized prospective studies are required to determine whether ARFI data are influenced by underlying liver disease.

One meta-analysis reviewed 36 reports on ARFI im-

aging of CHB patients and those with other liver diseases. In terms of diagnosis of patients of F grade ⩾ 3, the mean AUROCs of studies including and excluding HBV patients were 0.87 (95%CI: 0.85-0.90) and 0.92 (95%CI: 0.89-0.95), respectively[73]. Thus, AFRI afforded good diagnostic accuracy when used to detect cirrhosis.

Advantages and disadvantages

In contrast to TE, ARFI yields real-time information on the extent of liver stiffness during observation using B-mode ultrasonography. Also, liver tissue can be directly targeted, with exclusion of small non-parenchymatous areas (for example, blood vessels). If a patient has a large right-side tumor or ascites, it is difficult to measure LSM using TE. In such instances, ARFI can be employed to diagnose cirrhosis. As ARFI is performed using a conventional ultrasound instrument, ARFI and standard ultrasound examination may be performed using the same machine in the same session. This is convenient for CHB patients, who are usually examined ultrasonographically once or twice per year.

The major disadvantage of ARFI is that no long-term follow-up validation studies have been performed. Also, ARFI values, in contrast to TE values, have a narrow range (0.5-4.4 m/s). This makes it difficult to define precise cutoff values that can be used to make decisions on patient management.

Cassinotto *et al*[55] reported that ARFI was (like TE) less efficient in obese patients. This is because the ultrasound beam travels poorly through thick fatty soft tissue. Thus, ARFI cannot be used as a first-line examination modality for obese patients.

An advantage of ARFI is the ability to measure LSM in both the right and left lobes of the liver. However, upon simultaneous liver biopsy of both lobes, a between-lobe difference of at least one fibrosis stage was noted in only 33% of patients, and liver cirrhosis was evident in only one biopsy in 14.5% of cases[11].

Acute cellular infiltration, increased central venous pressure, and cholestasis, can cause the extent of liver fibrosis to be overestimated. These possibilities should be considered when interpreting elastography data[74-76]. As is true of TE measurements, food intake also increases ARFI-measured liver stiffness[77].

REAL-TIME ELASTOGRAPHY

Measurement of liver stiffness

RTE is a novel noninvasive ultrasound modality measuring liver elasticity, and has recently been used to quantitatively assess the extent of liver fibrosis[78-81]. As with ARFI, RTE uses a modified version of standard ultrasound equipment. RTE propagates a shear wave through the liver and echo signals are captured in real time. Friedrich-Rust *et al*[79] and Kanamoto *et al*[82] examined the utility of RTE in evaluation of liver fibrosis.

No special skills are required. A patient is placed in a supine position with the right arm extended above the head to stretch the intercostal muscles. After B-mode examination, the elastographic mode is selected. It is easy to verify the position of the liver because the elastographic images may be superimposed on B-mode reference images. The probe pressure may be varied manually. A small compression plate is usually attached to the ultrasonic probe so that stable tissue compression is attained and the stress field transmitted more uniformly. Tissue elasticity is color-coded (red: soft; blue: hard) in terms of magnitude and superimposed on conventional two-dimensional (2D) images[83], allowing anatomical correspondence to be noted.

Diagnostic performance

Only a few reports on use of RTE to evaluate CHB patients have appeared. Xie *et al*[84] used RTE to calculate elastic strain ratios (elastic strain values of liver tissue in the ROI/strain values of intercostal muscle in the ROI) in patients with different degrees of liver fibrosis. The diagnostic cut-off ratios were 1.10 in patients with substantial liver fibrosis and 0.60 in those with cirrhosis. The detection sensitivities for substantial fibrosis and cirrhosis were 77.8% and 50.0%, respectively, and the specificities 80.0% and 96.7%. The positive predictive values were 80.0% and 71.4% and the negative predictive values 77.8% and 92.2%.

Wang *et al*[85] described the diagnostic performance of an elasticity index obtained using RTE, and the aspartate aminotransferase (AST) to platelet ratio index (APRI) used to stage liver fibrosis in CHB patients. The AUROCs for detection of cirrhosis were 0.66 and 0.23, respectively. The diagnostic accuracies of both tests used to assess the stage of liver fibrosis (except cirrhosis) in CHB patients were high. In terms of elasticity index distribution by Metavir fibrosis stage, the cut-off value was 90.31 for cirrhosis.

Advantages and disadvantages

RTE reveals relative tissue strain in real time by measuring tissue displacement. RTE can be used (as can ARFI) to evaluate liver fibrosis patients with ascites or who are severely obese. Eleven stiffness parameters are measured, and changes in tissue stiffness are thus evaluated systematically and sensitively.

However, RTE requires further clinical validation. Although strain images are usually clear, artifacts include multiple reflections at the surface of the liver; echo-free areas filled by thick blood vessels, ribs and lungs; and lack of wave penetration. Also, if the probe pressure is excessive, the elastic relationship will vary (elasticity is nonlinear). In addition, pressure is not uniformly transmitted to the liver because that tissue is slightly deformed by heartbeats. However, good images can be obtained simply by applying the probe lightly to right-side intercostal regions. New quantitative analysis systems can measure tissue compression caused by the rhythmic beats of the heart and blood vessels; no manual compression is required. This minimizes the capacity for human error.

MAGNETIC RESONANCE ELASTOGRAPHY

Measurement of liver stiffness

MRE is not yet widely available. The technique uses a modified phase-contrast method to image the propagative characteristics of a shear wave traversing the liver[86]. A probe is placed against the patient's back. Measurements are obtained from the anterior segment of the right lobe (the region of the liver that is usually biopsied). Liver stiffness is measured by delineating an ROI of at least 100 mm^2 in the liver parenchyma, avoiding the edges of the tissue, large vessels (> 3 mm in diameter), and fissures. Low-frequency vibrations are emitted and the MRI spin echo sequence is used to gather data. Mean liver stiffness is expressed in kPa using a formula deriving a shear modulus, which is one-third the modulus of Young used in TE evaluation[87].

Diagnostic performance

From a clinical viewpoint, MRE affords excellent accuracy when used to differentiate significant fibrosis from mild fibrosis or absence of fibrosis, and cirrhosis from other stages of liver fibrosis[88,89]. MRE has a high negative predictive value (97%) when used to exclude the presence of fibrosis[90]. A meta-analysis reviewing five studies employing MRE showed that the technique performed well diagnostically when used to evaluate liver cirrhosis attributable to different causes. The sensitivity of the technique was 92% and the specificity 96% when used differentiate F0-2 fibrosis from fibrosis of grades F3-4[91].

Venkatesh *et al*[92] found that MRE was an accurate non-invasive method when used to detect and stage fibrosis in CHB patients. The diagnostic performance of MRE was significantly higher than those of serum fibrosis markers. The optimally discriminatory cutoff MRE value was 4.33 kPa for diagnosis of cirrhosis in CHB patients, with a sensitivity of 100%, a specificity of 100%, a PPV of 91.3%, and an NPV of 100%. Use of MRE was associated with a 14.3% error rate. Therefore, MRE is a valuable non-invasive test when used to detect and stage liver fibrosis in CHB patients.

Interestingly, the MRE cutoff value for diagnosis of cirrhosis in CHB patients (4.33 kPa) was lower than that reported for CHC patients (6.20 kPa)[93] but similar to that associated with diagnosis of advanced fibrosis in NASH patients (4.3 kPa)[94]. This suggests that the cutoff values of diagnostic MRE data differ for diseases that vary in etiology. However, such results require confirmation in large-scale studies.

Advantages and disadvantages

MRE affords a significantly higher accuracy and technical success rate than other non-invasive modalities. MRE scans the entire liver and thus does not select an acoustic window. MRE can be used to evaluate obese patients or those with ascites. MRE can be easily incorporated into routine liver MRI; clinicians can simultaneously assess structural disease, liver stiffness, and fat and iron levels.

Serum ALT level is a known confounding factor when fibrosis is detected using TE; liver stiffness is positively correlated with serum ALT levels[95,96]. However, MRE has exhibited no such association[89], probably because MRE and TE assess different mechanical properties.

However, MRE is quite expensive, and is not yet widely available. MRE cannot be used to evaluate patients who are claustrophobic or fitted with heart pacemakers. MRE is more time-consuming than are other ultrasound-based elastographic methods. MRE cannot be performed on iron-overloaded livers because of signal-to-noise limitations.

SERUM BIOMARKERS

Measurement of liver stiffness

Diagnostic serum biomarkers of liver fibrosis and cirrhosis have been well-validated. Although marker levels are highly reproducible, they are not specific for liver disease and do not allow easy discrimination of intermediate stages of fibrosis[95]. Score panels based on combined blood test data are also of limited utility in differentiating the stages of fibrosis. Biomarkers detect cirrhosis more readily than intermediate stages of fibrosis[97,98]. However, the clinical utility of markers in diagnosis of liver cirrhosis requires further verification.

Diagnostic performance

Several serum markers have been evaluated in terms of the ability to diagnose liver cirrhosis. These include the FibroTest, the APRI, the prothrombin index (PI), the AST/ALT ratio (the AAR), the Lok index, and the Goteborg University cirrhosis index (the GUCI)[99,100]. The FibroTest and biopsy data performed similarly when used to diagnosis significant fibrosis in CHC patients[98,101-104]. One large study (of 913 CHC and 284 HBV patients) prospectively compared the most popular patented tests (the FibroTest, the Fibrometre, and the Hepacore) with a nonpatented test (the APRI); the AUROC values for cirrhosis ranged from 0.77 to 0.86 and no significant among-test differences in scores were evident[105]. Although nonpatented tests such as the Forns index, the FIB-4, and the APRI may lack the performance of the patented tests, the former tests are inexpensive, easy to perform, and widely available. However, few serum biomarkers have been evaluated in terms of the ability to define the stage of liver cirrhosis in CHB patients. The results of studies on CHC patients cannot be directly applied to CHB patients because CHB infection is associated with a specific type of pathogenesis. Thus, dedicated validation of marker utility in CHB patients is required.

The FibroTest has been studied extensively over the last 5 years and is currently the best-understood serum marker panel used to detect fibrosis[106]. Application of

the FibroTest to CHB patients was associated with AUROCs of 0.77-0.78 for detection of significant fibrosis and cirrhosis, with 85.0% sensitivity and 52.0% specificity[107,108].

The APRI is a simple test that combines AST level with platelet count to predict the occurrence of significant fibrosis and cirrhosis in CHC patients. Venkatesh *et al*[92] showed that serum AST levels differed significantly between patients with low-grade and advanced fibrosis, but ALT levels did not. The APRI was helpful for discriminating CHB patients with advanced fibrosis from those with mild-to-moderate conditions. When the APRI cutoff was set at 0.5 for CHB patients, the AUROC for prediction of significant fibrosis was 0.673[109] and the PPV and NPV 30% and 87%, respectively. In other words, the APRI can reliably be used to exclude the presence of significant fibrosis.

Hui *et al*[109] developed a predictive model based on body mass index (BMI) and the data of three routine laboratory tests, which had an AUROC of 0.79 when used to assess the fibrosis stage of CHB patients. The laboratory data were bilirubin, albumin, platelet, and ALP levels. The PPV was 38% when a cutoff of 0.15 was applied and 53% when the cutoff was 0.5. The results suggest that the model should be used primarily to identify patients lacking significant fibrosis.

Sebastiani *et al*[110] reported that sequential use of the APRI, the FibroTest, and liver biopsy data, greatly improved diagnostic performance compared to use of a single non-invasive test in CHB patients. The sequential combination was developed to detect cirrhosis in HBV patients. The need for liver biopsy was reduced by 50%-80%. All of the FibroTest, the APRI, the AAR, and the GUCI were only 77.5%-86.1% accurate in terms of cirrhosis detection when used individually. The FibroTest showed the best PPV, NPV, and AUROC for cirrhosis detection (90%, 87.1%, and 0.76, respectively). However, the sequential combination afforded excellent accuracy (100%) in terms of detection. Stepwise combination algorithms featuring the APRI, the FibroTest, and biopsy, afforded excellent performance (0.95 AUROC and 98.3% NPV for cirrhosis).

Montazeri *et al*[111] suggested that serum hyaluronate level might predict extensive liver fibrosis and inflammation in CHB patients. Zhang *et al*[112] suggested that use of the APRI test in combination with hyaluronic acid (HA) measurement could serve to detect moderate-to-severe fibrosis in CHB patients. When the APRI was used alone to this end, the sensitivity, specificity, PPV, and NPV were 44.7%, 84.3%, 41.3%, and 84.7%, respectively. The diagnostic performance of the APRI was low in terms of discrimination of fibrosis stages in such patients. However, when an APRI score of $\geqslant$ 1.5 was used in combination with an HA cutoff of > 300 ng/mL, moderate-to-severe fibrosis was accurately predicted in CHB patients (98.9% of specificity and 93.7% of PPV).

Lebensztejn *et al*[113] reported that the AUROC of combined hyaluronan and laminin measurements was 0.84. Zeng *et al*[114] developed a non-invasive combination model including age and measurements of serum alpha-2-macroglobulin, hyaluronan, and γ-glutamyl transpeptidase. The AUROCs were 0.77-0.84. The expected rate of misdiagnosis was around 20%, similar to that reported in hepatitis C patients. However, it may be inappropriate to apply the test in real clinical settings. Recently, "proteome" technology has been used to study liver fibrosis. A total of 30 features predictive of significant fibrosis and cirrhosis were identified in 46 CHB patients. The AUROCs were high, being 0.906 and 0.921 for detection of advanced fibrosis and cirrhosis, respectively[115]. However, the method is rather complicated and may not be applicable to large-scale testing.

A meta-analysis[116] of data from 30 studies using the FibroTest and biopsy (3501 CHC and 1457 CHB patients) found that the mean standardized AUROC for diagnosis of significant fibrosis was 0.84, and did not differ significantly between patients with CHC (0.85) and CHB (0.80).

Advantages and disadvantages

The inter-laboratory reproducibilities of the FibroMeter, FibroTest, and APRI tests, and combinations thereof, are excellent[117]. The APRI test is inexpensive and widely available (being nonpatented). However, these tests perform less well than does TE in terms of cirrhosis diagnosis. Also, test results are not immediately available[95]. The tests are not specific for liver disease and do not discriminate among the intermediate stages of fibrosis. Test results can be influenced by comorbidities, and critical data interpretation is required. False-positive FibroTest and Hepascore results are yielded by patients with Gilbert's syndrome or hemolysis, because such patients exhibit hyperbilirubinemia[118]. Similarly, acute hepatitis can produce false-positive results in the APRI, Forns index, FIB-4, or Fibrometer tests.

DIAGNOSIS OF DECOMPENSATED CIRRHOSIS

Recently, noninvasive ultrasound- and MRI-based elastographic techniques have allowed the relationships between liver or spleen stiffness and portal pressure to be explored. Use of TE revealed a statistically significant association of these parameters with the hepatic venous pressure gradient of CHC patients[119,120], and a correlation with the grade of esophageal varices was evident[121]. Talwalkar *et al*[122] used MRE to show that splenomegaly, mean spleen stiffness, and serum platelet count were potentially associated with the presence of esophageal varices. Neither the mean liver stiffness value nor the mean spleen volume was significantly associated with the presence of esophageal varices. A cutoff spleen stiffness value of over 10.5 kPa identified all cirrhotic patients with esophageal varices.

Ye *et al*[123] reported that the liver stiffness cutoff value of ARFI was 1.88 m/s for diagnosis of liver cirrhosis in

CHB patients (AUROC = 0.97; sensitivity, 95.7%; specificity, 91.8). Interestingly, spleen stiffness values were also determined in the cited study, to aid in diagnosis of cirrhosis and esophageal varices. The spleen stiffness cutoff value was 2.72 m/s for diagnosis of liver cirrhosis (AUROC = 0.96; sensitivity, 88.4%; specificity, 93.2%). The optimal spleen stiffness cutoff predicting varices was 3.16 m/s (AUROC = 0.83). In this report, portal hypertensions could not be graded according to liver stiffness, but a significant linear correlation was evident between spleen stiffness and varices grade.

COMPARISONS OF MODALITIES USED TO DIAGNOSE CIRRHOSIS

TE and ARFI

Lupsor *et al*[66] found that both ARFI and TE data were strongly correlated with the stage of fibrosis, and TE was superior when used to predict early-stage disease in patients with chronic hepatitis C. However, Ebinuma *et al*[65] found that the diagnostic powers of the two techniques were almost identical. Neither mode detected low-grade fibrosis effectively, but diagnostic capacities rose as the stage of fibrosis advanced.

ARFI and TE were compared in a meta-analysis of eight studies involving 518 patients. TE afforded a slightly higher diagnostic accuracy for cirrhosis (mean AUROC difference: 0.04)[124]. The mean AUROC of all published studies on the use of ARFI to detect cirrhosis in CHB patients was 0.90. However, most studies had small sample numbers and the study populations were heterogeneous.

Friedrich-Rust *et al*[69] found that ARFI and TE performed similarly when used to diagnose liver fibrosis in CHB patients. ARFI afforded excellent performance when used to diagnose advanced fibrosis, but differentiation of mild fibrosis was in need of improvement. When a low cutoff of 1.03 m/s was used to exclude significant fibrosis, and a high cutoff of 1.39 m/s to confirm significant fibrosis, 38% of patients were correctly classified using ARFI.

Cassinotto *et al*[55] compared the use of ARFI, TE with the M and XL probes, and the FibroTest, in evaluation of 321 patients (39 with HBV infections). In terms of cirrhosis diagnosis, no significant difference was evident between ARFI elastography and TE with the M or XL probes. However, the diagnostic performance of TE was significantly better than that of the FibroTest.

Comparison of TE and MRE

In a comparative study, MRE was superior to both TE and APRI when used to assess liver fibrosis, affording accuracies of over 98% in diagnosis of all categories of fibrosis[89]. The AUROC of MRE was 0.998 for diagnosis of liver cirrhosis and the technical success rate was higher than that associated with ultrasound elastography (AUROC = 0.930), APRI (AUROC = 0.820), and a combination of TE with APRI (AUROC = 0.944).

Comparison of RTE and other elastographic modalities

Colombo *et al*[125] compared the utility of TE, RTE, and ARFI in diagnosis of liver fibrosis. TE and ARFI exhibited high diagnostic accuracies (AUROCs 0.9) when used to diagnose cirrhosis. All three methods afforded fair (AUROC = 0.7) to good (AUROC = 0.8) diagnostic accuracy when used to diagnose all fibrosis (F1-4 Metavir grades) and significant fibrosis (F2-4 Metavir grades). Of the various modalities, TE exhibited the best diagnostic performance (AUROCs of 0.878 for fibrosis and 0.897 for significant fibrosis, respectively). TE and ARFI were highly accurate when used to diagnose cirrhosis.

Friedrich-Rust *et al*[79] found that the AUROCs for diagnosis of significant fibrosis and cirrhosis using TE and RTE were 0.84 *vs* 0.69, and 0.97 *vs* 0.65, respectively. RTE was less accurate than TE when used to predict liver fibrosis. However, upon direct comparison of TE, RTE, and ARFI in 74 Korean patients, the cut-off values for diagnosis of cirrhosis were 8.60 kPa (AUROC = 0.786; sensitivity, 81.0%; specificity, 64.2%); 1.39 m/s (AUROC = 0.807; sensitivity, 90.5%; specificity, 66.0%); and 2.79 (AUROC = 0.767; sensitivity, 81.0%; specificity, 64.2%), respectively[126]. TE and ARFI, rather than RTE, are the best modalities for non-invasive assessment of liver fibrosis. However, all three methods reliably predict cirrhosis.

Comparison of elastography and serum biomarker measurements

In terms of detection of cirrhosis, AUROC analysis indicated that the APRI test was superior to the elastic strain ratio determined using real-time elastography, and the Forns index[84]. This suggests that the APRI should be used to diagnose cirrhosis in preference to calculation of the elastic strain ratio. When the AUROCs of spleen stiffness measurement and APRI were compared, the overall diagnostic performance of the former test in prediction of liver fibrosis stages was superior to that of the APRI[68]. The diagnostic accuracy of the FibroTest was comparable to those of elastographic methods. APRI scoring has been shown to be inferior to FibroTest- and TE-based evaluations[127,128].

Other methods for diagnosis of liver cirrhosis

Hu *et al*[129] measured newly maximal accumulative respiration strain (MARS) values obtained from hepatic tissue image analysis of CHB patients. Each value represents the average strain of hepatic tissue in the ROI at different stages of the respiratory cycle. The MARS values were correlated with fibrotic stage. The diagnostic accuracy rate for cirrhosis had an AUROC of 0.75. However, the performance of MARS was inferior to that of TE.

COMBINATION OF LSM WITH OTHER MODELS OF FIBROSIS PREDICTION

The use of combination models has been proposed to

Table 1 Diagnostic performance of non-invasive diagnostic methods in predicting cirrhosis with chronic hepatitis B

Ref.	Year	Modality	Patients (*n*)	Cut-offs ≥ F4	Standard reference	AUROC	Se	Sp	Accuracy	PPV	NPV
Myers *et al*[107]	2003	Biomarkers	209	0.2[1]	Biopsy	0.780	85.0%	52.0%	-	43.0	92.0
				0.4[1]			54.0%	80.0%	-	53.0	81.0
				0.8[1]			18.0%	99.0%	-	92.0	75.0
Hui *et al*[109]	2005	Biomarkers	235	> 0.15[1]	Biopsy	0.791	88.0%	50.0%	93.0	38.0	92.0
				> 0.5[1]		0.791	37.0%	88.0%	-	53.0	81.0
Zeng *et al*[114]	2005	Biomarkers	200	> 3.0[1]	Biopsy	0.770	94.8%	44.1%	-	70.1	86.1
				> 8.7[1]		0.770	35.3%	95.2%	-	91.1	51.6
Marcellin *et al*[20]	2009	TE	173	18.2 kPa[2]	Biopsy	0.930	57.0%	97.0%	94.0	67.0	96.0
				11 kPa[2]	Biopsy	0.930	93.0%	87.0%	38.0	38.0	99.0
Chan *et al*[19]	2009	TE	161	13.4 kPa[2]	Biopsy	0.930	75.0%	93.0%	89.0	78.0	92.0
				12 kPa[3]			79.0%	92.0%	-	76.0	93.0
Kim *et al*[18]	2009	TE	91	10.3 kPa	Biopsy	0.803	59.0%	78.0%	-	68.0	72.0
Kim *et al*[52]	2010	TE	104	10.1 kPa	Biopsy	0.849	86.7%	88.1%	-	87.5	77.1
		TE	52	15.5 kPa		0.867	66.7%	100.0%	-	100.0	72.9
Kim *et al*[138]	2010	LSPI[4]	330	38[5]	Biopsy	0.956	98.0%	69.2%	-	82.9	95.7
				62[5]			85.9%	93.8%	-	95.5	81.3
				42[6]			96.3%	67.4%	-	73.3	95.1
				94[6]			67.5%	97.7%	-	96.4	76.4
Hu *et al*[129]	2010	MARS	28	20.32%	Biopsy	0.750	100.0%	-	-	-	-
Ye *et al*[123]	2011	ARFI	264	1.88 m/s	Biopsy	0.970	95.7%	91.8%	-	-	-
		Spleen ARFI		2.72 m/s		0.960	88.4%	93.2%	-	-	-
Xie *et al*[84]	2012	RTE[7]	71	-0.6	Biopsy	0.797	50.0%	96.7%	-	71.4	92.2
		APRI	71		Biopsy	0.930	-	-	-	-	-
Wang *et al*[85]	2012	RTE	75	90.31	Biopsy	0.660	71.4%	80.0%	-	93.8	40.0
		APRI	75		Biopsy	0.930	-	-	-	-	-
Cassinotto *et al*[55]	2013	TE[8]	285	14.1 kPa	Biopsy	0.910	77.0%	92.0%	89.0	74.0	93.0
		TE[9]	254	10.1 kPa	Biopsy	0.880	85.0%	82.0%	82.0	76.0	96.0
Friedrich-Rust *et al*[69]	2013	ARFI	92		Biopsy	0.970	-	-	-	-	-
		TE	92		Biopsy	0.930	-	-	-	-	-
Venkatesh *et al*[92]	2013	MRE	64	4.33 kPa	Biopsy	0.980	100.0%	95.2%	-	91.3	100.0

[1]Cut-off value for significant fibrosis; [2]Cut-off value with maximum of diagnosis accuracy; [3]Cut off value with a maximum sum of sensitivity and specificity; [4]Liver stiffness measurement × spleen diameter/platelet count; [5]Cut-off value with normal alanine aminotransferase (ALT); [6]Cut-off value with high ALT; [7]Elastic strand ratio; [8]M probe; [9]XL probe. LSPI: Liver stiffness measurement - spleen diameter to platelet ratio index; TE: Transient elastography; ARFI: Acoustic radiation force impulse imaging; RTE: Real-time elastography; MRE: Magnetic resonance elastography; MARS: Maximal accumulative respiration strain; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUROC: Area under the receiver operating characteristic.

increase the diagnostic performance of tests for liver cirrhosis, although elastography alone accurately predicts histological cirrhosis. The combined use of the Enhanced Liver Fibrosis (ELF) test with either ARFI or TE in patients with multi-origin cirrhosis increased the diagnostic accuracy of liver cirrhosis[130]. The PPV and NPV of ARFI-plus-ELF were 73% and 100%, and those of TE-plus-ELF 64% and 100%, respectively.

The ultimate validation of liver fibrosis as a marker of liver injury is the prognostic value thereof in terms of morbidity and mortality. In recent studies on non-CHB patients, the TE test and measures of serum fibrosis markers were prognostically more valuable than liver biopsy data[131-133]. However, long-term follow-up studies on ARFI are required.

Chung *et al*[126] explored the efficacies of combinations of LSM modalities (TE, ARFI, and RTE) with platelet count in terms of increasing the diagnostic power for liver disease of varying etiology. Cutoff ratios of LSM/(platelet count) used to predict cirrhosis were no more effective than the LSM data alone. However, the ratios predicted significant fibrosis (grade ≥ F2) more effectively than did LSM data alone.

The diagnostic performance of TE in CHB patients can be improved by combining test data with those of fibrosis serum marker levels derived using the APRI, Fib-4, Fibrometer and FibroTest[134-136]. Wong *et al*[137] reported that agreement between a high LSM and a high Forns index value improved diagnostic specificity (from 99% to 100% and from 87% to 98% in training and validation cohorts, respectively). Kim *et al*[138] derived optimal predictive threshold values of the LSM/spleen diameter to platelet ratio index (LSPI; LSM × spleen diameter/platelet count) for detection of cirrhosis in terms of ALT level. The AUROC was 0.956, thus slightly higher than the AUROC of 0.919 afforded by use of TE data alone. When used to diagnose cirrhosis in patients with normal ALT levels, use of an LSPI predictive threshold value of 38 was associated with 98.0% sensitivity and 69.2% specificity, whereas a threshold value of 62 was associated with 85.9% sensitivity and 93.8% specificity. Similarly, in a high-level ALT group, an LSPI predictive threshold value of 42 was associated with 96.3% sensitivity and 67.4% specificity, whereas a threshold of 94 was associated with 67.5% sensitivity and 97.7% specificity.

Kim *et al*[46] found that optimal LSM cutoff values varied with ALT level, and use of an index (a ratio) combining data on age, spleen size, and platelet level, enhanced

LSM performance when used to diagnose cirrhosis in CHB patients (AUROCs = 0.917 in patients with ALT ⩽ upper ULN; 0.909 in those with ALT ⩽ 2 × ULN; and 0.894 in all patients).

Evaluation of hepatitis B patients using elastography increased the accuracy of these measures when used to diagnose cirrhosis. Increasing acceptance of the utility of combination modalities is expected to reduce the requirement for liver biopsy. The combination methods enhance diagnostic accuracy and reduce the number of liver biopsies needed to evaluate cirrhotic CHB patients. An optimal choice of diagnostic modality requires the conduct of large-scale validation studies in a variety of patient populations. Also, the cost-effectiveness of combination models requires future attention.

CONCLUSION

The last decade has seen significant progress in development of noninvasive liver disease assessment in CHB patients (Table 1). It has become accepted that liver biopsy has limitations when used to diagnose cirrhosis. TE, ARFI, RTE, and MRE are valuable for early diagnosis of cirrhosis in CHB patients because the AUROC values associated with use of these techniques are in excess of 0.8[139].

Routine use of TE in management of hepatitis C patients has significantly reduced the need for liver biopsy[140], but reliable detection of mild fibrosis and accurate differentiation of fibrotic stages remain problematic[141-143]. In addition, accurate measurements may be difficult to obtain from obese patients, those with ascites, and those exhibiting severe hepatic atrophy. Acoustic radiation force impulse imaging is a novel form of ultrasound providing *in vivo* information on the local mechanical properties of tissue[144,145]. However, ARFI cannot be used to evaluate patients who are obese or cirrhotic patients with very stiff tissue because imaging results are automatically rejected if detection of low-frequency acoustic wave propagation is inadequate[63]. RTE does not have such limitations and can be used to image almost all patients, including those who cannot be assessed by TE or ARFI. MRE was more accurate than either ultrasound-based elastography or measurement of serum markers of fibrosis[88,89,93]. However, most previous studies on RTE and MRE included patients with chronic liver disease of various etiologies. RTE and MRE should be validated in CHB cohorts (with macronodular cirrhosis) in particular.

TE is the most widely used method for diagnosis of cirrhosis in CHB patients, but ARFI and MRE may be equally valuable. Novel techniques including supersonic shear imaging[146,147] or measurement of spleen stiffness[148] may also be valuable in diagnosis of liver cirrhosis. A combination of elastography and biomarker measurements may improve diagnostic performance during surveillance of CHB patients for development of liver cirrhosis. However, clinical benefit must be weighed against cost when a combined approach is planned.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Hepatitis B virus mutations related to liver disease progression of Korean patients

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Abstract

Hepatitis B virus (HBV) infection is a global health problem and more than 350 million people worldwide are chronic carriers of the virus. Despite the recent dramatic decline in HBV chronic patients through successful programs of hepatitis B surface antigen vaccination, South Korea is still recognized as an endemic area of HBV infection. HBV infections in South Korea exhibit several distinct features in epidemiologic and clinical aspects. In this review paper, we summarize the distinct HBV mutation patterns related to clinical severity and the molecular epidemiologic traits in Korean chronic patients based on previous reports. Generally, several lines of evidence, including our previous results, have led to the conclusion that a combination of the exclusive predominance of genotype C2, which is prone to mutations, the high prevalence of basal core promoter double mutations, and the presence of distinct immune responses against HBV proteins in the Korean population may generate the distinct HBV variants rarely or not encountered in other areas, which results in distinct clinical manifestations in Korean chronic patients. This may provide a novel insight into the relationships between clinical severity, HBV genotype distribution, and HBV naturally occurring variants.

Key words: Hepatitis B virus; Mutation; South Korea; Hepatocellular carcinoma; Genotype C2

Core tip: In this review paper, we summarize the distinct hepatitis B virus (HBV) mutation patterns related to clinical severity and the molecular epidemiologic traits in Korean chronic patients based on previous reports. Generally, several lines of evidence have led to the conclusion that a combination of the exclusive predominance of genotype C2, which is prone to mutations, the high prevalence of basal core promoter double mutations, and the presence of distinct immune responses against HBV proteins in the Korean population may generate the distinct HBV variants rarely or not encountered in other areas, which results in distinct clinical manifestations in Korean chronic patients.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people worldwide are chronic carriers of the virus[1]. The infection is associated with a large spectrum of clinical manifestations ranging from acute or fulminant hepatitis to various forms

of chronic infection, including asymptomatic carriers, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[2]. HBV vaccination was first introduced into the Korean population in 1983[3], and it dramatically reduced the prevalence of hepatitis B surface antigen (HBsAg) positive chronic carriers from more than 10% to 3.7% in 2007 during a period of approximately 30 years[4].

Despite the recent dramatic decline in HBV chronic patients through the successful program of HBsAg vaccination, South Korea is still recognized as an endemic area of HBV infection. HBV infections in South Korea have exhibited some distinct features from both epidemiologic and clinical aspects. First, among the Organisation for Economic Co-operation and Development nations, South Korean infections have exhibited the highest incidence of HCC that is primarily induced by HBV infection[5]. Second, relatively lower antiviral responses against alpha interferon and/or lamivudine were found in Korean patients compared with patients in other countries[6]. Third, of particular note, it was reported that only genotype C2 infections, which are known to be more prone to mutations and are related to greater severity in liver diseases compared with genotype B[7], were found in an exclusive manner in this area[8], which contribute to the distribution of characteristic HBV mutation patterns related to the progression of liver diseases. These features lead to the hypothesis that there may be a distinct naturally occurring HBV mutation related to clinical severity and lower antiviral responses in chronic patients in South Korea. In order to prove this hypothesis, we have attempted to determine the HBV variants related to the progression of liver diseases, particularly HCC, and to analyze the mutation frequency in HBV encoding antigens from Korean patients[9-22].

In this review paper, we summarize the distinct HBV mutation patterns related to the clinical severity and molecular epidemiologic traits in Korean chronic patients, primarily focusing on the mutation patterns of 4 regions, preS[9,15,16], surface (S)[12,13,20], precore/core (preC/C)[11,17], and X[14,18,22].

DISTRIBUTION OF HBV GENOTYPES IN KOREAN CHRONIC PATIENTS

HBV has been divided into eight genotypes, types A-H, based on one of the following criteria: an intergroup divergence of 8% or greater in terms of its complete genome nucleotide sequence or a 4.1% divergence or greater for the surface antigen gene[23-25]. These genotypes reflect the geographical distribution of HBV. It has also been suggested that the area-specific localization of HBV genotypes is associated with anthropologic history[26]. In addition, remarkable differences have been reported in the clinical and virological characteristics of patients infected with different genotypes[27]. For example, in Asia, genotype C has been found to have a greater ability to induce disease than genotype B[7]. Our previous molecular epidemiologic study based on the direct sequencing protocol targeting the partial *S* gene (541 bp) found that all HBV strains from 209 Korean chronic patients belonged to genotype C2 (100%)[8]. Other studies based on serology[28] and polymerase chain reaction (PCR) restriction fragment length polymorphism analysis, or genotypic-specific PCR[29], also support these results. The exclusive predominance of genotype C infection without coexistence with other genotypes is the most distinct epidemiologic trait shown in Korean chronic patients[8], and this may affect the clinical manifestations of Korean chronic patients as well as the virological traits such as mutation frequency.

MUTATION FREQUENCY AND PATTERNS IN THE PRES REGION IN KOREAN CHRONIC PATIENTS

The envelope of HBV is composed of three forms of HBsAg sharing 226 amino acids at the C-terminus: the large (coded using the *preS1/S2/S* gene), middle (the preS2/S gene), and small (the *S* gene) envelope proteins. During the viral life cycle, at least two essential functions have been attributed to the preS domain: attachment to the hepatocyte membrane and budding of the virus at the endoplasmic reticulum (ER)[30,31]. Thus far, several lines of evidence that mutants occurring naturally in the preS region correlate with more progressive forms of liver disease have been documented[32-34]. The mutations, particularly deletions, in the preS region may affect the ratio between the small and large envelope proteins, which results in the ER stress associated with the aggravation of liver disease. Furthermore, integration of the truncated large or middle envelope protein into the host chromosome enhances the potential development of HCC by increasing the transactivating capacity[35].

Our report regarding the prevalence of preS deletions in Korean chronic patients demonstrated that a relatively high level of preS deletions was found in Korean chronic patients (30.8%, 37/120 patients)[16]. The comparisons of the clinical information between chronic patients with and without preS deletions indicated that patients with deletions were older (54.3 ± 12.7 *vs* 45.1 ± 18.2, P = 0.006), had more severe liver disease (liver cirrhosis and HCC; 73% *vs* 41%, P = 0.001), and had a higher HBV DNA level (378.4 *vs* 70, P = 0.009) than those without the deletion. These results suggest that the acquisition of preS deletions may contribute to the progression into severe types of disease such as HCC and liver cirrhosis, at least in genotype C-infected Korean chronic patients[16].

Although preS deletion in Korean chronic patients was significantly associated with severe forms of liver diseases, a difference between the preS1 and preS2 deletions in relation to HCC and liver cirrhosis was found. For example, preS1 deletions were observed more frequently in HCC patients than in patients with liver cirrhosis [32.5% (13/40 patients) *vs* 19.9% (4/21 patients)], and the opposite was observed in preS2 deletion variants [15.0% (6/40

Table 1 Mutations in 4 hepatitis B virus regions related to the progression of liver diseases in Korean chronic patients

Region		Mutations related to severe liver diseases	*P* value	Ref.
PreC/C	PreC	W28* [HCC 12/35 (34.3%) *vs* LC+ CH + C 5/35 (14.3%)]	0.093	Kim *et al*[11]
	C	P5H/L/T [HCC 5/35 (14.3%) *vs* LC + CH + C 0/35 (0%)]	0.020	Kim *et al*[11]
		E83D [HCC 4/35 (11.4%) *vs* LC + CH + C 0/35 (0%)]	0.039	Kim *et al*[11]
		I97F/L [HCC 13/35 (37.1%) *vs* LC + CH + C 4/35 (11.4%)]	0.024	Kim *et al*[11]
		L100I [HCC 6/35 (17.1%) *vs* LC + CH + C 1/35 (2.9%)]	0.046	Kim *et al*[11]
		Q182K/* [HCC 4/35 (11.4%) *vs* LC + CH + C 0/35 (0%)]	0.039	Kim *et al*[11]
PreS	preS1	W4P/R [HCC 13/96 (13.5%) *vs* CH 0/32 (13.2%)]	0.028	Lee *et al*[9]
		PreS1 start deletion [HCC 9/40 (22.5%) *vs* C 1/38 (2.6%)]	0.048	Mun *et al*[16]
	preS2	F141L [HCC 26/99 (26.3%) *vs* LC 2/52 (3.8%)]	0.001	Mun *et al*[15]
		PreS2 deletion [HCC 35/99 (35.4%) *vs* CH 6/45 (13.3%)]	0.020	Mun *et al*[15]
S	S	W182* [HCC + LC 56/176 (31.8%) *vs* CH + C 17/99 (17.2%)]	0.010	Lee *et al*[12]
X	X	V5M/L [HCC 30/60 (50.0%) *vs* LC 11/42 (26.2%)]	0.024	Kim *et al*[22]
		P38S [HCC 13/60 (21.7%) *vs* C 2/41 (4.9%)]	0.023	Kim *et al*[22]
		H94Y [HCC 24/60 (40.0%) *vs* C 2/41 (4.9%)]	< 0.001	Kim *et al*[22]
		I127T/N [HCC 22/60 (36.7%) *vs* CH 6/41 (14.6%)]	0.023	Kim *et al*[22]
		KV130MI [HCC 52/60 (86.7%) *vs* CH 25/41 (61.0%)]	0.004	Kim *et al*[22]

HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; CH: Chronic hepatitis; C: Carrier.

patients) *vs* 38.1% (8/21 patients)], which suggests that the preS1 and preS2 deletions cause different patterns of disease progression, at least in Korean chronic patients[16]. Furthermore, a discrepancy between the two deletion groups according to hepatitis B e antigen (HBeAg) serostatus was also observed. While the preS1 deletion was not related to the HBeAg serostatus (HBeAg negative *vs* HBeAg positive; 21.3% *vs* 18.6%), the frequency of preS2 deletions was positively related to the HBeAg negative serostatus (HBeAg negative *vs* HBeAg positive; 23% *vs* 6.8%, $P = 0.02$), which implies that preS2 may be more sensitive to the host immune response than preS1[16].

A total of four types of specific mutations in the preS region, *i.e.*, two types in the preS1 region (preS1 start codon deletion[16] and W4P/R mutation[9]) and two types in the preS2 region (preS2 deletion[16] and F141L mutation[15]), were related to disease progression in Korean chronic patients (Table 1). The deletion type of the preS1 start codon, which leads to the deletion of 11 amino acids at the N-terminus of the large surface protein characteristic of genotype D, exhibited a very high prevalence in HCC patients [22.5% (9/40) HCC *vs* 5.3% (1/38) asymptomatic carriers, $P = 0.048$][16]. It has been reported that some genotype D strains lead to intracellular retention of surface proteins in mixed infections with genotype A, which could induce hepatic carcinogenesis through activating the ER stress pathway[36]. Although the exact mechanism remains to be elucidated, the possible cause of HCC in the deletions of the preS1 start codon might be similar to the genotype D case described above. This deletion type was also found in two of three Korean HCC patients in a previous report[21], but it has rarely been found in chronic patients from other countries. Therefore, the possibility that the deletion of the preS1 start codon might be prevalent among Korean patients cannot be excluded. Our recent report[10] that the preS1 start deletion was found with a high frequency in Korean patients related to HBV occult infection also strongly supports this hypothesis.

Furthermore, our recent molecular epidemiologic study based on real time PCR introduced novel preS1 substitutions (W4P/R) that were significantly related to severe liver diseases in Korean chronic patients infected with genotype C [HCC and liver cirrhosis (12.4%, 19/153 patients) *vs* chronic hepatitis and carrier (1.1%, 1/94 patients), $P < 0.001$], which changes the tryptophan to proline or arginine at the 4th codon from the preS1 start. Surprisingly, all W4P/R mutants (20 patients) were found in male patients only, which implies that the W4P/R mutation may occur predominantly in males[9]. Therefore, our study led to the conclusion that W4P/R may make an important contribution to the disease severity in male chronic patients infected with genotype C. It may also provide a partial explanation as to the relatively high ratio of male to female incidence of HCC generation in Korean chronic patients. To our knowledge, W4P/R is the first mutation of the virus gene associated with gender disparity[9].

Our previous molecular epidemiologic study based on the Mbo Ⅱ PCR restriction fragment length polymorphism analysis method proved that two types of preS2 mutations, *i.e.*, the preS2 deletion and F141L mutation, were significantly related to severe forms of liver diseases in Korean chronic patients[15]. Of these two mutations, several lines of evidence have already suggested that the preS2 deletion correlates with more progressive forms of liver disease through affecting the ratio between the small and large envelop proteins, which results in the ER stress associated with the aggravation of liver disease[37]. The relationship of the F141L mutation to disease progression was first introduced by our study. Our data demonstrated that F141L mutations, but not preS2 deletion, are significantly prevalent in HCC patients compared with patients with any other stage of liver disease and even LC patients, which suggests that F141L and preS2 deletion affect different stages in the progression of liver disease[15]. While pre-S2 deletions may have a function in the transition from chronic hepatitis to liver cirrhosis, the F141L mutation have

a pivotal function in the progress from liver cirrhosis to HCC. Using a functional study based on the stable cell lines, we proved that large surface proteins with the F141L mutation could contribute to the pathogenesis of HCC through the induction of cell proliferation and transformation[15].

It should also be noted that two types of preS deletion related to disease progression in Korean chronic patients, i.e., the deletion in preS1 start codon and preS2 deletion, were also significantly prevalent in HBV occult subjects compared with chronic patients at the carrier stage[10], which indicates that they may induce a mechanism such as the defect in the secretion of virions or HBsAg leading to ER stress, which can explain both occult infection and disease progression.

MUTATION FREQUENCY AND PATTERNS IN THE S REGION IN KOREAN CHRONIC PATIENTS

The *S* gene of HBV contains a dominant neutralizing epitope, termed the "a" determinant, in the major hydrophilic region (MHR) of the *S* gene, which spans amino acid positions 100-160. The "a" determinant is widely regarded to be located between amino acids 124 and 147 of HBsAg[38]. Mutations in MHR, particularly the "a" determinant, are known to be associated with the generation of vaccine escape variants or persistent infection by reducing the binding affinity between the HBsAg and antibody to the HBsAg[39]. It is important that the prevalence and types of variants of the *S* gene found in endemic populations should be monitored, because this will affect policy decisions relating to vaccine and diagnostic reagents design[40,41]. When compared with previous results obtained in Japanese patients with genotype C by Ogura *et al*[42], our previous report demonstrated that there were several distinct epidemiologic traits in the prevalence of S variants in Korean chronic patients. First, an unexpectedly higher prevalence of naturally occurring MHR variants (46.5%, 47/101) was observed in the Korean patients compared with 24% prevalence in Japanese patients. Second, a relatively higher mutation frequency (37.3%, 22/59) and unique mutation patterns were observed in positions outside the "a" determinant region, while most mutations in Japanese patients were concentrated inside the "a" determinant region[20]. These traits observed in Korean patients may result from the exclusive predominance of the genotype C prone to mutations, which could influence the virological aspects of the HBV populations in the region[43]. Furthermore, the possibility of the presence of distinct immune pressures against HBV antigens in Korean patients cannot be excluded, and these could induce the distinct HBV mutation patterns that are not encountered in other areas. Our recent report regarding HBV occult infections demonstrating the presence of several novel HBsAg variants related to occult infections in Korean patients strongly supports this hypothesis[10].

Recently, we introduced a novel mutation type in the outside MHC regions of *S* gene (*sW182**) that resulted in a premature stop at codon 182 in the *S* gene of genotype C[12]. Our molecular epidemiologic study based on a multi-probe real time PCR proved that the prevalence of sW182* was significantly higher in patients with progressive forms of the disease (HCC and liver cirrhosis) than in patients with less severe forms of the disease (chronic hepatitis and carrier) [31.8% (56/176 patients) *vs* 17.2% (17/99 patients), $P = 0.010$][12] (Table 1). Furthermore, an *in vitro* study based on the stable cell lines stable expressing the S protein with sW182* also strongly supported its relationship with HCC[12]. Interestingly, sW182* has been reported to be the most frequently encountered mutation among occult infections related to HBsAg mutations in Korean patients, which suggests the possibility of its horizontal transfer among the Korean population[10]. Comparison of the clinical data between patients with and without the *sW182** mutation demonstrated that the HBV DNA levels of patients with variants were significantly lower than those with wild types, which indicates that there may be mechanisms that lower the DNA level itself. One potential mechanism is that the truncated S protein could interrupt the formation of normal virions, leading to a loss of infectivity and, in turn, DNA levels. The study using the full HBV genomic DNA harboring the *sW182** mutation proved that it failed to form normal HBV virions, which also provided a likely explanation as to its prevalence in subjects with HBV occult infections as well as patients with severe types of liver diseases[12].

MUTATION FREQUENCY AND PATTERNS IN THE PREC/C REGION IN KOREAN CHRONIC PATIENTS

The HBV C protein (HBcAg), which is the protein shell of the virus core, is 183 residues long, of which 149 residues of the N-terminal are the assembly domain[44]. HBcAg is the principal target for the host immune response, particularly cytotoxic T lymphocyte attacks, in which non-synonymous mutations may lead to the production of immune escape variants, resulting in the persistence of HBV[45,46]. Moreover, since the mutation of HBcAg can lead to simultaneous mutations in HBeAg, which is a key HBV immune-regulatory protein, and can profoundly affect the natural course of HBV chronic infection[47].

Our previous report regarding the mutations in the precore/core (preC/C) region from 70 Korean chronic patients led to several significant results. First, a positive relationship between the preC/C mutation frequency and old age [wild type (36.9) *vs* mutation (51.9), $P = 0.001$] was found[11], which indicates that the accumulation of preC/C mutations during the natural course of chronic hepatitis B contributes to the persistent infection of HBV in areas where vertical infection is predominant. Second, the preC/C mutations were found more frequently in immuno-active regions than in immuno-inactive regions

(2.2% *vs* 1.7%, $P = 0.016$)[11], which implies that the host immune pressure at the T cell level is the significant driving force of the preC/C mutations[48,49]. Notably, a significant higher level of mutation rates in the major histocompatibility complex (MHC) class Ⅱ restricted region (2.3% *vs* 1.7%, $P = 0.009$), but not in the MHC class I restricted region, was found when compared with the immuno-inactive region, which indicates that former, *i.e.*, the target of the CD4 T helper cell, is more prone to mutations induced by the host immune response than the latter, *i.e.*, the target of the CD8 cytotoxic T cell[11]. Third, five mutations in the C region (C-P5H/L/T, C-E83D, CI97F/L, C-L100I, and C-Q182K/*) and one in the preC (preC-W28*), which is known to be a HCC-related preC mutation at nucleotide 1896 (G→A)[17,50], were found to be related to HCC patients compared with patients in other stages of the disease[11] (Table 1). It should be noted that four of the five HCC-related C mutations, *i.e.*, C-P5H/L/T, C-E83D, C-I97F/L, and C-L100I, were located in the MHC class Ⅱ restricted regions (one at aa 1-20 and three at aa 81-105), which implies that evasion of the CD4 T cell-mediated immune response, primarily through mutations in the "hot spot" region of aa residue 81-105, has a function in the hepatocarcinogenesis of chronic patients infected with genotype C[11]. Of the five HCC-related mutations in the C region, two types (C-L100I and C-Q182K/*) were introduced for the first time in that study[11]. Collectively, our data indicates that the HBV variants in the C region, particularly in the MHC class Ⅱ restricted regions, may contribute to the HCC progress in chronic patients infected with genotype C via immune evasion of the CD4 T cell-mediated immune response. It also implies the presence of a distinct immune pressure at the CD4 T cell level against HBcAg in the Korean population, which results in contributing to the HBV persistent infections via the generation of immune evading HBcAg variants[11].

MUTATION FREQUENCY AND PATTERNS IN THE X REGION IN KOREAN CHRONIC PATIENTS

HBV X protein (HBx) has been the focus of significant attention in recent years because it is strongly implicated in hepatocarcinogenesis. It is a 154-amino acid protein with an N-terminal negative regulatory domain and a C-terminal transactivation domain. The HBx protein is multifunctional and affects gene transcription, signaling pathways, genotoxic stress responses, cell-cycle control, and apoptosis; it also has an essential function in viral replication[51,52]. Several reports have demonstrated that specific point mutations in the HBx gene are related to severe forms of liver disease, such as cirrhosis of the liver and/or HCC[22,53]. In addition, deletions, especially COOH terminal truncations or insertions, have also been frequently detected in tissues and sera samples in HCC patients[14].

Our previous report using a cohort of 267 Korean patients demonstrated that the prevalence of deletions or insertions in the X region was significantly higher in patients with severe liver disease, HCC, or cirrhosis of the liver (7.2%, 10/132) compared with patients who were carriers or had chronic hepatitis (1.5%, 2/135) ($P = 0.017$)[14], which implies that the deletions or insertions in the X region may contribute to disease progression in Korean patients with genotype C infection.

Our other report regarding mutations in the X regions from a cohort of 184 Korean patients demonstrated that a total of five mutation types (V5M/L, P38S, H94Y, I127T/N, and K130M and V131I) affecting the six codons were related to clinical severity[22] (Table 1). Several noteworthy findings regarding these mutations are as follows. First, the V5M/L mutation first discovered during the present study was always significantly more frequent in HCC patients than in other patients, even patients with liver cirrhosis (Table 1) [HCC (50%) *vs* liver cirrhosis (26.2%), $P = 0.024$][22], which implies that it has a pivotal function in the progression from liver cirrhosis to HCC. Second, three mutations (H94Y, I127V/I, and K130 and V131) were also related to mutational "hot spots" of the overlapped enhancer II (H94Y: C→T of nt 1653) or BCP (I127T/N:T→V of nt 1753, K130M and V131I: A →T of nt 1762 and G→A of nt 1764). In particular, the double mutations of K130 and V131 overlapped in the BCP mutations were observed with the highest frequency (66.1%, 123/184 patients), which strongly supports the previous result of the very high prevalence of BCP in Korean chronic patients[22]. The recent study using a large cohort of Taiwanese showed that the prevalence of the BCP double mutation was significantly higher in patients infected with HBV genotype C than in those infected with HBV genotype B (43.0% *vs* 21.4%; $P < 0.001$)[54]. But, even the prevalence of BCP double mutation in genotype C infected Taiwanese (43.0%) is lower than that in genotype C infected Korean (66.1%).

Third, two types of mutation, *i.e.*, V5M/L [HBeAg negative (40%) *vs* HBeAg positive (19.1%), $P = 0.004$] and H94Y [HBeAg negative (30.4%) *vs* HBeAg positive (22.6%), $P = 0.087$], were related significantly and nearly significantly to HBeAg negative serostatus, respectively[22]. Fourth, three mutation types, *i.e.*, V5M/L [K130M and V131I (36.6%) *vs* no mutation (8.2%), $P < 0.001$], H94Y [K130M and V131I (35%) *vs* no mutation (6.6%), $P < 0.001$], and I127T/N [K130M and V131I (31.7%) vs no mutation (11.5%), $P = 0.003$], were strongly related to the BCP mutations[22]. This implies that the subsequent substitutions in specific codons of the X region following BCP double mutations may have a pivotal function in the progression of liver disease, at least in Korean chronic patients. Our previous report that the deletions of long lengths and amino acid substitutions followed by BCP double mutations[19] might contribute to the diversity of HBV quasispecies strongly supports this hypothesis.

In summary, our data suggest that an accumulation of mutations in the X region, in particular the subsequent

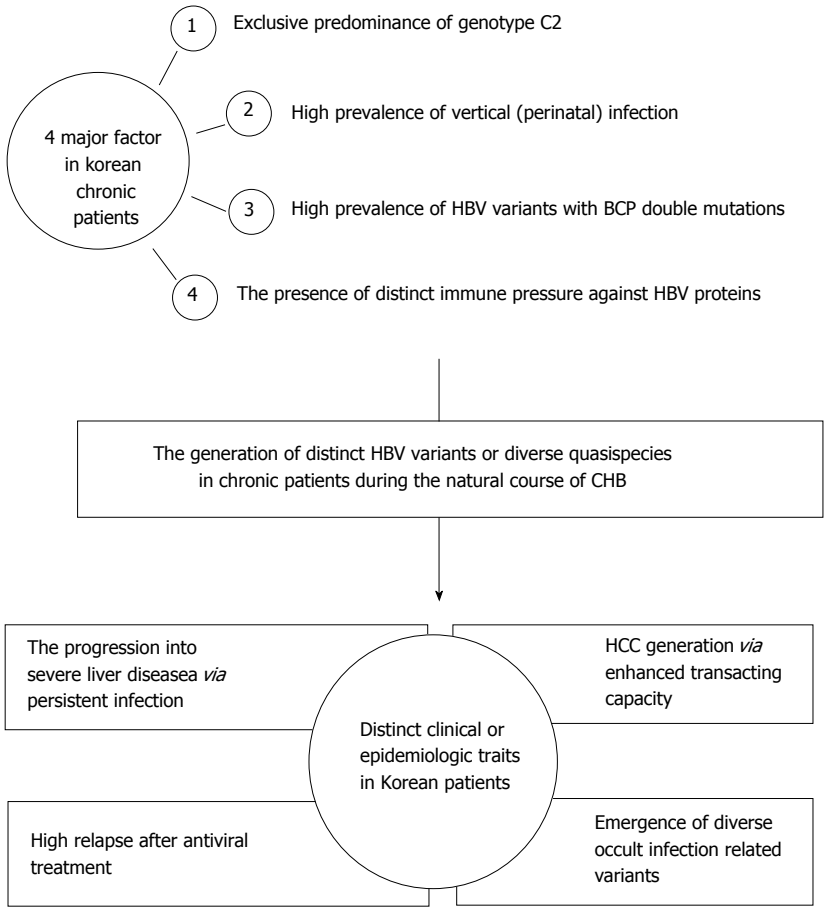

Figure 1 The 4 major factors in Korean chronic patients leading to distinct clinical or epidemiologic traits for hepatitis B virus. Combinatorial effect of 4 major factors (1) exclusive predominance of genotype C2; (2) high prevalence of perinatal infection; (3) high prevalence of basic core promoter mutations; and (4) the presence of distinct immune pressure) could generate distinct hepatitis B virus (HBV) variants or diverse quasispecies during the natural course of chronic hepatitis B, resultantly leading to distinct clinical or epidemiological traits in Korean chronic patients. HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.

mutations in specific codons following the BCP double mutations, contributes to disease progression in Korean patients with chronic genotype C infections (Figure 1).

CONCLUSION

Several reports regarding HBV mutations from Korean chronic patients have led to the conclusion that the combination of four main factors, *i.e.*, the exclusive predominance of only genotype C2 prone to mutations[8], the predominance of a perinatal infection route providing sufficient time for the generation of variants[55], the high prevalence of BCP double mutations leading to subsequent mutations with high frequency[19,22,50], and the presence of distinct immune responses against HBV in Korean population[11], may lead to the generation of the distinct HBV variants rarely or not encountered in other areas in the course of CHB (Figure 1). The production of HBV variants may contribute to clinical or epidemiologic manifestations that are distinct in Korean chronic patients. First, it could contribute to the progression into severe types of liver disease through persistent infection by evading host immune responses[9-22]. Second, several types of mutation such as sW182* in the *S* gene[12] and F141L in the preS1[15] could contribute to the HCC generation via enhanced transacting capacity. This provides a potential explanation for the high relapse after antiviral treatments being observed in Korean chronic patients compared with other countries[6]. Finally, it may be responsible for the generation of highly diverse HBV variants related to occult infections that have been observed in Korean patients[10] (Figure 1).

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Evolution of hepatitis B management in kidney transplantation

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Abstract

Chronic hepatitis B virus (HBV) infection adversely influences the clinical outcomes of renal transplant recipients owing to increased hepatic complications. Management of HBV infection in kidney transplant recipients presents a challenge to clinicians, especially in endemic regions. Interferon precipitates renal allograft dysfunction. Treatment with lamivudine, the first oral nucleoside analogue available, resulted in effective viral suppression, reduced liver-related complications, and improved patient survival so that medium-term data showed comparable patient survival rates between hepatitis B surface antigen-positive and HBsAg-negative kidney transplant recipients in the era of effective antiviral therapies. Entecavir has replaced lamivudine as first-line therapy for treatment-naïve subjects in view of the propensity for drug resistance with the latter. Management of HBV infection in kidney transplant patients needs to take into consideration the nephrotoxicity of nucleoside/tide analogues such as adefovir and tenofovir. Prevention of HBV-related complications in kidney transplant recipients starts much earlier prior to transplantation, with vaccination of patients with chronic kidney disease and donor-recipient matching with regard to HBV status. In addition to anti-viral treatment, patients with chronic HBV infection must have regular surveillance for liver cancer and assessment for the development of cirrhosis.

Key words: Hepatitis B; Kidney transplantation

Core tip: Treatment with oral nucleoside/tide analogues brought a new paradigm in the management of hepatitis B surface antigen-positive kidney transplant recipients, resulting in effective viral suppression, reduced hepatic complications, and improved patient survival, without compromising renal allograft outcome. Entecavir has replaced lamivudine as first-line therapy for treatment-naïve subjects given the propensity of lamivudine for selecting resistance. Due to the nephrotoxicity of adefovir and tenofovir, the optimal management of drug-resistant hepatitis B virus (HBV) remains to be defined. Other important measures to prevent HBV-related complications in renal transplant patients include early vaccination in non-immune subjects, donor-recipient matching of HBV status, and surveillance for liver cancer and cirrhosis.

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INTRODUCTION

Hepatitis B virus (HBV) infection confers a significantly negative impact on the clinical outcomes of kidney allograft recipients. The inferior patient survival in hepatitis B surface antigen-positive ($HBsAg^+$) renal transplant patients compared with the HBsAg-negative counterparts

is attributed to increased hepatic complications such as chronic hepatitis and cirrhosis, fibrosing cholestatic hepatitis, and hepatocellular carcinoma[1-10]. Prevention and management of HBV infection in patients with renal failure is a major issue in endemic regions such as Asia, when the HBV carrier rate in the general population can exceed 10%. The reported prevalence of HBV infection among dialysis patients in the United States is often below 1.0%, whereas the prevalence rate is between 7.0% and 15% in the Asian-Pacific region[11-13]. While the incidence of HBV infection among dialysis patients has declined significantly over the past three decades because of widespread implementation of infection control measures, reduced need for transfusion and adherence to safe transfusion practices, and the introduction of HBV vaccination to neonates in many countries, over 350 million subjects worldwide carry HBV and thus it will remain a significant clinical issue for some time[14]. In this context, a considerable number of HBsAg-positive patients will undergo kidney transplantation[15].

PREVENTION OF *DE NOVO* HBV INFECTION IN RENAL TRANSPLANT RECIPIENTS

An effective immunization program in dialysis and chronic kidney disease patients is the cornerstone to prevent de novo HBV infection in renal transplant recipients. HBV vaccination should be given early in the course of chronic kidney disease owing to the relatively poor response in patients with significant renal impairment[16]. In the dialysis population, higher doses of vaccine are recommended, with post-vaccination and subsequent annual testing and booster administration if anti-HBs titer falls below 10 IU/L[17]. Intra-dermal injection may be considered in non-responders to enhance the vaccination efficacy[18]. The donor-recipient matching with regard to their HBV serological status significantly affects the risk of *de novo* HBV infection post-transplant. One must not transplant an HBsAg-positive allograft into a recipient who is negative for both HBsAg and anti-HBs, or de novo infection would occur and the course is often aggressive[19]. The risk of HBV transmission from HBsAg-negative anti-HBc positive donors to HBsAg-negative recipients is low, and the risk is even lower if the recipient is anti-HBs positive[20]. Accumulating experience suggests that it is safe to transplant an HBsAg-positive kidney to an HBsAg-negative recipient who has anti-HBs antibody under HBV immunoglobulin cover[21].

CLINICAL OUTCOMES OF HBSAG-POSITIVE RENAL TRANSPLANT RECIPIENTS IN THE PRE-ANTIVIRAL NUCLEOSIDE/TIDE ANALOGUE ERA

The clinical manifestations and diagnosis of HBV infection in kidney transplant recipients are generally similar to patients without renal disease, but due to the immunosuppressed state these individuals are more susceptible to progressive liver disease and severe life-threatening complications like fibrosing cholestatic hepatitis[10]. The significantly inferior survival of HBsAg-positive kidney transplant recipients in the "no-treatment" era was regarded unavoidable, and much of the mortality occurred relatively early, due to severe progressive liver disease[2-7,9]. In a meta-analysis of six observational studies, HBsAg-positivity was associated with a 2.49-fold risk of death after renal transplantation[22]. Liver-related complications were significantly increased in subjects with detectable serum HBV DNA or were HBeAg-positive[23]. The 10-and 20-year patient survival rates in HBsAg-positive kidney transplant recipients without anti-viral therapy were 85% and 71% respectively (*vs* 98% and 95% at 10 and 20 years in HBsAg-negative patients)[24].

Before the availability of oral nucleoside/tide analogues, chronic HBV infection was managed with interferon therapy. Interferons offer the advantage of sustained response with a finite duration of therapy in both HBeAg-positive and HBeAg-negative patients[25]. However, there has been data suggesting that the efficacy of interferon might be lower in endemic regions where most patients contract the infection during infancy, compared to non-endemic areas where the infection is contracted during adulthood[26,27]. Moreover, interferon should be avoided in kidney allograft recipients as it commonly precipitates allograft dysfunction and rejection[28,29], although one study suggested that interferon treatment might not be associated with acute rejection in HCV-positive kidney transplant recipients with low rejection risk[30]. With the advent of oral nucleoside/tide analogues which suppress HBV replication effectively, there was a dramatic change in the clinical course of HBsAg-positive kidney transplant recipients and a new paradigm of therapeutic management.

IMPACT OF NUCLEOSIDE/TIDE ANALOGUE THERAPY ON THE OUTCOMES OF HBSAG-POSITIVE RENAL TRANSPLANT RECIPIENTS

The current options of nucleoside/tide analogues include lamivudine, entecavir, telbivudine, adefovir and tenofovir (Table 1). The objective of treatment is to prevent HBV-related complications in these immunosuppressed individuals, and the indication to start treatment is based on the commencement of immunosuppressive therapy (the "prophylactic" approach) or the evidence of impending HBV reactivation (the "pre-emptive" approach). Due to a paucity of data, the optimal duration of antiviral treatmentin HBsAg-positive kidney transplant recipients remains undefined. Preliminary experience suggests that while most patients would require lifelong anti-viral suppression discontinuation may be cautiously attempted

Table 1 The major clinical trials regarding the use of oral nucleoside/tides for HBsAg-positive kidney transplant recipients

Oral Nucleoside/tides	Study design	*n*	Major treatment outcomes
Lamivudine			
Rostaing *et al*[32] (1997)	Prospective	6	LAM as initial Rx → ALT normalization and HBV DNA undetectability in 4/6 patients
Chan *et al*[1] (2002)	Prospective	11	LAM as initial Rx → ALT normalization and HBV DNA undetectability in all patients; e-seroconversionrate (21.4%); markedly improved patient survival when compared to historical controls who had no anti-viral Rx ($P < 0.001$)
Fabrizi *et al*[33] (2004)	Meta-analysis	184	LAM as initial Rx → HBV-DNA undetectability [91% (95%CI: 86%-96%)], ALT normalization [81% (95%CI: 70%-92%)] and LAM-resistance [18% (95%CI: 10%-37%)] after 12 mo; e-seroconversion rate (0%-46%) in 4 trials
Thabut *et al*[34] (2004)	Prospective	14	LAM as initial Rx → HBV undetectability (57%) andALT normalization (57%) after 3 mo; LAM-resistance (57%) after median of 15 mo
Filik *et al*[31] (2006)	Prospective	15	LAM as initial Rx → HBV DNA undetectability (46.7%) after 2 yr
Yap *et al*[24] (2010)	Retrospective	38	LAM as initial Rx → LAM-resistance (64%) after 4 yr; improved long-term patient survival (83% *vs* 34% at 20-yr, $P = 0.006$) when compared to historical controls who had no anti-viral Rx
Adefovir			
Fontaine *et al*[37] (2005)	Prospective	11	ADV as mono-therapy for LAM-resistant KTR → 5 log ↓ HBV DNA after 1 yr, only 1 patient had transient deterioration of allograft function
Kamar *et al*[40] (2009)	Prospective	11	ADV for LAM-resistant KTR → significant ↓ in HBV DNA ($P = 0.01$) and ALT normalization after 12 mo, ↑serum creatinine and proteinuria after 24 mo ($P = 0.02$)
Tse *et al*[43] (2010)	Retrospective	4	ADV for LAM-resistant KTR → 4 log ↓ HBV DNA and significant ↓ ALT levels ($P = 0.029$) after 18 mo, no significant change in allograft function
Lampertico *et al*[41] (2011)	Prospective	11	ADV as add-on Rx to LAM for LAM-resistant KTR → HBV undetectability (88%) after 3 yr; no significant changes in renal function and proteinuria
Lai *et al*[42] (2012)	Retrospective	14	ADV as mono- ($n = 5$) or add-on ($n = 9$) therapy in LAM-resistant KTR → HBV DNA undetectability [5 (35.7%) and 6 (42.8%) patients]after 12 and 24 mo with no virological breakthrough; ALT normalization in 13 patients (92.8%) after 1 yr; moderate to severe renal insufficiency (29%)
Entecavir			
Kamar *et al*[48] (2008)	Prospective	10	ETV for ADV-resistant ($n = 9$) or LAM-resistant ($n = 1$) KTR → HBV DNA undetectability (50%) after 16.5 mo
Hu *et al*[47] (2012)	Prospective	27	ETV in KTR patients without LAM-resistance → HBV DNA undetectability (96% and 100%) after 12 and 24 mo, with no virological breakthrough
Tenofovir			
Daudé *et al*[52] (2011)	Prospective	3	TFV as mono-therapy → HBV DNA undetectability (43%); no changes in allograft function

ADV: Adefovir; ALT: Alanine transaminase; ETV: Entecavir; LAM: Lamivudine; KTR: Kidney transplant recipients; TFV: Tenofovir; HBV: Hepatitis B virus.

after stabilization, with success, in carefully selected low-risk patients[1].

Lamivudine

Since lamivudine is the first amongst this class of drugs available for clinical use, it has yielded the majority of data on the management of HBsAg-positive renal transplant recipients. Lamivudine given as either prophylactic or pre-emptive treatment was proven superior to salvage therapy when liver dysfunction is evident[11,31]. Data from our group and other investigators have demonstrated that lamivudine was effective in suppressing HBV DNA and improving liver transaminase levels[1,22,32]. A meta-analysis which pooled data from 14 prospective clinical trials (a total of 184 patients) supported these observations[33]. With lamivudine as initial treatment, the mean rate of effective HBV DNA suppression, HBeAg clearance, alanine transaminase (ALT) normalization, and lamivudine-resistance was 91% (95%CI: 86%-96%), 27% (95%CI: 16%-39%), 81% (95%CI: 70%-92%), and 18% (95%CI: 10%-37%) respectively after a mean duration of 14 mo. The frequency of HBeAg seroconversion and lamivudine resistance correlated positively with treatment duration. Most importantly, treatment with lamivudine was associated with significantly improved patient survival[1,10,24]. With the use of lamivudine, the 10-year patient survival rate in HBsAg-positive renal transplant recipients was 81% and such results were nearly comparable to HBsAg-negative patients[24]. Although antiviral treatment has led to reduced mortality as a result of decreased hepatic complications ($P = 0.036$), liver-related deaths still accounted for 40% of mortalities in HBsAg-positive patients in the era of effective antiviral therapies, and 22.2% of all deaths that occurred in patients who had received antiviral treatment[24]. Prolonged treatment with lamivudine is associated with progressive increase in drug resistance and the cumulative probability of developing lamivudine-resistance was approximately 60% after 69 mo[24,33-35]. The emergence of lamivudine resistance can be associated with liver dysfunction, although one recent study showed that drug resistance did not have a significant negative impact on liver stiffness score, rate of HBeAg seroconversion rate, incidence of liver failure or hepatocellular carcinoma, or patient survival over 10-14 years of follow-up when rescue antiviral therapies are available[24].

Adefovir

Adefovir has similar activity against both wild-type and lamivudine-resistant HBV, this drug is nephrotoxic and the major clinical application of this antiviral agent is for the management of lamivudine-resistance[36]. Data regarding the management of lamivudine-resistance in kid-

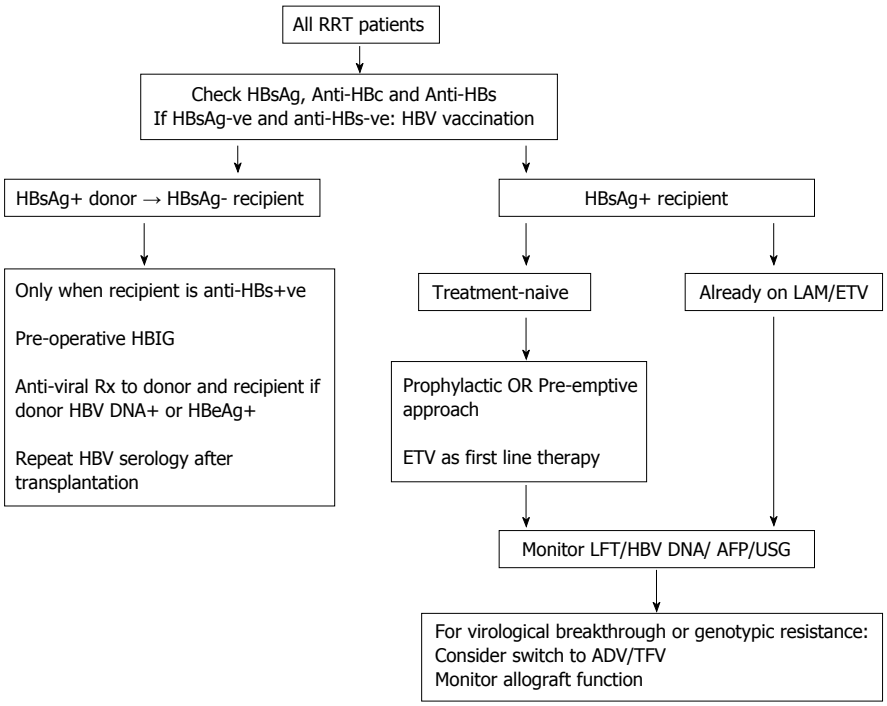

Figure 1 Management algorithm of hepatitis B virus infection in renal transplant recipients. ADV: Adefovir; AFP: Alpha-fetoprotein; ETV: Entecavir; HBIG: Hepatitis B hyperimmune globulin; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; LAM: Lamivudine; LFT: Liver function test; RRT: Renal replacement therapy; TFV: Tenofovir; USG: Ultrasonography.

ney transplant recipients is relatively limited[37-39]. Prior to the availability of alternative nucleoside/tide analogues, it was the usual practice to continue lamivudine in patients who had developed lamivudine-resistance. Since the introduction of adefovir, there have been reports on its short-term efficacy either as mono- or add-on therapy in kidney transplant recipients[37-42]. The study by Fontaine *et al*[37] examined the use of adefovir as monotherapy in 11 post-kidney transplant patients with dosage adjustment according to renal function. Adefovir treatment led to a significant decline in serum HBV DNA with no virological breakthrough at one year and the drug was well-tolerated. Others have reported that adefovir as add-on therapy to lamivudine resulted in undetectable HBV DNA levels in 35.7%, 42.8% and 88.0% of lamivudine-resistant renal transplant recipients after 12, 24 and 36 mo[41,42]. There was no virological breakthrough and normalization of ALT was achieved in 92.8% of patients after 12 mo of treatment[42]. However, the virological response could be variable and relatively slow when compared with treatment-naïve subjects[43]. Nevertheless, rescue therapy with adefovir resulted in significantly better viral suppression and liver biochemistry compared with continuation of lamivudine (75% *vs* 14.3% had persistent normalization of ALT), and the clinical response was sustained for at least 24 mo[24]. Evidence of nephrotoxicity was observed in 30%-50% of renal allograft recipients despite dosage adjustment, and could necessitate treatment discontinuation[41,42]. In our experience, using adefovir in patients with serum creatinine below 150 μmol/L or creatinine clearances above 40 mL/min appeared safe, without evidence of worsening of renal allograft function during follow-up[24]. However, one must appreciate that the antiviral activity of adefovir at the currently approved dose is relatively weak, and efficacy could be further reduced with dose adjustment according to renal dysfunction.

Entecavir, tenofovir and telbivudine

Entecavir is effective in both treatment-naïve and lamivudine-resistant patients[44,45]. In immunosuppressed treatment-naïve post-renal transplant patients who required prolonged antiviral administration, entecavir is preferred due to its high resistance barrier and favorable safety profile[44,46]. A recent 2-year prospective study showed that the use of entecavir in treatment-naïve renal transplant recipients resulted in undetectable HBV DNA levels in 70%, 74%, 96% and 100% of patients after 12, 24, 52 and 104 wk respectively[47]. In this study, entecavir was associated with a more potent response than lamivudine and the tolerability profile was favorable. Experience regarding the use of entecavir in renal transplant recipients who had developed lamivudine- or adefovir-resistance had been examined in a small study with 10 solid organ transplant recipients (8 kidney allograft recipients)[48]. Treatment with entecavir resulted in an appreciable drop in HBV DNA levels and a 50% HBV undetectability in both HBeAg-positive and HBeAg-negative patients after 16.5 mo of therapy. Previously we had also reported the efficacy and tolerability of entecavir in lamivudine-resistant kidney allograft recipients, and showed that the virological response could be variable and relatively slower compared with treatment-naïve subjects[24,43]. Thus

the response to entecavir in lamivudine-resistant subjects, and the subsequent emergence of entecavir-resistance, should be carefully monitored[49].

Tenofovir shows high efficacy in the treatment of treatment-naïve or lamivudine-resistant HBV infection[45,50]. There is little data in the renal transplant setting, and there is concern on its potential nephrotoxicity[51]. Daudé *et al*[52] reported the favorable short-term virological response and renal function stability in 7 solid organ transplant recipients (3 kidney allograft recipients) with a follow-up of 12 mo. Larger studies with longer follow-up duration are warranted to ascertain the long-term efficacy and effect on kidney allograft function. There is currently no data on the use of telbivudine in renal transplant recipients but it would be worthwhile to explore the use of this agent in treatment-naïve kidney allograft recipients given its relatively low resistance rate, lack of nephrotoxicity, and the relatively lower cost compared with other nucleoside/tide analogues[53,54].

CONCLUSION

The outcome and management of HBsAg-positive kidney transplant recipients have changed dramatically over the past few decades (Figure 1). Prior to the advent of effective and safe therapy, HBV infection had such a severe negative impact on patient survival that some centres regarded HBsAg sero-positivity as a contraindication against kidney transplantation. In the era of effective nucleoside/tide analogue therapy the 8-10 year survival rate of HBsAg-positive kidney transplant recipients is approaching that of HBsAg-negative subjects. The access to optimal therapy is limited by the cost of drugs in some places, unfortunately often in endemic regions when the treatment is needed most. The management of patients with drug resistant HBV infection remains a challenge, as is the nephrotoxic impact of some effective anti-viral agents. Apart from the treatment of HBV infection with anti-viral agents, the importance of regular surveillance for liver complications cannot be over-emphasized. In this regard, the data clearly shows that early detection of liver tumour with ultrasound and alpha-fetoprotein level measurement markedly increases the resection rate and patient survival[55-57].

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REVIEW

Limitations of liver biopsy and non-invasive diagnostic tests for the diagnosis of nonalcoholic fatty liver disease/ nonalcoholic steatohepatitis

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Abstract

It is estimated that 30% of the adult population in Japan is affected by nonalcoholic fatty liver disease (NAFLD). Fatty changes of the liver are generally diagnosed using imaging methods such as abdominal ultrasonography (US) and computed tomography (CT), but the sensitivity of these imaging techniques is low in cases of mild steatosis. Alanine aminotransferase levels may be normal in some of these patients, warranting the necessity to establish a set of parameters useful for detecting NAFLD, and the more severe form of the disease, nonalcoholic steatohepatitis (NASH). Although liver biopsy is currently the gold standard for diagnosing progressive NASH, it has many drawbacks, such as sampling error, cost, and risk of complications. Furthermore, it is not realistic to perform liver biopsies on all NAFLD patients. Diagnosis of NASH using various biomarkers, scoring systems and imaging methods, such as elastography, has recently been attempted. The NAFIC score, calculated from the levels of ferritin, fasting insulin, and type Ⅳ collagen 7S, is useful for the diagnosis of NASH, while the NAFLD fibrosis score and the FIB-4 index are useful for excluding NASH in cases of advanced fibrosis. This article reviews the limitations and merits of liver biopsy and noninvasive diagnostic tests in the diagnosis of NAFLD/NASH.

Key words: Nonalcoholic fatty liver disease; Liver biopsy; Steatosis; Fibrosis; Nonalcoholic steatohepatitis

Core tip: Liver biopsies remain a gold standard, although the procedure has several limitations for the diagnosis of nonalcoholic steatohepatitis (NASH). The NAFIC score, calculated from the levels of ferritin, fasting insulin and type Ⅳ collagen 7S, is useful for diagnosing NASH, while the nonalcoholic fatty liver disease fibrosis score and the FIB-4 index are useful for excluding NASH in cases of advanced fibrosis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent form of chronic liver disease in the world. According to a cooperative study group comprised of 10 institutions in Japan [Japan Study Group of NAFLD (JSG-NAFLD)], 29.7% of health checkup examinees (41.0%

of men and 17.7% of women) had NAFLD[1], making it a major national disease of the 21st century. The long-term outcomes of NAFLD patients have been reported in several studies. Compared with matched control populations, NAFLD patients have an increased overall mortality, with the most common cause of death being cardiovascular disease (28% of total deaths). In addition, there is an increased risk of death from a variety of extrahepatic malignancies (25% of total deaths) and from liver disease (13% of total deaths), which is the third leading cause of death for these patients and the eleventh leading cause in the general population[2]. NAFLD can be classified as either nonalcoholic steatohepatitis (NASH) or simple steatosis. NASH carries a high risk of liver disease-related mortality such as deaths from hepatic cirrhosis and hepatocellular carcinoma. Simple steatosis, however, has a low risk of liver disease-related mortality. NASH can be differentiated from simple steatosis only by liver biopsy and is diagnosed when all of the following 3 criteria are met: (1) macrovesicular fatty change of hepatocytes; (2) inflammatory cell infiltration; and (3) ballooning degeneration of hepatocytes. However, liver biopsy is invasive, has drawbacks such as sampling error and cost and is not possible for all NAFLD patients. Thus, it is necessary to establish a method to efficiently detect progressive NASH in NAFLD patients to decrease liver disease-related mortality. This review summarizes the current limitations and problems of liver biopsy and noninvasive diagnostic methods for NAFLD/NASH in Japan and other countries and outlines future prospects for improved diagnostic practices.

NAFLD DIAGNOSIS

According to the latest guidelines established by the American Association for the Study of Liver Diseases (AASLD)[3], NAFLD is diagnosed when the following 4 criteria are met: (1) fatty change of the liver is observed by imaging or histologically; (2) no marked alcohol drinking habit is present (ethanol intake of < 210 g/wk for men and < 140 g/wk for women); (3) no presence of other factors inducing fatty change of the liver; and (4) no concomitant factors causing chronic liver disease are present. This section of the review focuses on diagnostic imaging methods and scoring systems for fatty change of the liver.

Usefulness and limitations of imaging methods in diagnosing fatty change of the liver

Simple, minimally invasive ultrasonography (US) is used for the imaging diagnosis of fatty liver in many cases. However, the sensitivity is low in mild cases with a fatty change of less than 20%-30%[4,5]. The dependency of the diagnosis on the subjective judgments of operators is also problematic[6]. Computed tomography (CT) is objective and capable of measuring the amount of visceral fat[6,7], but radiation exposure and cost are negative aspects of this methodology. Moreover, although fatty liver is diagnosed when the liver-to-spleen CT ratio (the L/S ratio) is below 0.9, the sensitivity is not high, and fatty liver cannot be ruled out even if the L/S ratio is 0.9 or higher[8]. Particularly, in cases of obesity and metabolic syndrome and in the absence of other factors inducing abnormal liver function, NAFLD/NASH should be considered even if fatty liver is not evident by imaging. It has been revealed that NAFLD/NASH is latently present in patients who are monitored for liver disorder of unknown causes. When liver biopsy was performed in 354 patients with abnormal liver function and in whom the disease could not be definitely diagnosed serologically, 64% had NAFLD[9]. In another study, liver biopsy was performed in 81 patients with chronic abnormal liver function of unknown cause, and simple steatosis and NASH were observed in 41 and 26 patients, respectively[10], suggesting the importance of performing liver biopsy. The severity of fatty change is not correlated with the advancement of fibrosis; rather, it decreases with the progression of fibrosis in NASH. Therefore, the grade of fatty change from imaging analysis should not be employed as an evaluation criterion for NAFLD severity. Magnetic resonance (MR) spectroscopy is reportedly the most accurate method for the quantification of fatty change[7,11-13], but currently, its use is limited to research.

The usefulness of US for the diagnosis of NAFLD is evaluated, to some extent, because of its simplicity. Recently, quantification of fatty change using US to supplement elastography has also occasionally been reported, and further development of this application is expected[14]. It is impossible to differentiate between NASH and simple steatosis using any imaging methods. At the same time, certain US and CT findings, such as irregularity of the liver surface, blunt margins of the liver, and splenomegaly, suggest the presence of chronic liver diseases, including NASH with advanced fibrosis, and can indicate the need for further attention. It has recently been reported that the differentiation between NASH and simple steatosis is possible using contrast-enhanced US[15].

Scoring systems for diagnosing fatty change of the liver

Because imaging has limited diagnostic value for NAFLD, as described above, the prediction of fatty change of the liver from general laboratory test values has been investigated. As shown in Table 1, various indices have been proposed, including the fatty liver index (FLI)[16], NAFLD liver fat score, hepatic steatosis index (HSI)[17], and Steato Test (ST)[18]. According to a report from Italy[19], FLI, calculated from the body mass index (BMI), waist circumference, and γ-glutamyl transferase (γGT) and triglyceride (TG) levels, is an independent risk factor for liver-related mortality. HSI, formulated based on data from approximately 10000 Korean patients, is a simple index calculated only from BMI, the aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR), sex, and the presence or absence of diabetes mellitus (DM)[17] Both the sensitivity and specificity of HSI are favorable compared to that observed in other scoring systems. A validation study involving Japanese patients is expected.

Table 1 Indexes for the prediction of liver steatosis

Index	Author (nation)	Paper (yr)	No. of subjects (fatty liver/ non-fatty liver)	Parameters	Cutoff values	Sensitivity	Specificity	AUROC	Diagnostic methods for hepatic steatosis
FLI	Bedogni (Italy)	*BMC Gastroenterology* (2006)	228/268	BMI, waist circumference, triglyceride, γGT	< 30 > 60	87.0% 61.0%	64.0% 86.0%	0.84	US
NAFLD liver fat score[1]	Kontronen (Finland)	*Gastroenterology* (2009)	470	MetS, type Ⅱ diabetes, IRI, AST, AST/ALT ratio	-0.64	86.0%	71.0%	0.87[2] 0.86[3]	MRS
HSI	Lee (South Korea)	*Dig Liver Dis* (2010)	5362/5362 (sex- and age-matched)	8 × AST/ALT ratio + BMI + (+ 2 for females, + 2 for diabetes)	< 30 > 36	93.1%	92.4%	0.81[2]	US
ST	Poynaud (France)	*Comp Hepatol* (2005)	744/140	12 parameters[4]	0.30 0.72	90%	90.0%	0.79[2] 0.80[3] 0.86[3] 0.72[3]	Biopsy
	Park (South Korea)	*Korean J Hepatol* (2011)	145/311	ALT/AST > 1.5 (= 1 point) γGT > 50 IU/L (= 1 point) TG > 150 mg/dL (= 1 point) BMI 23-24.9 (= 2 points) ⩾ 25 (= 3 points)	3	71.7%	75.9%	0.797	US
	Bajaj (India)	*Indian J Med Res* (2009)	39/82	IRI +1.6 × BMI + 1.9 × FPG	1.6	84.6%	76.0%	0.76	US

[1]PNPLA3 did not improve diagnostic accuracies; [2]Estimation group; [3]Validation group; [4]Alanine aminotransferase (ALT), α2-macroglobulin, apolipoprotein A-I, haptoglobin, total bilirubin, γ-glutamyl transferase (γGT), cholesterol, triglycerides, glucose, age, gender and body mass index (BMI). ROC: Receiver operating characteristics curve; US: Ultrasonography; MetS: Metabolic syndrome; MRS: Magnetic resonance spectroscopy, IRI: Immuno-reactive insulin; FPG: Fasting plasma glucose. FLI: Fatty liver index; HSI: Hepatic steatosis index; ST: Steato Test; AST: Aspartate aminotransferase; NAFLD: Nonalcoholic fatty liver disease; AUROC: Area under the receiver operating characteristic curve.

CURRENT STATUS AND PROBLEMS OF LIVER BIOPSY FOR DIAGNOSIS OF NASH: IS LIVER BIOPSY NECESSARY?

Pros and cons of liver biopsy for NAFLD

There is some controversy surrounding whether liver biopsy should be actively performed to make a definite diagnosis of NAFLD and its prognosis and to differentiate it from other diseases or if it should be avoided as much as possible[20]. Liver biopsy is essential to the definite diagnosis of NASH and is considered very useful in differentiating NASH from other diseases, making a prognosis, and judging the effects of therapeutic intervention. However, liver biopsy is inefficient in many non-advanced cases and has several drawbacks, such as sampling error and high cost, as described below. Furthermore, pathologists differ in their diagnosis and recognition of liver biopsy results, and there is no established treatment method for NASH even when it is diagnosed by liver biopsy. In the guidelines recently published by the AASLD, liver biopsy is suggested for complications of metabolic syndrome and a high serum ferritin level in patients with NASH, as well as in those suspected of having advanced fibrosis[3].

Limitations of liver biopsy

Sampling error: Only 1/50000 of the whole liver tissue is sampled during a liver biopsy, for which sampling error is of concern. To prevent sampling errors, it is essential to collect a sufficient amount of tissue; the use of a thick needle[21] and collection of 2 or more samples with a sufficient length are recommended. Making an accurate diagnosis of NASH is dependent on the length of the specimens[22], with a necessary length of 15-16 mm or longer to accurately evaluate fibrosis[23]. Ratziu *et al*[24] excised and compared two percutaneous liver biopsy samples from each of 51 NAFLD patients and observed that the consistency in fatty change was relatively high (78%), but the fibrosis stage was different between the two samples in 41% of the patients. In 35% of the cases with bridging fibrosis observed in one sample, only mild or no fibrosis was noted in the other sample. The inconsistency in ballooning degeneration of hepatocytes, an essential feature for the diagnosis of NASH, was 18%, suggesting that NASH may be overlooked when only one sample is collected. In other reports, the results differed by one or more stages between specimens biopsied from the left and right lobes in 30% of the patients[25], and the inflammatory findings were more inconsistent than those of fatty change and fibrosis between biopsied specimens from the left and right lobes[26]. The assessment criteria for the pathological diagnosis recently proposed by the AASLD specify that the right lobe should be biopsied first, and when the left lobe is biopsied before treatment, a sample should also be biopsied from the left lobe after treatment to judge the therapeutic effect[27].

Inter- and intra-observer variability: Inter- and intra-observer variability also presents a serious problem for the pathological diagnosis of NAFLD. Younossi *et al*[28] reported that the evaluations of fatty change (κ = 0.64) and fibrosis (κ = 0.60) were highly consistent among observers, but that the evaluation of inflammatory activity was inconsistent at a high rate (κ =0.33). It has also been

Table 2 Pathological criteria for the diagnosis of nonalcoholic steatohepatitis

Criteria (yr)	Classifications	Definitions of NASH	Characteristics
Matteoni (1999)	Type 1: steatosis alone	Type 3 or 4	Depend on the subjective judgments of observers
	Type 2: steatosis with inflammation		(existence of hepatocyte balloning)
	Type 3: steatosis with hepatocyte balloning		Well correlation with liver-related mortality
	Type 4: Type 3 plus MDB or fibrosis		Inflammation is not included
NAS (2005)	Steatosis (0-3)	Total scores: 5 to 8	Numerical score
	Inflammation (0-3)		Low sensitivity, NAS ⩾ 4 may be better
	Hepatocyte balloning (0-2)		Fibrosis is not included
			No significant correlation with liver-related mortality
	Total: 0 to 8		Recommended use for assessing the therapeutic effect during clinical studies
Younossi (2011)	Steatosis	Steatosis	Inflammation is not included
	Hepatocyte balloning	+ Hepatocyte balloning	Well correlation with Matteoni's classification
	MDB	or + MDB	Can diagnose so-called burned-out NASH
	Fibrosis	or + Fibrosis	Essential validation study

MDB: Mallory-Denk bodies; NAS: Nonalcoholic fatty liver disease activity score; NASH: Nonalcoholic steatohepatitis.

shown that inter-observer variability remained even when training in histopathological observation was provided in an effort to reduce these inconsistencies[29]. In that study, the post-intervention κ value (0.39) was not significantly different from the pre-intervention κ value (0.27). Measures to solve this problem are needed.

Risk and complications: Regarding the complications of liver biopsy, the incidence of pain is reportedly 20%, but it increases to 84% when a mildly unpleasant feeling is included in the assessment. The incidence of serious complications and mortality has been reported to be 0.3%-0.57% and 0.01%, respectively[30-32]. To decrease complications, operators that are trained by an instructor with sufficient experience should perform biopsies, and operation with a US guide and the use of an aspiration-type biopsy needle are recommended[33,34].

Problems with pathological diagnosis: The pathological features of typical NASH, in addition to fat deposition in hepatocytes, include inflammatory cell (neutrophil and lymphocyte) infiltration in lobules, ballooning degeneration of hepatocytes, Mallory-Denk bodies, pericellular fibrosis, sinusoidal fibrosis, giant mitochondria, eosinophilic necrosis, and iron deposition. However, few NASH patients show all of these typical findings, and there are no integrated criteria to diagnose NASH based on them. Matteoni *et al*[35] classified NAFLD into 4 types: type 1, fat deposition alone; type 2, fat deposition and inflammatory cell infiltration in the parenchyma; type 3, fat deposition and ballooning degeneration of hepatocytes; and type 4, type 3 criteria plus Mallory-Denk bodies or fibrosis. The authors observed that the liver disease-related mortality during an approximately 8-year follow-up period was only 1.7% in the type 1-plus-type 2 group, but significantly increased to 11% in the type 3-plus-type 4 group. They proposed the definition of types 3 and 4 as NASH from a prognostic viewpoint (Table 2). Later, Rafiq *et al*[36] followed the course for a longer period and reported that liver disease-related mortality was only 2.7% in the first group but was 17.5% in the second group. Based on these findings, the following consensus has been reached in Japan: the gold standard for the diagnosis of NASH is liver biopsy, and NASH is diagnosed when all of the following 3 pathological findings are observed (*i.e.*, those in types 3 and 4 of Matteoni's classification): (1) macrovesicular fatty change of hepatocytes; (2) inflammatory cell infiltration; and (3) ballooning degeneration of hepatocytes. However, the differentiation between types 2 and 3 of Matteoni's classification depends on the judgment of ballooning degeneration of hepatocytes, which is subjectively made by observers. Thus, the Nonalcoholic Steatohepatitis Clinical Research Network (NASH-CRN) proposed the classification of these two types by scoring the severities of fatty change (0-3 points), inflammation (0-3), and ballooning degeneration of hepatocytes (0-2) (0-8 points in total) by a system termed the NAS scoring system[37]. Cases with a score of 5 or higher or 2 or lower are regarded as NASH and non-NASH, respectively, and those with a score between these values are regarded as borderline cases. A NAS validation study was performed at NASH-CRN-affiliated institutions, and the utility of the system was reported in the United States. However, some researchers deem a NASH threshold of 5 points or higher as too insensitive, and they believe that it should be set at 4 or higher[38]. NAS is markedly reproducible, requires no special staining, is applicable for pediatric NASH, and is useful for assessing therapeutic effects in clinical studies. However, NAS is incapable of diagnosing NASH in patients with burned-out NASH, in whom fatty changes and inflammatory cell infiltration resolving in fibrosis has progressed; *i.e.*, inflammatory findings have been improved by treatment and only fibrosis remains. Moreover, a divergence has been reported in pathological diagnosis using NAS between general and liver-specialized pathologists[39]. It has recently been reported in the United States that Matteoni's classification scheme more faithfully reflects the diagnosis and prognosis of NASH than NAS[40]. In the future, NAS may be used as an index for judging therapeutic effects rather than as a diagnosis tool for NASH. Therefore, it is desirable to employ Matteoni's classification when a diagnostician skilled in

diagnosing NASH is present. Matteoni's classification is useful for routine clinical practice, single-facility clinical studies, and investigation of long-term prognosis, such as carcinogenesis. However, NAS is useful for multicenter clinical studies involving several diagnosticians, many patients, and evaluation of the short-term therapeutic effects of drugs. According to a new definition of NASH proposed by Younossi *et al*[41], NASH is diagnosed for (1) any degree of steatosis along with centrilobular ballooning and/or Mallory-Denk bodies or (2) any degree of steatosis along with centrilobular pericellular/perisinusoidal fibrosis or bridging fibrosis. Younossi's criteria almost perfectly agree with Matteoni's classification, and these two definitions of NASH correlated significantly with the prediction of a higher liver-related mortality rate. Younossi's criteria, which placed high importance on the presence of fibrosis, would enable the diagnosis of burned-out NASH in patients. Finally, Younossi's criteria are now accepted by the NAFLD/NASH clinical practice guideline committee (under the chairmanship of Prof. Sumio Watanabe, Juntendo University) organized by the Japan Society of Gastroenterology. In Japan, the diagnosis of NASH will be based on the presence of hepatic steatosis plus ballooning, Mallory-Denk bodies, or fibrosis in the near future (Table 2).

Miscellaneous: Liver biopsies may be performed at outpatient clinics to reduce costs overseas, but biopsy patients are hospitalized for several days in Japan. Performing liver biopsies for all NAFLD patients in Japan, estimated at 10 million, would be prohibitively expensive, and no cost-benefit analysis has been performed to date. In regard to the follow-up after liver biopsy, Toyoda *et al*[42] reported a very low follow-up rate in NAFLD patients compared with that in viral hepatitis patients, suggesting the need for more patient education.

NONINVASIVE DIAGNOSTIC METHODS FOR NASH

Several extensive reviews from Western countries have previously discussed noninvasive diagnostic methods for NASH or advanced fibrosis[43-45]. However, most of these papers described a simple enumeration of noninvasive tests. Thus, we here review biomarkers or scoring systems with critical appraisal to establish diagnostic algorithms that can be applicable even for Asian patients with NAFLD in clinical practice. Various parameters of oxidative stress, inflammation, apoptosis, and fibrosis have been reported to be useful for the noninvasive diagnosis of NASH[46]. Interest in cytokeratin in viral and nonviral hepatitis has been rapidly increasing during recent years, especially as proposed circulating biomarkers of hepatic necrosis and apoptosis[47]. Among those, circulating levels of cytokeratin-18 (CK18) fragments have been investigated extensively as novel biomarkers for the presence of steatohepatitis in patients with NAFLD. A recent meta-analysis, consisting of 10 studies with 838 patients, showed that CK18 fragments may be a useful biomarker for screening NASH[48]. Although these are very encouraging results, currently, this assay is not commercially available. Furthermore, as each study utilized a study-specific cut-off value, there is not an established congruent cut-off value for identifying steatohepatitis. According to the AASLD guidelines, CK18 is not recommended in routine clinical practice[3].

Differentiation between NASH and simple steatosis

Yilmaz *et al*[45] extensively reviewed biochemical diagnostic tests for differentiating simple steatosis from NASH. Here, we summarize scoring systems including multiple serum tests. The first evaluation of NASH was the HAIR scoring system, reported from Australia. This system comprises three scored components-hypertension (HTN), ALT level, and insulin resistance (IR)-that were established based on data from 105 weight loss surgery-treated obese patients[49]. Later, Palekar *et al*[50] of the Mayo Clinic investigated 80 NAFLD patients and reported the use of six criteria - age $\geqslant$ 50 years old, female sex, BMI $\geqslant$ 30 kg/m^2, AST $\geqslant$ 45 IU/L, AAR $\geqslant$ 0.8, and hyaluronic acid $\geqslant$ 55 ng/mL - of which any three, when met, allowed the diagnosis of NASH with a sensitivity and specificity of 74% and 66%, respectively. The NashTest, developed in Europe, predicts the disease on the basis of 13 parameters[51]. A recently proposed equation (2.627 × ln [AST] + 2.13 for DM) comprises only 2 items, AST and the presence or absence of DM, attaching greater importance to simplicity[52]. Campos *et al*[53] proposed a NASH clinical scoring system composed of HTN, type 2 DM, AST $\geqslant$ 27 IU/L, ALT $\geqslant$ 27 IU/L, sleep apnea syndrome, and race (other than blacks). Nice's French group recently reported the Nice model, in which CK18, ALT, and the presence or absence of metabolic syndrome is scored[54]. However, it is unclear whether these scoring systems are applicable for Japanese NAFLD patients because these reports from Western countries were based on severely obese patients treated with bariatric surgery, and no validation study has been adequately performed.

In Japan, Shimada *et al*[55] reported that early NASH and simple steatosis could be differentiated by a combination of 3 values: adiponectin ($\leqslant$ 4.0 μg/mL), homeostasis model assessment of insulin resistance (HOMA-IR) ($\geqslant$ 3.0), and type 4 collagen 7S ($\geqslant$ 5.0 ng/mL). However, adiponectin cannot be measured at general practice sites. JSG-NAFLD proposed the NAFIC score, which comprises three items-ferritin, fasting insulin, and type 4 collagen 7S - for the screening of NASH. These three variables were extracted as factors independently contributing to NASH in an analysis of 177 NAFLD patients. The NAFIC system assigns one point for 200 (female) or 300 (male) ng/mL or higher ferritin, one point for 10 μU/mL or higher fasting insulin, and two points for 5.0 ng/mL or higher type 4 collagen 7S. The total of these points is regarded as the NAFIC score (Table 3), and the possibility of NASH is high when the NAFIC score is 2 or higher. The usefulness of this scoring system has been verified in a validation study involving 442 patients[56].

Table 3 Scoring systems for picking up nonalcoholic steatohepatitis or severe fibrosis in nonalcoholic fatty liver disease

Index	NAFIC score		NAFLD fibrosis score		FIB4 index	
Object	Predicting NASH		Excluding severe fibrosis (stage 3-4)			
Formula	Ferritin > 200 (female), 300 (male) ng/mL (= 1 point) Fasting insulin > 10 μU/mL (= 1 point) Type 4 collagen 7S > 5.0 ng/mL (= 2 points) Total: 0-4 points		-1.675 + 0.037 × age (yr) + 0.094 × BMI (kg/m^2) + 1.13 × impaired fasting glycemia /DM (yes = 1, no = 0) + 0.99 × AAR – 0.013 × PLT (× 10^9/L) - 0.66 × Alb (g/dL)		Age (yr) × AST (IU/L)/(PLT (10^9/L) × √ALT (IU/L)	
Cut-off values	1	2	-1.455	0.676	1.30	2.67
Sensitivity	94%[1]	66%[1]	82%[1]	51%[1]	74%	33%
	88%[2]	60%[2]	77%[2]	43%[2]		
Specificity	48%[1]	91%[1]	77%[1]	98%[1]	71%	98%
	43%[2]	87%[2]	71%[2]	96%[2]		
Positive predictive value	31%[1]	90%[1]	56%[1]	90%[1]	43%	80%
	66%[2]	85%[2]	52%[2]	82%[2]		
Negative predictive value	86%[1]	67%[1]	93%[1]	85%[1]	90%	83%
	75%[2]	64%[2]	88%[2]	80%[2]		

[1]Estimation group; [2]Validation group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST/ALT ratio; BMI: Body mass index; DM: Diabetes mellitus; PLT: Platelets; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

The three variables constituting the NAFIC score are parameters associated with the pathology of NASH, such as oxidative stress, IR and fibrosis. The relevance of the scoring parameters to NASH pathology and the fact that no complex calculation is required are advantageous. However, there are also problems to be addressed, such as the scoring of insulin-treated patients, usefulness for races other than Japanese, cost, and coverage by national health insurance. No established scoring system to screen for NASH is currently available, but the utility of the NAFIC score is expected to be investigated by a large-scale study in Japan.

Diagnosis of NASH with advanced fibrosis

Noninvasive diagnosis of liver fibrosis is one of the most rapidly evolving fields in recent years. A recent extensive review mentioned noninvasive diagnostic tests, including routine clinical parameters, fibrosis biomarkers, and imaging techniques in chronic hepatitis C, alcoholic liver disease, and NAFLD/NASH[44]. The stage of fibrosis has generally been diagnosed according to Brunt's criteria[57] or Kleiner's classification as proposed by NASH-CRN[37]. According to Brunt's criteria, the severity of hepatic fibrosis is defined in terms of the following stages: Stage 1, zone 3 perisinusoidal fibrosis; Stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; Stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and Stage 4, cirrhosis[57]. Kleiner's classification differs from Brunt's criteria in that Stage 1 is subdivided into three substages: Substages 1a and 1b are zone 3 perisinusoidal and differ only by the character of collagen disposition (delicate or dense, respectively), and Substage 1c is portal or periportal (representing the pediatric pattern)[37]. Advanced fibrosis is classified as Stage 3 or 4.

A French group proposed the BAAT score (0-4 points) as a system to predict the grade of fibrosis, in which 1 point each is assigned to BMI ⩾ 28 kg/m^2, ALT 2 or more times greater than the normal upper limit, age ⩾ 50 years old, and TG ⩾ 1.7 mmol/L. For the differentiation of patients with Stage 2 or higher fibrosis, the negative predictive value (NPV) of a 0-1 point score was 100%. The same group developed the FibroTest, which is composed of bilirubin, γGT, γ globulin, haptoglobin, and α2-macroglobulin. From the United States, the Mayo Clinic proposed the NAFLD fibrosis score (NFS) [= -1.675 + 0.037 × age (year) + 0.094 × BMI (kg/m^2) + 1.13 × IFG/DM (with = 1, without = 0) + 0.99 × AAR-0.013 × platelets (PLT) (× 10^9/L) - 0.66 × Alb (g/dL)], calculated from readily measured routine parameters such as the age, PLT, albumin (Alb) level, AAR, fasting hyperglycemia (impaired fasting glucose, or IFG) or DM, and BMI (Table 3)[58]. NFS has been confirmed to be useful in predicting the progression of fibrosis regardless of whether the ALT level is normal or abnormal, even in bariatric surgery-treated obese patients. NFS is advantageous because it contains no items that require a special test and has been validated in many studies. The latest AASLD guidelines recommend the use of NFS for decision making for the application of liver biopsy[3]. Although NFS contains no items that require a special test, the calculation is complex, and the score is intermediate (NFS = -1.455 to 0.676) in approximately 25%-30% of patients [between low (NFS < -1.455) and high (NFS > 0.676) scores][2] for whom a liver biopsy is still unavoidable. The results of a validation study of NFS performed in China have been recently published[59], and the NPV of a low score was favorable and useful for the exclusion of advanced cases. However, the positive predictive value (PPV) of a high score was low, showing that the usefulness of NFS for detecting advanced cases in Asians remains questionable.

In the United States, Harrison *et al*[60] proposed a simple system, the BARD score, assigning one, two, and one point to BMI ⩾ 28 kg/m^2, AAR ⩾ 0.8, and DM, respectively, and reported that the possibility of Stage 3 or 4 is very high when the total score is 2 or higher. The NPV was high, and the results were favorable in validation studies performed in Poland and Argentina. However,

the usefulness of the BARD score for Japanese populations is questionable because the Japanese have lower BMIs than Western populations[61].

The FIB-4 index, calculated as: [age (year) × AST (IU/L)]/[PLT (10^9/L) × ALT (IU/L)], was proposed as a parameter of the progression of fibrosis in patients superinfected with human immunodeficiency virus/hepatitis C virus and was also investigated with regard to application for NAFLD (Table 3)[62]. Unlike other scoring systems, the FIB-4 index has the ability to identify Stage 3 or higher fibrosis. This index is advantageous because it is based on test values that are routinely measured in health checkups, the number of items is small, and the index is not influenced by the BMI. In a study performed by JSG-NAFLD involving Japanese subjects, the FIB-4 index was the most useful in differentiating patients with advanced fibrosis[63]. Furthermore, the usefulness for patients with normal ALT is comparable to that for patients with abnormal ALT[64]. Similar findings were confirmed in England: the FIB-4 index value was low in approximately 80% of the patients diagnosed with NAFLD during a health checkup, whereas a high value was noted in only approximately 1% of patients. As a parameter used alone, PLT is expected to be useful but carries the caveat that the counts are relatively high when fibrosis is severe. It has been shown that advanced fibrosis patients can be simply excluded using a combination of PLT and AAR (PAAR) (the possibility of Stage 3 or higher fibrosis is very low when the platelet count is 1950000 or greater with an AAR below 0.8)[65]. The AST to platelet ratio index (APRI) {[(AST level/upper limit of normal AST)/PLT (10^9/L)] × 100}, originally developed for hepatitis C patients, has also been suggested as a useful strategy for predicting significant fibrosis due to NASH. McPherson *et al*[66] compared five scoring systems, AAR, APRI, BARD, NFS, and the FIB-4 index, in a study involving 145 English NAFLD patients. Evaluation based on area under the receiver operating characteristic curve (AUROC) demonstrated that the FIB-4 index was the most favorable (0.86), followed by AAR (0.83), NFS (0.81), BARD (0.77), and APRI (0.67), and the PPVs of the FIB-4 index and of NFS were 75% and 79%, respectively. On the basis of these results, the authors recommended the FIB-4 index and NFS. The Nippon score was reported from a multicenter study performed with Japanese subjects in Nagasaki, Japan. The score was calculated by assigning one point each to the following characteristics: female sex, an age ≥ 60 years old, and the presence or absence of type 2 DM and hypertension (4 points in total). Although this system is very simple, it has not been confirmed to be superior to other scoring systems. Other scoring systems such as FibroMeter have been proposed as tests for the probability of advanced fibrosis, but additional studies are necessary for their validation. The above information suggest that, overall, the NFS and the FIB-4 indexes are the most recommendable scoring systems that are expected to be useful for Japanese patients because these systems have been relatively well validated in Japan and in other countries. However, both systems require further evaluation by performing prospective multicenter validation studies. The usefulness of elastography has attracted attention recently. FibroScan is very useful in predicting the progression of fibrosis in NAFLD patients[67], and is covered by national health insurance in Japan as of October 2011.

Scoring systems useful for predicting liver carcinogenesis and making a prognosis

There has been no study on the association of liver diseases with carcinogenesis, but Kawamura *et al*[68] reported that the annual liver carcinogenic rate in NAFLD patients was 0.043% and that APRI was useful in predicting liver carcinogenesis. It was recently reported that the scores derived from the fibrosis-predicting scoring systems NFS, APRI, and FIB-4 also serve as prognostic factors[69]; however, the prognostic value of these scores still requires verification in Japan.

Proposal of a diagnostic algorithm for NAFLD

There have been no established algorithms for the diagnosis of NAFLD/NASH. An algorithm for the management of NAFLD was suggested by Rafiq *et al*[70]. Liver biopsies should be considered if NAFLD patients show potential signs of cirrhosis, such as a hard edge of the liver, AST > ALT, and low albumin or platelets, or have abnormal ALT levels for more than 6 mo in spite of undergoing diet change and exercise therapy.

Based on the results of the multicenter study performed by JSG-NAFLD, it can be concluded that the FIB-4 index is useful for excluding advanced fibrosis patients[63], whereas the NAFIC score is useful for detecting NASH[56]. Thus, we would like to propose a diagnostic algorithm for NAFLD based on these data, as shown in Figure 1. First, the FIB-4 index is applied to every NAFLD patient. If the FIB-4 index is higher than 2.67, liver biopsy should be performed immediately. If the FIB-4 index is lower than 1.30, follow-up is recommended. If the FIB-4 index is between indeterminate ranges, an NAFIC score should be calculated. If the NAFIC score is above 2 points, liver biopsy should be considered. In our cooperative study with institutions performing health checkups, the FIB-4 score was high, intermediate, and low in approximately 1%, 19% and 80% of the NAFLD patients, respectively[65]. Accordingly, patients other than those with a low FIB-4 score, *i.e.*, approximately 20% of the NAFLD patients, will be treated by hepatologists.

GENETIC PREDISPOSITION ASSOCIATED WITH THE PATHOGENESIS OF NAFLD/NASH

Genome-wide association studies (GWAS) offer a powerful technique for discovering novel associations between single-nucleotide polymorphisms (SNPs) and disease

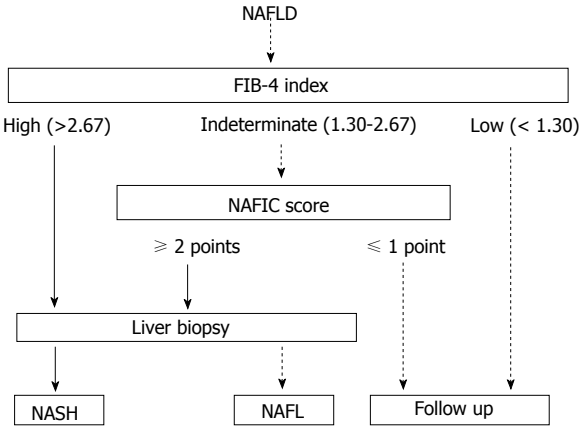

Figure 1 Proposed diagnostic algorithms combining non-invasive methods and liver biopsy. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NAFL: Nonalcoholic fatty liver.

phenotypes. Romeo *et al*[71] first reported that a SNP in patatin-like phospholipase domain-containing protein 3 (PNPLA3) (rs738409 [G], encoding I148M), also termed adiponutrin, on chromosome 22 was strongly associated with increased hepatic fat levels, as well as with hepatic inflammation. This allele was most commonly observed in Hispanics, the group most susceptible to NAFLD among the 2111 subjects that comprised a mixed study population of Hispanics, African Americans, and European Americans. PNPLA3 is highly expressed in adipose tissue as well as in the liver, and the overexpression of PNPA3 promotes lipogenesis in mouse primary hepatocytes. In humans, hepatic PNPLA3 messenger RNA expression appears to be correlated with hepatic triglyceride content. Association studies[72-76], including one meta-analysis[76], confirm that the I148M polymorphism is also a strong modifier of NASH and progressive hepatic injury in various populations throughout the world. In addition to PNPLA3, other SNPs associated with NAFLD include neurocan, lysophospholipase-like 1, glucokinase regulatory protein, protein phosphatase 1 regulatory subunit 3b, and apolipoprotein C3[77-79]. However, it is unknown whether screening for these SNPs can facilitate the diagnosis of NASH or advanced fibrosis.

CONCLUSION

Currently, liver biopsy is essential for the diagnosis of NASH, but in the future, combining scoring systems and imaging methods may efficiently diagnose NAFLD/NASH. Whether these scoring systems reflect the long-term prognosis and carcinogenesis potential remains to be investigated. The development of an improved scoring system that will prove useful for efficiently detecting NASH and reducing liver disease-related deaths is expected in the future.

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Molecular targeting agents associated with transarterial chemoembolization or radiofrequency ablation in hepatocarcinoma treatment

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer in the world. According to Barcelona Clinic Liver Cancer modified criteria, patients with early stage disease are candidate to radiofrequency ablation (RFA), while patients with intermediate stage HCC are usually treated by transarterial chemoembolization (TACE). TACE and RFA induce a transient devascularisation effect followed by strong neo-angiogenic stimulus. In fact, after these procedures, it has been demonstrated an up-regulation of pro-angiogenic and growth factors such as vascular endothelial growth factor-A, which might contribute to accelerated progression in patients with incomplete response. Several studies have demonstrated that MAP-kinase and AKT pathways, in addition to neo-angiogenesis, have an important role in the development of HCC. In advanced HCC, anti-angiogenic therapy and tyrosine kinases inhibitors showed potential clinical benefit. Actually, a number of clinical studies are ongoing testing these agents in combination with TACE or RFA. In this paper, we have reviewed the most recent preclinical and clinical results of such trials.

Key words: Hepatocellular carcinoma; Molecular targeting agents; Angiogenesis; Chemoembolization therapeutic; Radiofrequency treatment; Sorafenib

Core tip: The outcome of patients (with early or intermediate stage according to Barcelona Clinic Liver Cancer) treated with loco-regional approach alone [radiofrequency ablation (RFA) or transarterial chemoembolization (TACE)] is disappointing because the rebound of vascular endothelia growth factors induced by tissue hypoxia. On this basis there is a strong preclinical background to associate TACE or RFA with anti-angiogenic agents. We summarized the crucial role of angiogenesis and the pathways involved in hepatocellular cancer progression, underscoring the consequences of pro- and anti-angiogenic factors produced after loco-regional therapy. We explored preclinical and clinical results of trials combining molecular targeting agents plus TACE or RFA.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer in the world[1]. To treat this tumor, it's necessary a multidisciplinary approach due to the underlying cirrhosis.

According to the Barcelona-Clinic Liver Cancer Classification (BCLCC), subset of patients in the very early and early stage could be treated with radiofrequency ablation (RFA)[1]. Patients with intermediate stage could be considered for transarterial chemoembolization (TACE)[1].

In classical TACE the blood supply to the tumor is blocked from chemotherapeutic agent (doxorubicin, mitomycin) plus lipiodol[2]. Chemoembolization with drug-eluting beads (DEB-TACE) combines the drug with the embolization device by using microsphere[2]. It's important to underline that ischemia, induced by TACE, strongly stimulates the expression of angiogenic factors, in particular vascular endothelial growth factor-A (VEGF-A)[3]. Consequently, HCC angiogenesis and progression are stimulated in patients with an incomplete response[3]. Also, insufficient or incomplete RFA could promote angiogenesis in residual HCC, inducing a rapid tumor regrowth[4].

Here, we have reviewed the pathways involved in HCC development, underscoring the angiogenic rebound following loco-regional therapy, and clinical trials employing the association between loco-regional therapy and molecular targeting agents.

MAIN PATHWAYS INVOLVED IN THE DEVELOPMENT OF HCC

Fundamental pathways involved in HCC proliferation are the RAS-mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) (Figure 1).

The RAS-MAPK pathway is the most important cascade leading to tumor cell progression[5]. Vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) are transmembrane tyrosine-kinase receptors that, binding several growth factors, activate the RAS GTPase proteins[5]. The increased activation of this pathway may be due to: (1) RAS proteins mutation; (2) overexpression of intracellular RTKs; and (3) a number of growth factors, such as VEGF or PDGF[5,6]. The main RAS effector pathway is the RAF-MAP/extracellular signal-regulated kinase (ERK) kinase. Once RAF proteins are activated and phosphorylated by different protein kinases, they phosphorylate MEK that, in turn, activates ERK1 and ERK2 that modulates gene expression via the phosphorylation of transcription factors, which have profound effects on tumorigenesis[6]. RAF activation is crucial in HCC progression; in fact overexpression of RAF is very frequent[6]. Interestingly, it was reported that hepatitis B and C virus infection are able to activate the RAS-MAPK pathway in HCC[7]. Therefore, this pathway represents the ideal molecular target of several anti-angiogenic therapies, such as sorafenib[2]. In addition, the signaling inhibition mediated by these receptors can be realized by various monoclonal antibodies (cetuximab, trastuzumab, ramucirumab, bevacizumab), and by "small" molecules (erlotinib, lapatinib)[8].

The RAS-MAPK pathway is a regulator of the phosphoinositide-3-kinase PI3K/AKT/mammalian target of rapamycin (mTOR) pathway[9]. This pathway stimulates the Janus kinase/signal transducer and the pathway, which represent a central regulator of proliferation[9]. The PI3K/AKT/mTOR pathway is activated in a subset of HCC and its blockade by rapamycin and/or everolimus, demonstrated to inhibit the growth of HCC cell lines[10-12].

POTENTIAL BENEFITS OF THE ASSOCIATION OF MOLECULAR TARGETING AGENTS PLUS TACE

TACE is used in the treatment of: (1) single lesion (> 3 cm); (2) multifocal HCC; and (3) awaiting liver transplantation[1]. HCC receives prolonged contact time with chemotherapeutic agent, by its infusion directly into vessels supplying the tumor, and subsequently obstructing these vessels with an embolization material[1]. Therefore, chemoembolization induces necrosis, prolonging the exposition between cancer cells and chemotherapy[2]. However, ischemia correlates with expression of angiogenic factors, such as VEGF-A and angiopoietin, and stimulates angiogenesis (resulting in the formation of a rich vascular bed in residual tumors), which may allow the surviving cancer cells to proliferate[13]. In fact, after TACE, the tumor microenvironment becomes deranged with increased hypoxia, leading to an up-regulation in hypoxia inducible factor-1 (HIF-1), which in turn up-regulates VEGF and also PDGFR, further increasing tumor angiogenesis[13]. During tumor angiogenesis, cancer and endothelial cells secrete PDGF-β, which acts stimulating pericytes to express VEGF[13].

In patients in whom TACE failed, due to incomplete embolization or partial recanalization, the secretion of pro-angiogenic factors by residual tumor or surrounding tissue might contribute to HCC progression[3]. In fact, TACE was associated with a high rate of disease recurrence, with 67% of post-therapy deaths due to tumor progression[3]. High serum VEGF levels are frequently expressed in HCC and increased serum VEGF levels are correlated with liver function, tumor size, tumor number, microscopic venous invasion, distant metastasis, reduced overall survival (OS), and recurrence of HCC after treatment[13-15].

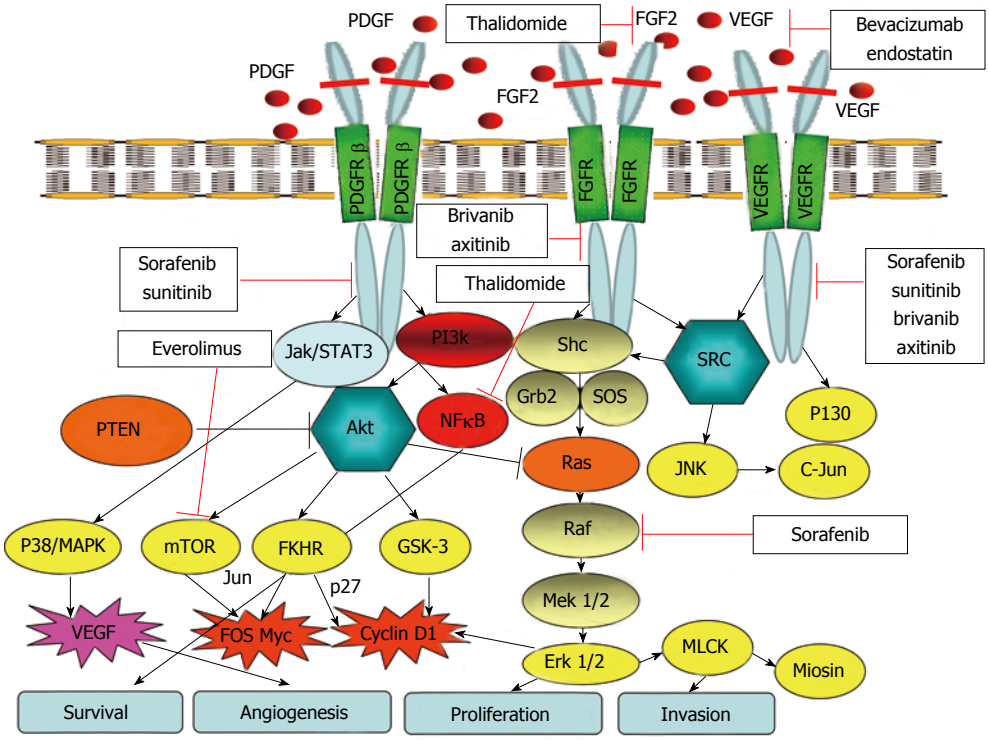

Figure 1 Fundamental pathways involved in development of hepatocellular cancer and their molecular targeting agents. VEGF: Vascular endothelial growth factor; PDGF: Platelet derived growth factor; VEGFR: VEGF receptor; PDGFR: PDGF receptor; FGF2: Fibroblast growth factor 2; PI3k: Phosphoinositide 3-kinase; NF-κB: Nuclear factor-κB; SRC: Steroid receptor coactivator; JNK: c-JUN terminal kinase; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin-homologue; STAT 3: Signal transducer and activator of transcription; MAPK: Mitogen-activated protein kinase; GSK-3: Glycogen synthase kinase-3.

Preclinical and clinical data suggested that a monitoring of VEGF-A (determined by ELISA) one day before and 7 d after TACE might be used to predict HCC growth and that an increase of serum VEGF levels from the first to the third day post-TACE could be related to poor prognosis[3,16-18].

According to these evidences, the treatment with multikinase inhibitors pre/after TACE could be both anti-proliferative, anti-angiogenic and, at the same time, may prolong time to recurrence, improve survival, and target lesions distal to the TACE site. In fact, preclinical models combining bland transarterial embolization with anti-angiogenic agents have observed a reduction in tumor volume and vessel density, as well as a prolongation in survival compared with transarterial embolization alone[19].

POTENTIAL BENEFITS OF THE ASSOCIATION OF MOLECULAR TARGETING AGENTS PLUS RFA

The mechanisms of rapid growth of residual HCC after RFA or the mediators are still poorly understood. It was hypothesized that insufficient RFA, due to a not sufficiently high local ablative temperature, could promote proliferation and angiogenesis of residual HCC[4]. Moreover, after RFA in the residual tumor ablation HIF-1α levels[20] are increased and, consequently, the tumor might exhibit an aggressive phenotype with unfavourable prognosis[21].

TACE WITH MOLECULAR TARGETING AGENTS

All clinical studies that analyzed molecular targeting drugs (Figure 1) with predominantly anti-angiogenic effect in combination with TACE are summarized in Table 1.

Sorafenib

Sorafenib is an oral multitargeted receptor TKI, blocking RAF/MEK/ERK pathway, VEGFR-2/3 and PDGFR-β, stem cell factor receptor, fms-like tyrosine kinase 3, and rearranged during transfection[22]. Sorafenib (at dose 800 mg/d) was approved from the United States Food and Drugs Administration (FDA) for the treatment of advanced HCC since November 2007[23,24].

The addition of sorafenib to TACE compared to TACE alone (Figure 2A and B) in patients with advanced or intermediate unresectable HCC and good liver function is under clinical investigation.

In regard to sequential timing of anti-angiogenic and loco-regional therapy, Strebel *et al*[25] proposed three different schedules: sequential, interrupted, continuous of combining TACE with sorafenib. In the first approach sorafenib was administered after chemoembolization. In a second model, sorafenib was given throughout, and it

Table 1 Clinical studies that analyzed targeted therapy in combination with transarterial chemoembolization

Author, Ref. or No. of clinical trials	Phase	Drug	Patients (*n*)	Type of TACE	Timing	Primary endpoint
Dufour *et al*[26]	I	Sorafenib (from 400 to 800 mg/d)	14	Classical (doxorubicin, mytomicin-C)	7 d before TACE and continuously	Safety
NCT01042041	I	Sorafenib (800 mg/d)	18 TR	Classical (cisplatin, doxorubicin, and mitomycin-C)	2 wk before TACE and continuously	Dose adjustment
Pawlik *et al*[27]	II	Sorafenib (800 mg/d)	35	DEB-TACE (doxorubicin)	1 wk before TACE and continuously	Safety, toxicity
Sieghart *et al*[28]	Pilot trial	Sorafenib (800 mg/d)	15	Classical (bilirubin-adjusted doxorubicin doses)	2 wk before TACE and continuously	Safety
Chung *et al*[29] START study	II	Sorafenib (800 mg/d)	165	Classical (doxorubicin)	After TACE (interrupted schedule)	Safety, efficacy
Park e*et al*[30]	II	Sorafenib (800 mg/d)	50	Classical (doxorubicin)	3 d after TACE for up to 24 wk	Safety, TTP
Sansonno *et al*[31]	RCT	Sorafenib (800 mg/d)	62 (with HCV infection)	Classical (doxorubicin and mytomicin C)	30 d after TACE (sequential schedule)	TTP
NCT01556815	II	Sorafenib (800 mg/d)	40 TR (with HBV infection)	Classical (doxorubicin)	1 wk after TACE (sequential schedule)	TTP
Kudo *et al*[32]	III	Sorafenib (800 mg/d)	458	Classical (epirubicin, cisplatin, doxorubicin, mitomycin-C)	After TACE (sequential schedule)	TTP
Lencioni *et al*[33] SPACE trial	II	Sorafenib (800 mg/d)	307	DEB-TACE (doxorubicin)	3-7 d before TACE and continuously	TTP
TATICS trial NCT01217034	II	Sorafenib (from 400 to 800 mg/d)	TR not specified	Classical (drugs not specified)	Before TACE (interrupted schedule)	TTUP
ECOG 1208 trial NCT01004978	III	Sorafenib (800 mg/d)	TR not specified	Classic (doxorubicin, mitomycin-C, cisplatin) or DEB-TACE (doxorubicin)	2 wk before TACE (interrupted schedule)	PFS
Meyer *et al*[34] TACE 2 trial	III	Sorafenib (800 mg/d)	412 TR	DEB-TACE with doxorubicin	Together with TACE and continuously	PFS
Hoffmann *et al*[75]	III	Sorafenib (800 mg/d)	208 (waiting LT)	Classical (carboplatin)	Together with TACE and continuously	TTP
Britten *et al*[37]	Pilot trial	Bevacizumab 10 mg/kg every 14 d	23	DEB-TACE (doxorubicin, cisplatin, mitomycin-C)	1 wk before TACE beyond week 16	Neovessel by angiography
AVATACE-1 NCT00280007	II	Bevacizumab 5 mg/kg *iv* every 14 d for 52 wk	32	Classical (drugs not specified)	After TACE for 52 wk	Effectiveness
NCT00049322	II	Bevacizumab (10 mg/kg) every 14 d	31	Classical (doxorubicin, cisplatin, mitomycin-C)	Before TACE continuously	Neovessel formation by angiography
NCT00335829	II	Bevacizumab (dose not specified)	26	Classical (drugs not specified)	Before TACE in weeks 1, 3, 5 (up to a maximum of 5 courses)	Median PFS
NCT00518557	II	Recombinant human endostatin	60 TR	Classical (epirubicin)	During TACE (via hepatic artery)	Safety, tolerability, mortality
Hao *et al*[45]	RCT	Thalidomide (200 mg/d)	108	Classical (gemcitabine, oxaliplatin, floxuridine)	Before TACE and continuously for 3–6 mo	Median OS
NCT00006016	II	Thalidomide (dose not specified)	75 TR	Classical (doxorubicin)	Before TACE (interrupted schedule)	Feasibility, potential activity of thalidomide
NCT00921531	III	Thalidomide (from 200 mg/d to 400 mg/d)	200 TR	Classical (5-fluorouracil, oxaliplatin, mitomycin-C)	After TACE continuously	OS
NCT01009801	I - II	Everolimus (10 mg/d)	98 TR	DEB-TACE (doxorubicin)	Before TACE for up to 12 mo	Dose-limiting toxicity, PFS
TRACER study NCT01379521	II	Everolimus (dose not specified)	80	Classical (drugs not specified)	Together with TACE continuously	TTP
SATURNE trial NCT01164202	II - III	Sunitinib (50 mg/d on days 1-28 before TACE)	190 TR	Classical (drugs not specified)	7-10 d before TACE and after TACE (every 6 wk for 1 year)	Unacceptable bleeding or hepatic failure, OS

NCT00524316	Ⅱ	Sunitinib (50 mg/d on days 1-1 and-15-35 in course 1 before TACE and on days 1-28 after TACE)	16 TR	Classical (doxorubicin)	7 d before TACE (interrupted schedule)	RR, PFS

TR: Target recruitment; RCT: Randomized clinical trial; TTUP: Time to untreatable progression (defined as time from randomization to untreatable progression and will be evaluated every 8 wk); LT: Liver transplantation; PFS: Progression free survival; OS: Overall survival; TACE: Transarterial chemoembolization; RR: Risk ratio; TTP: Time to progression.

was interrupted only during TACE[25]. In the third model, sorafenib, started before TACE, was administrated continuously[25].

Several phase Ⅱ/Ⅲ studies used a continuous schedule, according to Dufour phase I study, that confirmed sorafenib anti-angiogenic action, due to significant decrease in VEGF-plasma concentration levels in HCC patients, against a good tolerability (only 4/14 patients have interrupted treatment for toxicity)[26].

Also, a phase Ⅱ trial showed a good safe of sorafenib started before doxorubicin DEB-TACE -from 1 to 5 cycles- with a median of 71 d of therapy with a disease control rate (DCR) of 95% [according to Response Evaluation Criteria in Solid Tumors Group (RECIST)] with an objective response of 58% (according to European Association for the Study of the Liver -EASL)[27].

Conversely, a pilot study evaluating safety of TACE (with bilirubin-adjusted doxorubicin doses) plus sorafenib, administered according to continuous schedule, was terminated prematurely for toxicity evidence, such as dermatologic toxicity (47%), and diarrhoea (47%)[28].

The interim analysis of phase Ⅱ START study showed in Asian patients treated with TACE (TACE cycles were repeated every 6-8 wk on demand, up to a maximum of 6 TACE cycles) plus sorafenib (400 mg *bid* on day 4 to day 7 after the first TACE and stopped 4 d before each next TACE), as an interrupted schedule, a DCR of 91.2%, an overall response rate of 52.4%, a median progression free survival (PFS) and a time to progression (TTP) of 9 mo, with an OS probability upper 90% at 18 mo[13]. The side effects (mainly gastrointestinal and dermatologic) were mild or moderate[13].

In a Korean phase Ⅱ trial patients treated with sorafenib, as interrupted schedule, presented an overall median TTP was 7 mo[30]. The 6-mo PFS rate, based on RECIST criteria, was 52% and the median OS was 20.8 mo. Nevertheless the use of interrupted approach, it was required sorafenib dose reduction in 70% of patients because of toxicity[30].

In an European study, in which sorafenib was administered 30 d after TACE in HCC patients with chronic hepatitis C virus infection, the TTP was 9 mo, without unexpected side effects ($P < 0.001$), in fact a similar number of patients (belonging 25% to sorafenib and 23% to placebo group) were withdrawn from the trial because of toxicity[31].

Conversely, a phase Ⅲ Asian trial concluded that sorafenib, given in patients who responded to TACE, did not significantly improve median TTP (sorafenib plus TACE: 5.4 mo *vs* TACE: 3.7 mo), because a long time interval elapsed before giving sorafenib after TACE. In fact, 60% of patients had a treatment lag upper 9 wk prior to randomization[32].

Actually, four ongoing randomized trials (SPACE, ECOG1028, TACE2 and TACTICS) are addressing the mode and timing to add sorafenib to TACE or DEB-TACE with a continuous dosing of sorafenib, as in the SPACE or TACE2 studies[33,34] *vs* interrupted dosing in ECOG 1208 or TATICS trials (ClinicalTrials.gov Identifier: NCT01217034; NCT01004978).

Bevacizumab

Bevacizumab is a humanized monoclonal anti-VEGF-A antibody approved by the United States FDA for several metastatic tumors, such as colon cancer, non-small cell lung cancer, renal cancer, glioblastoma multiforme, and approved by the European Medicines Agency (EMA) for breast cancer[35].

Based on preclinical evidences that demonstrated in HCC models a strong neo-angiogenesis inhibition after bevacizumab[36] the addition of bevacizumab to TACE compared to TACE alone in patients with unresectable HCC and good liver function is under clinical investigation.

A pilot study showed in patients treated with bevacizumab (administered intravenous according to continuous schedule) plus TACE a statistically significant improvement in PFS at 16 wk [0.19 in TACE group *vs* 0.79 in combination arm ($P = 0.021$)][37]. Overall, bevacizumab was well tolerated, not showing an increased risk of bleeding. In fact, only 3 patients, who received bevacizumab, had grade 2/3 treatment-related gastrointestinal bleeding, which was not life threatening[37]. In conclusion, the Authors claimed that the addition of bevacizumab to TACE was safe and feasible in selected HCC patients and that this combination was able reduce neo-vessel formation (as showed by angiography), in particular, at week 14[37].

A phase Ⅱ AVATACE-1 trial will evaluate tumor progression in patients treated with bevacizumab (after one year treatment) plus TACE measured by liver magnetic resonance imaging (MRI) and positron emission tomography-scanning and several angiogenesis markers (circulating endothelial progenitors, pro-angiogenic hematopoietic cells, HGF levels) (ClinicalTrials.gov Identifier: NCT00280007).

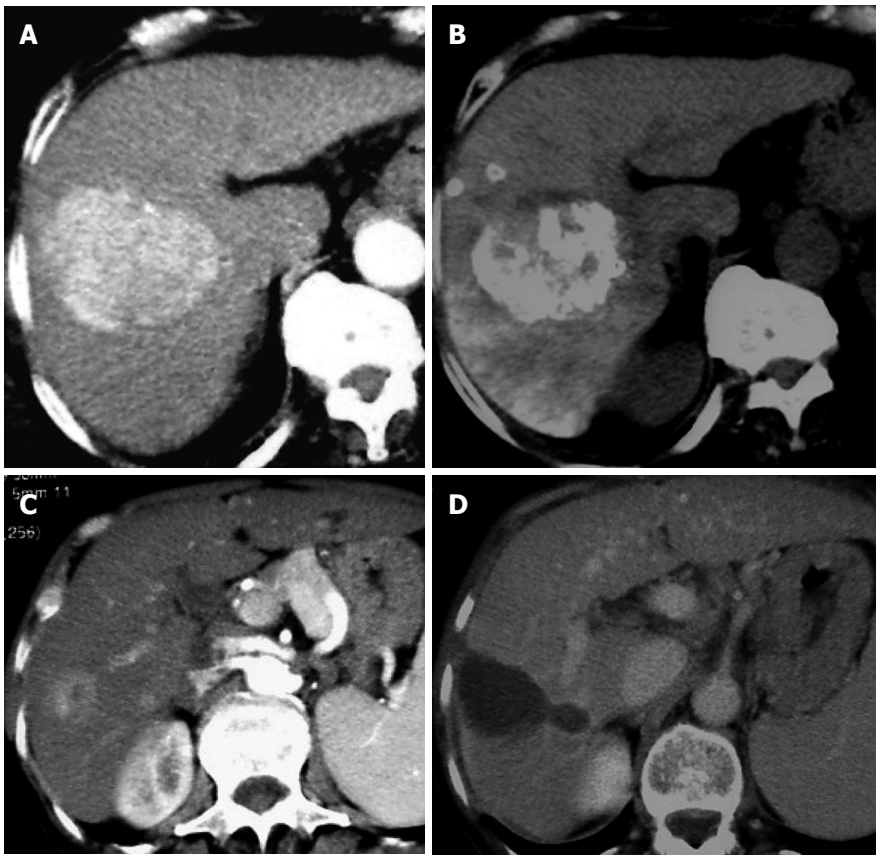

Figure 2 Computed tomography scan. A: An intermediate hepatocellular cancer (HCC) is showed at liver segment Ⅶ before transarterial chemoembolization (TACE). Note the HCC hyperdensity in arterial phase; B: The same tumor showed in A was observed 1 mo after TACE. Note the lipiodol impregnation of the tumor in computed tomography scan without intravenous contrast; C: An early hepatocellular cancer is showed at liver segment Ⅴ before radiofrequency ablation (RFA). Note the HCC hyperdensity in arterial phase; D: The same tumor showed in C was observed 1 mo after RFA. Note the cavitation of the tumor as an image with no density.

Endostatin

The precise mechanisms of antitumor activity have not yet been fully clarified. It's known that endostatins had been utilized to modify 12% of the human genome to down regulate pathological angiogenesis and inhibit 65 tumor types in animal models, including HCC[38].

According to preclinical evidences that showed anti-angiogenic activity of endostar (a recombinant human endostatin expressed and purified in Escherichia coli with an additional nine-amino acid sequence and forming another his-tag structure) blocking VEGF-induced tyrosine phosphorylation of KDR/FLK-1 in endothelial cells[39], and a tumor reduction ($P < 0.01$) 2 wk after treatment with both TACE plus endostar (arterially administrated at dose of 0.25 mg/kg) in HCC models[40], the addition of endostar to TACE in patients with unresectable HCC and good liver function is under investigation.

Two clinical studies are considering safety of endostatin plus TACE and endostatin haematic level as biomarker of HCC recurrence (ClinicalTrials.gov Identifiers: NCT00834028; NCT00518557).

Thalidomide

Thalidomide is an inhibitor of angiogenesis induced by fibroblast growth factor immunomodulatory and a drug able to potentiate the cytotoxic activity of immune system by several mechanisms: (1) inhibition of the production of interleukin-6 (IL-6); (2) activation of caspase 8; (3) induction of c-JUN terminal kinase (JNK)-dependent release of cytochrome-c and Smac; (4) direct activation of T cells to produce IL-2; and (5) increasing the activity of NK-dependent[41].

United States FDA approved thalidomide in 2006 in combination with dexamethasone and in 2007 EMA approved this drug in combination with melphalan plus prednisone for the treatment of multiple myeloma[42].

In advanced HCC two Asian studies demonstrated that thalidomide (from 200 mg/d to maximum tolerated dose) had good safety, but only modest response[43,44].

Hao *et al*[45] assessing the association of thalidomide to TACE *vs* TACE alone in HCC patients, showed a median OS of 28 mo (95%CI: 12-24) in the thalidomide arm and of 13 mo (95%CI: 10-16) in the control group ($P < 0.05$), with an OS at 2-year of 51.0% and 24.6%, respectively. Moreover, adverse events in patients taking thalidomide were mild and infrequent.

Actually, two phases Ⅱ and Ⅲ trials are evaluating the effectiveness of thalidomide plus TACE as adjuvant or neoadjuvant therapy with an interrupted schedule (Clini-

calTrials.gov Identifiers: NCT00006016; NCT00921531).

Everolimus

Everolimus (RAD-001) is an oral derivative of sirolimus that inhibits mTOR. Everolimus was approved by United States FDA in several tumors: advanced kidney cancer, subependymal giant cell astrocytoma associated with tuberous sclerosis, advanced pancreatic neuroendocrine tumors not surgically removable, and metastatic breast cancer (hormone-receptor positive, HER2-negative type) in combination with exemestane[46].

A randomized phase Ⅰ-Ⅱ trial is investigating on the dose limiting toxicity of everolimus given in combination to DEB-TACE (ClinicalTrials.gov Identifier: NCT01009801). The phase Ⅱ multicentric TRACER trial will evaluate TTP of everolimus in patients with unresectable HCC (ClinicalTrials.gov Identifier: NCT01379521).

Sunitinib

Sunitinib is an oral multitargeted receptor TKI, approved by the United States FDA for the treatment of metastatic renal cell carcinoma, imatinib-resistant gastrointestinal stromal tumor[47] and unresectable, locally advanced or metastatic pancreatic neuroendocrine tumors[48].

A phase Ⅱ-Ⅲ SATURNE trial will assess safety (bleeding or hepatic failure at 10 wk post-treatment) of sunitinib (at interrupted schedule before and after TACE) in combination with TACE (ClinicalTrials.gov Identifier: NCT01164202) in about 200 patients with unresectable HCC.

A phase Ⅱ study will evaluate the RR and PFS at 4 mo of patients treated with sunitinib (at interrupted schedule before and after TACE) plus TACE (ClinicalTrials.gov Identifier: NCT00524316).

RFA WITH MOLECULAR TARGETING AGENTS

Clinical studies that analyzed targeted therapy in combination with RFA are summarized in Table 2.

Sorafenib

Patients with small HCC candidate to RFA have an OS at 5 year of 50%-70%[1] (Figure 2C and D).

Considering preclinical data that demonstrated after sorafenib plus RFA a reduced neovascularisation[49], an increased time to recurrence (TTR) (inhibiting HIF-1α and VEGF-A expression) and an inhibited proliferation in HCC models[50], the combination of sorafenib to RFA in patients with unresectable HCC and good liver function is under investigation.

All clinical studies achieving the efficacy of sorafenib addition to RFA are ongoing.

A phase Ⅱ trial will evaluate the anti-angiogenic properties of sorafenib in limiting tumor blood flow measured through a novel MRI technique (ClinicalTrials.gov Identifier: NCT00813293).

The phase Ⅱ SORAMIC trial is considering the benefit of sorafenib addition to RFA in prolonging TTR compared to RFA plus placebo in patients who were candidates for RFA (local ablation group)(ClinicalTrials.gov Identifier: NCT01126645). However, the study will evaluate the benefit of adding radioembolization with yttrium-90 microspheres (SIRT) to sorafenib in comparison to sorafenib alone, in those patients in which RFA is not appropriate and who are not candidate for TACE (palliative group)(ClinicalTrials.gov Identifier: NCT01126645). This study will confirm the non-inferiority or superiority of Gadoxetic acid-based MRI contrast agent (Primovist®-enhanced MRI) compared with contrast-enhanced computer tomography in stratifying patients to a palliative *vs* local ablation treatment strategy (ClinicalTrials.gov Identifier: NCT01126645).

The phase Ⅲ STORM trial will assess efficacy in recurrence free survival of sorafenib after surgical resection or local ablation of HCC, as adjuvant therapy within 4 mo from potentially curative treatment (ClinicalTrials.govIdentifier: NCT00692770).

Bevacizumab

There are only two preclinical studies showing the synergistic interaction between bevacizumab and RFA to reduce pro-angiogenic effect and HCC progression, that occur after insufficient local ablation[4,51].

The most significant study demonstrated whether hyperthermia could induce sublines of a human hepatoma cell line (HepG2 cells) with rapid proliferation and enhanced pro-angiogenic effect through a HIF-1α/VEGF-A dependent mechanism[4]. A subline of HepG2 cells, selected for higher viability and significant heat tolerance after 47uC heat treatment, showed 18% increase in viability and enhanced pro-angiogenic effect compared with parental HepG2 cells in which it was observed an up-regulated cellular protein levels of VEGF-A, HIF-1α and P-AKT, VEGF-A mRNA and secreted VEGF-A[4]. Bevacizumab, inhibiting VEGF-A effect, reduced the difference in pro-angiogenic effect between HepG2 k and parental HepG2[4]. Moreover, the Authors supposed that higher viability subline, hyperthermia-induced, exerted its stronger pro-angiogenic effect through overexpressed PI3K/AKT/HIF-1α/VEGF-A signaling pathway[4].

However, additional preclinical studies are required to confirm the involvement of PI3K/AKT/HIF-1α/VEGF-A pathway in the mechanism of tumor progression and more clinical studies demonstrating the efficacy of bevacizumab to prevent tumor recurrence after RFA.

Thalidomide

Actually, there is only one phase Ⅱ-Ⅲ trial ongoing with aims to evaluate PFS and morbidity of low-dose thalidomide after RFA for unresectable HCC (ClinicalTrials.gov Identifier: NCT00728078).

NEW AGENTS IN DEVELOPMENT

4-[3,5-bis (trimethylsilyl) benzamide] benzoic acid (TAC-101)

Table 2 Ongoing clinical studies that analyzed targeted therapy in combination with radiofrequency ablation

Number of clinical trials	Phase	Drug	Patients (*n*)	Timing	Primary endpoint
NCT01470495	Randomized study	Sorafenib (dose non specified)	200 TR	Together with RFA continuously	TTP
NCT00813293	Ⅱ	Sorafenib (800 mg/d)	20	9 d before RFA	Effectiveness
SORAMIC trial NCT01126645	Ⅱ	Sorafenib (dose non specified)	1500	After RFA or SIRT	Time to recurrence, OS
STORM trial NCT00692770	Ⅲ	Sorafenib (800 mg/d)	1114	After RFA continuously	RFS
NCT00728078	Ⅱ-Ⅲ	Thalidomide (150 mg/d)	200	After RFA for 6 mo	PFS, morbidity

TR: Target recruitment; RFS: Recurrence free survival; RFA: Radiofrequency ablation; TTP: Time to progression; OS: Overall survival; PFS: Progression free survival.

is an oral synthetic retinoid with a specific binding activity on retinoid acid receptor-α[52]. This interaction induces the inhibition of activated protein-1, a transcription factor, which normally activates the expression of metastasis-related genes, including urokinase-type plasminogen activator, matrix metalloproteinase-9, and VEGF[52]. Preclinical models have shown that TAC-101 (at dose established of 20 mg/d) have an antitumor activity in HCC. A recent Japanese phase I study on TAC-101, observed a disease control rate of 38.5% in patients with advanced HCC[53]. Despite of an acceptable toxicity profile, the most frequent side effects were fatigue, headache, and skin toxicity[53].

Actually, a phase Ⅱ study will evaluate the time to appearance of new lesions in patients with advanced HCC treated with TAC-101 (at dose 20 mg/d) plus TACE (ClinicalTrials.gov Identifier: NCT00667628).

Brivanib (BMS-582664) is an oral drug with anti-angiogenic activity, inhibiting VEGFR1-2-3 and fibroblast growth factor receptors (FGFR1-2-3). In a subanalysis performed to evaluate the effects of brivanib (at dose of 800 mg/d), as first-line therapy between Asian and non-Asian patients, the median OS was 10.6 mo in Asian patients and 5.7 mo in non-Asian patients. In contrast, in patients receiving brivanib as second-line therapy, the median OS was comparable between Asian and non-Asian patients (9.8 mo *vs* 9.4 mo, respectively)[54]. A phase Ⅲ BRISK TA trial will evaluate the benefits of brivanib (at dose 200 mg/d), as adjuvant therapy, and TACE association in patients with unresectable HCC (ClinicalTrials.gov Identifier: NCT00908752).

Axitinib is an oral multitargeted receptor TKI, binding VEGFR1-2-3, PDGFR, and c-KITR. Axitinib was approved by FDA in January 2012 for pretreated patients with advanced renal cell carcinoma[55]. A phase Ⅱ Chinese study will achieve two-year survival rate of patients with unresectable HCC, treated with axitinib (5 mg/d for 6 cycle) plus TACE, followed by axitinib alone (ClinicalTrials.gov Identifier: NCT01352728).

Orantinib (TSU-68) is an oral multitargeted receptor TKI, binding VEGFR-2, PDGFR, FGFR and c-KITR[56]. The Asian phase Ⅲ ORIENTAL trial will assess OS in patients with unresectable HCC, treated with orantinib (200 mg/d *bid*) in combination with TACE (ClinicalTrials.gov Identifier: NCT01465464).

DISCUSSION

The combination of loco-regional treatments of HCC plus molecular targeting agents is a recent approach, and a number of questions still remain open: (1) the best sequential timing of targeted therapy and loco-regional therapy; (2) the number of TACE cycles to be performed; (3) the best criteria to evaluate the clinical outcome; (4) the best imaging technique to evaluate response; (5) the best targeted drug to use in combination with loco-regional treatment; and (6) the most correct primary endpoint of the studies.

With regard to the first point, there are three potential schedules: sequential, interrupted or continuous[25]. According to few actual evidences, we don't really know which is the optimal schedule to administer sorafenib in addition to TACE or RFA.

Concerning the second point, there is not a magic number of TACE cycles, in fact TACE is to be repeated as many times as requested. However, it is also true, that after 5 to 6 treatments, the tumor progression usually does not allow further loco-regional approaches, and a systemic treatment alone should be preferred.

About the third point, the only agreement in literature is that RECIST criteria are inadequate[57,58], but, if these criteria should be replaced by EASL, CHOI or MASS criteria is still a matter of debate[59-62]. For what concern tumor necrosis induction, we think that its incorporation in evaluated criteria needs to await a prospective validation like the one that is part of the CALGB 80802 randomized phase Ⅲ trial evaluating sorafenib plus chemotherapy with doxorubicin *vs* sorafenib alone(ClinicalTrials.gov Identifier: NCT01015833).

Considering fourth point, all studies considered, as imaging technique to evaluate response classical MRI and/or contrast-enhanced CT, and only few studies now are investigating on the best imaging technique to assess clinical outcome (SORAMIC, AVATACE-1).

With regard to fifth point, there are more clinical studies that assessed safety and TTP given by the

addition of sorafenib to TACE. Globally, these studies obtained an increased TTP of 7-9 mo in favour to sorafenib adjunct. Considering thalidomide, although the exciting results reported by Asian studies[43,44], the perspective of this drug in the combined HCC approach may be limited because of its low activity as single agent[63]. Nevertheless, based on the favourable safety data, some Authors still think rationale to consider thalidomide in combination with loco-regional therapy, in order to increase outcome of HCC patients[45]. The same considerations of thalidomide apply to bevacizumab[64]. In fact, although this drug in combination with TACE was able to reduce VEGF serum levels, the disappointing results in terms of survival[64], raise some concern about the future of this anti-angiogenic drug. The sixth controversial point is the definition of efficacy. In that regard, the primary endpoints of the ECOG 1208 (ClinicalTrials.gov Identifier: NCT01004978) and SPACE[33] studies are different although both studies try to evaluate efficacy in HCC. The SPACE study has TTP as the primary endpoint and defines failure of therapy as an inability to achieve objective response after more than two TACE procedures in the treated tumor nodule. ECOG 1208 in contrast, using PFS as the primary endpoint, and integrating any progression of disease occurring in the liver and not just in the ablated lesion makes an evaluation more adherent to reality and patient overall health status. As a consequence of this disagreement, the optimal endpoint of the studies, actually, still remains unknown.

CONCLUSION

In early or intermediate stages, traditionally treated with RFA or TACE, the contribution of a systemic therapy may have the effect of reducing neoangiogenesis, thus prolonging time to recurrence and, possibly, survival[1,2,65,66].

It is our opinion that there should not be a long time elapsing between TACE and start of anti-angiogenic therapy, given as "adjuvant", to promote the synergistic interaction between local and systemic treatment, in order to prevent early rebound of VEGF, and, consequently, HCC relapse[13,67,68].

A field of research that should be further developed is related to haematic pro-angiogenic factors (such as VEGF) monitoring (before and after loco-regional therapy), which may predict the usefulness of the addition of targeted therapy in HCC patients[69-72].

In conclusion, the complexity of HCC and its different therapeutic strategies will require continued adjustments based on even more strict multidisciplinary approach and mainly on better knowledge of the biology of this disease[73-77].

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ORIGINAL ARTICLE

Deletion of *Gpr128* results in weight loss and increased intestinal contraction frequency

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Abstract

AIM: To generate a *Gpr128* gene knockout mouse model and to investigate its phenotypes and the biological function of the *Gpr128* gene.

METHODS: Bacterial artificial chromosome-retrieval methods were used for constructing the targeting vector. Using homologous recombination and microinjection technology, a *Gpr128* knockout mouse model on a mixed 129/BL6 background was generated. The mice were genotyped by polymerase chain reaction (PCR) analysis of tail DNA and fed a standard laboratory chow diet. Animals of both sexes were used, and the phenotypes were assessed by histological, biochemical, molecular and physiological analyses. Semi-quantitative reverse transcription-PCR and Northern blotting were used to determine the tissue distribution of *Gpr128* mRNA. Beginning at the age of 4 wk, body weights were recorded every 4 wk. Food, feces, blood and organ samples were collected to analyze food consumption, fecal quantity, organ weight and constituents of the blood and plasma. A Trendelenburg preparation was utilized to examine intestinal motility in wild-type (WT) and *Gpr128*$^{-/-}$ mice at the age of 8 and 32 wk.

RESULTS: *Gpr128* mRNA was highly and exclusively detected in the intestinal tissues. Targeted deletion of *Gpr128* in adult mice resulted in reduced body weight gain, and mutant mice exhibited an increased frequency of peristaltic contraction and slow wave potential of the small intestine. The *Gpr128*$^{+/+}$ mice gained more weight on average than the *Gpr128*$^{-/-}$ mice since 24 wk, being 30.81 ± 2.84 g and 25.74 ± 4.50 g, respectively (n = 10, P < 0.01). The frequency of small intestinal peristaltic contraction was increased in *Gpr128*$^{-/-}$ mice. At the age of 8 wk, the frequency of peristalsis with an intraluminal pressure of 3 cmH2O was 6.6 ± 2.3 peristalsis/15 min in *Gpr128*$^{-/-}$ intestine (n = 5) *vs* 2.6 ± 1.7 peristalsis/15 min in WT intestine (n = 5, P < 0.05). At the age of 32 wk, the frequency of peristaltic contraction with an intraluminal pressure of 2 and 3 cmH_2O was 4.6 ± 2.3 and 3.1 ± 0.8 peristalsis/15 min in WT mice (n = 8), whereas in *Gpr128*$^{-/-}$ mice (n = 8) the frequency of contraction was 8.3 ± 3.0 and 7.4 ± 3.1 peristalsis/15 min, respectively (2 cmH_2O: P < 0.05 *vs* WT; 3 cmH_2O: P < 0.01 *vs* WT). The frequency of slow wave potential in *Gpr128*$^{-/-}$ intestine (35.8 ± 4.3, 36.4 ± 4.2 and 37.1 ± 4.8/min with an intraluminal pressure of 1, 2 and 3 cmH_2O, n = 8) was also higher than in

WT intestine (30.6 ± 4.2, 31.4 ± 3.9 and 31.9 ± 4.5/min, n = 8, P < 0.05).

CONCLUSION: We have generated a mouse model with a targeted deletion of *Gpr128* and found reduced body weight and increased intestinal contraction frequency in this animal model.

Key words: G-protein-coupled receptors; *Gpr128*; Knockout mouse; Weight loss; Intestinal contraction frequency

Core tip: The Adhesion family is the second largest subfamily of the G-protein-coupled receptors (GPCR). The physiological function of the orphan Adhesion-GPCR *Gpr128* is unknown. In the present study, we generated *Gpr128* knockout mice and confirmed the selective expression of *Gpr128* in the intestinal tissues. Phenotypic analysis revealed that targeted deletion of *Gpr128* in the mouse resulted in reduced body weight gain and increased frequency of peristaltic contraction and slow wave potential in the small intestine. The physiological roles of *Gpr128* in the gastrointestinal tract and its potential as a therapeutic target for obesity and nutritional disorders warrant further investigation.

Ni YY, Chen Y, Lu SY, Sun BY, Wang F, Wu XL, Dang SY, Zhang GH, Zhang HX, Kuang Y, Fei J, Gu MM, Rong WF, Wang ZG. Deletion of *Gpr128* results in weight loss and increased intestinal contraction frequency. *World J Gastroenterol* 2014; 20(2): 498-508 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/498.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.498

INTRODUCTION

G protein-coupled receptors (GPCRs) constitute one of the largest protein families in humans[1,2] and play important roles in the transduction of intercellular signals across the plasma membrane *via* different G-proteins[3,4]. GPCRs respond to a large variety of extracellular signals including small molecules such as Ca^{2+}, hormones, peptides, chemokines and other factors as well as sensory stimuli such as vision, smell, taste and neuronal transmission in response to photons[5]. Due to their extremely diverse roles in biological processes, GPCRs represent important molecular targets for biomedical research and drug discovery[6].

The adhesion family of GPCRs (Adhesion-GPCRs) is the second largest subfamily of GPCRs, with over 30 members found in mammals[7,8]. These proteins are characterized by the dual presence of a secretin-like seven-transmembrane (7TM) domain and a long cell adhesion-like N-terminal domain, which typically consists of a functional GPCR proteolytic site domain (GPS domain) and one or more conserved domains[9,10]. Generally, the long N-termini bind various proteins that promote cell-to-cell and cell-to-matrix interactions[11]. However, some Adhesion-GPCRs were found to have a GPS domain but to lack the conserved domains. *HE6* and *GPR56* are two such members for which no N-terminal conserved domains have been identified, although they have both been shown to have adhesive properties. *HE6* attachment appeared to be required for the maturation of germ cells because mutation of this receptor resulted in male infertility in mice[12]. Mutations in *GPR56* have been shown to be associated with cortical malformation of the human brain[13,14] and to participate in tumor cell adhesion[15,16].

GPR128 is an orphan receptor of the Adhesion-GPCR family uncovered during BLASTP searches of the Celera database in 2003. *GPR128* is phylogenetically related to *HE6* and *GPR56* and lacks the conserved N-termini domains apart from the GPS domain[17]. The mouse *Gpr128* shares 69.9% homology with human *GPR128* and contains 16 exons.

GPCRs are expressed in virtually all tissue types in the body[18]. However, some GPCRs are expressed in specific tissues and therefore are important targets for drug discovery[19]. The tissue distribution of *GPR128*, as derived from the EST data or analysed by real-time quantitative polymerase chain reaction (RT-qPCR), shows specific patterns in human and mouse gastrointestinal tissue[20,21]. However, until the commencement of this study, there was little information regarding the ligand or the physiological function of *GPR128* in mammals. Using PCR, Northern blotting and immunofluorescence staining, we show that *Gpr128* might be exclusively expressed in mouse intestine tissue. To study the role of *Gpr128* in the intestine, we generated mice with a targeted deletion of *Gpr128*. We found that *Gpr128* knockout mice exhibited less body weight gain and an increase in intestinal contraction frequency compared with their wild-type (WT) counterparts.

MATERIALS AND METHODS

Construction of the Gpr128 targeting vector and electroporation of embryonic stem cells

The 129/Sv bacterial artificial chromosome (BAC) clone bMQ-239c21 was provided by the Sanger Institute. BAC-retrieval methods were used for constructing the targeting vector[22,23].

The sequence, including the GPS domain and a portion of the 7TM domain, was retrieved from the BAC clone using a retrieval vector containing two homologous arms.

A targeting vector was constructed by replacing the mouse *Gpr128* genomic fragment (8.4 kb) covering exons 10-12 with the 1.9-kb phosphoglycerate kinase-neomycin resistance (PGK-Neo) cassette for positive selection and was laid with an external herpes simplex virus-1-thymi-

dine kinase cassette for negative selection[24]. Additionally, this deletion causes an out-of-frame reading frame shift and thereby generates a loss-of-function allele.

The targeting vector contained 7.1 kb of homologous DNA upstream of the PGK-Neo cassette and 5.3 kb of homologous DNA downstream of the cassette as homologous recombination arms. After linearization, the targeting vector was electroporated into embryonic stem (ES) cells derived from 129/Sv G418- and GANC-resistant clones were selected using two pairs of PCR primers. The sequences of the primers used for identifying the recombinant clones are as follows: 5'-CCATAGGAAGAATAATATCAACCAATC-3' (forward primer P1), 5'-CTGAGCCCAGAAAGCGAAGGA-3' (reverse primer P2), 5'-ACAAAAGCAAAACAAGGTCTGGAAAG-3' (forward primer P3) and 5'-CCTCCCCCGTGCCTTCCTTGAC-3' (reverse primer P4).

Generation of Gpr128 knockout mice

Chimeric male mice were generated by injecting the recombinant ES cell clone into C57BL/6 blastocysts, which were subsequently implanted into pseudopregnant female recipient mice. Germ line transmission was monitored by a coat color marker. Heterozygous mice were generated by crossing chimeras with WT 129/Sv female mice and selected for sib mating to create WT (*Gpr128*$^{+/+}$), heterozygous (*Gpr128*$^{+/-}$) and homozygous mice (*Gpr128*$^{-/-}$) for further experiments.

The mice were genotyped by PCR analysis of tail DNA using two primer pairs, which allows the amplification of WT and targeted alleles. The forward primer P3 and reverse primer P4 were used to amplify the 3' targeted allele, which yields a 5.7 kb band. The sequences of the primers used to amplify the WT allele are as follows: 5'-TCTTCATCTCATTAGTTGGATGGGGTA-3' (forward primer P5) and 5'-ACAAAAGCAAAACAAGGTCTGGAAAG-3' (reverse primer P6). The length of the WT allele is 5.4 kb.

Semi-quantitative RT-PCR

All experiments involving animals were conducted under protocols approved by Institutional Animal Care and Use Committee of Shanghai Research Center for Model Organisms (Approval ID: 2010-0017), and the care of animals was in accord with the institution's guidelines.

The mice were anesthetized with ketamine and xylazine diluted in 0.9% saline, and all efforts were made to minimize animal suffering. Total RNA was extracted from adult mouse tissues using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. For RT-PCR analysis, total RNA was treated with RNase-free DNase I (Promega, Fitchburg, Wisconsin, United States) and quantitated. A 1-μg sample of total RNA was reverse-transcribed to cDNA with an RNA PCR kit (Takara, Dalian, Liaoning, China) according to the standard protocol. A fragment of *Gpr128* was amplified (25 cycles) with forward primer R1 (5'-GATTCCAACTTCATTACTCTG-3') and reverse primer R2 (5'-GGTCCATATCTGCCCACTG-3'). β-actin was amplified as a control. As shown in Figure 1D, the specific Gpr128 fragment from WT mice was amplified with forward primer R3 (5'-AACCACAAACTTT TCCAATCAA-3') and reverse primer R4 (5'-CCACT CAGGGCATAAATAC TCC-3').

Northern blotting analysis

Total RNA was extracted from adult mouse tissues using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Northern blotting was performed as described in the manual provided by the manufacturer (Northern Max-Gly; Ambion Inc., Carlsbad, CA, United States). A 1-μg aliquot was removed from each mRNA sample from adult WT mice for analysis. The probe used for *Gpr128* was a 715-bp DNA fragment prepared from mouse intestine cDNA using the PCR forward primer N1 (5'-AGAGTCGACAGACAGACCACTGAAGGGAAG-3') and reverse primer N2 (5'-TGGCA TCAAAATCTGACTC-3'). Probe DNA (25 ng) was labeled with [a^{32}P]-dATP using a Random Primer Labeling Kit (NEBlot Kit, NEB, Beverly, MA, United States) and subsequently purified by gel filtration.

Maintenance and body weight studies of Gpr128-deficient mice

All mice used in this study were on a mixed 129/BL6 background. The mouse colony was maintained in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle, and the mice were fed a standard laboratory chow diet with free access to water. The animals were maintained by crossing heterozygous progeny.

Beginning at the age of 4 wk, body weights were recorded every 4 wk. Animals of both sexes were used, but littermates were matched by gender.

Histology and immunofluorescence staining

The intestines of WT and *Gpr128*$^{-/-}$ mice at 8 wk of age were collected and fixed with 10% formalin for sectioning followed by hematoxylin and eosin (HE) staining. Sections (6 mm) were cut and stained with HE according to standard procedures. For immunofluorescence analysis, paraffin-embedded sections were deparaffinized with xylene and treated with gradually decreasing concentrations of ethanol. The sections were blocked for 1 h in 5% bovine serum followed by staining overnight at 37 ℃ with goat anti-GPR128 antibodies (sc-48208, Santa-Cruz Biotechnology Inc., Santa-Cruz, CA, United States) for human and mouse tissues and finally incubated with fluorescent-conjugated secondary antibody for 30 min. Finally, the slides were rinsed with PBS and mounted with VECTASHIELD mounting medium (H-1200, Vector Laboratories Inc., Burlingame, CA, United States).

Food consumption studies and fecal quantity analysis

At week 16 of the experimental diet period, the mice were

individually caged and given preweighed food for 5 d. During this period, the amount of food consumed was determined, and feces were quantitatively collected over a 24 h period. The results are expressed as grams of food consumed and feces excreted per day.

Analyses for the constituents of the blood and plasma

After the 32 wk experimental feeding period, the mice were fasted for 16 h and subsequently anesthetized with ketamine and xylazine diluted in 0.9% saline. Blood was removed by cardiac puncture into tubes containing 1 mmol/L EDTA. White adipose (epididymal and uterine fat pads) and brown adipose (intrascapular) tissue as well as the heart, liver, spleen, lungs, and kidneys were removed, and the wet weight of each was recorded.

Blood samples were collected for complete blood counts including white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, white-small cell rate, white-middle cell rate, and white-large cell rate using an automated hematology analyzer (Poch-100ivd, Sysmex, Kobe, Japan). Plasma was obtained by low-speed centrifugation of the blood samples for measurement of albumin/globulin, globulin, low-density lipoprotein cholesterol, albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, urea nitrogen, creatinine, glucose, high-density lipoprotein cholesterol, lactate dehydrogenase, total cholesterol, triglycerides and total protein using an automated chemistry analyzer (CHEMIX-180; Sysmex, Kobe, Japan).

Analysis of intestine motility

Male and female mice at 8 and 32 wk of age were sacrificed. A Trendelenburg preparation was utilized to examine intestinal motility in WT and *Gpr128*$^{-/-}$ mice. Briefly, the jejunum was removed and placed in pre-oxygenated Kreb's Ringer solution at room temperature. A segment of the jejunum (6 cm long) was placed into an organ bath and was superfused with oxygenated Krebs solution at 37 ℃. Both ends of the jejunum were catheterized. The proximal tube was connected to a syringe cylinder (for altering the resting intraluminal pressure) and a pressure transducer *via* a three-way stopcock. A glass micropipette (tip diameter approximately 50 μm) was placed on the intestinal wall to record the slow waves through gentle suction. The peristalsis and slow waves were fed into a computer through the Micro1401 interface (Cambridge Electronic Design, United Kingdom) and analyzed using the Spike2 program (CED, United Kingdom). The preparation was allowed to stabilize for at least 40 min before the experiments were started.

Statistical analysis

The data are presented as the mean ± SD. Differences between groups were determined by the 2-tailed Student *t* test. *P* values less than 0.05 were considered significant.

RESULTS

Targeted disruption of the Gpr128 gene

To investigate the potential roles of *Gpr128* in mice, we generated a targeted disruption of the mouse *Gpr128* gene in ES cells by homologous recombination. In the targeting vector, 3 exons (10, 11 and 12), which encode the GPS domain and a portion of 7TM domain, were replaced with a PGK cassette followed by the neomycin resistance gene (Figure 1A). After electroporating ES cells with the linearized targeting vector under positive-negative selection, we identified three targeted ES clones by PCR (Figure 1B). Two of these clones were microinjected into C57BL/6 blastocysts to obtain chimeras. Mice heterozygous for *Gpr128* showed normal development and were fertile, indicating that the targeted locus does not have detrimental dominant activity.

The genotypes of the offspring were analyzed by PCR to identify WT (+/+), heterozygous (+/-), and homozygous (-/-) mice. Amplification of the WT and targeted alleles produced bands of 5.4 and 5.7 kb, respectively (Figure 1C). As expected, the ratio of phenotypes was in accord with Mendelian frequency, indicating that there was no increased embryonic mortality in the mutant animals. Semi-quantitative RT-PCR and immunofluorescence staining demonstrated that *Gpr128* was not detected in the intestine of homozygous mice (Figure 1D and E), indicating that we have successfully established a *Gpr128* disruption mouse model.

Gpr128 is specifically expressed in the mouse intestine

We investigated the expression pattern of the WT *Gpr128* gene in adult mouse tissues by semi-quantitative RT-PCR, Northern blotting and immunofluorescence staining. *Gpr128* mRNA was highly and exclusively detected in the intestine (Figure 2A, B and D). RT-PCR was then performed to determine the presence of *Gpr128* mRNA throughout the digestive tract and at different postnatal development stages. *Gpr128* expression was detected prominently in the small intestine and colon from postnatal day 0 through 8 wk (Figure 2C). The distribution of Gpr128 protein in the mouse intestine was then analyzed by immunofluorescence staining. We found that the Gpr128 protein was confined to the mucosa. As shown in Figure 2D, Gpr128 expression was restricted to epithelial cells.

Gpr128$^{-/-}$ mice gained significantly less body weight than their WT counterparts

Mice lacking the *Gpr128* gene (*Gpr128*$^{-/-}$) grew normally and displayed normal reproductive functions on a standard mouse chow diet. We found no differences between *Gpr128*$^{+/+}$ and *Gpr128*$^{-/-}$ mice with respect to food intake or fecal excretion (Figure 3B and C). However, *Gpr128*$^{-/-}$ mice gained less weight on average than their *Gpr128*$^{+/+}$ littermates by 24 wk of age. The body weights of WT and *Gpr128*$^{-/-}$ mice were 30.81 ± 2.84 and 25.74 ± 4.50 g, respectively (Figure 3A, $n = 10$, $P < 0.01$). When separated by sex, both male and female *Gpr128*$^{-/-}$ mice gained

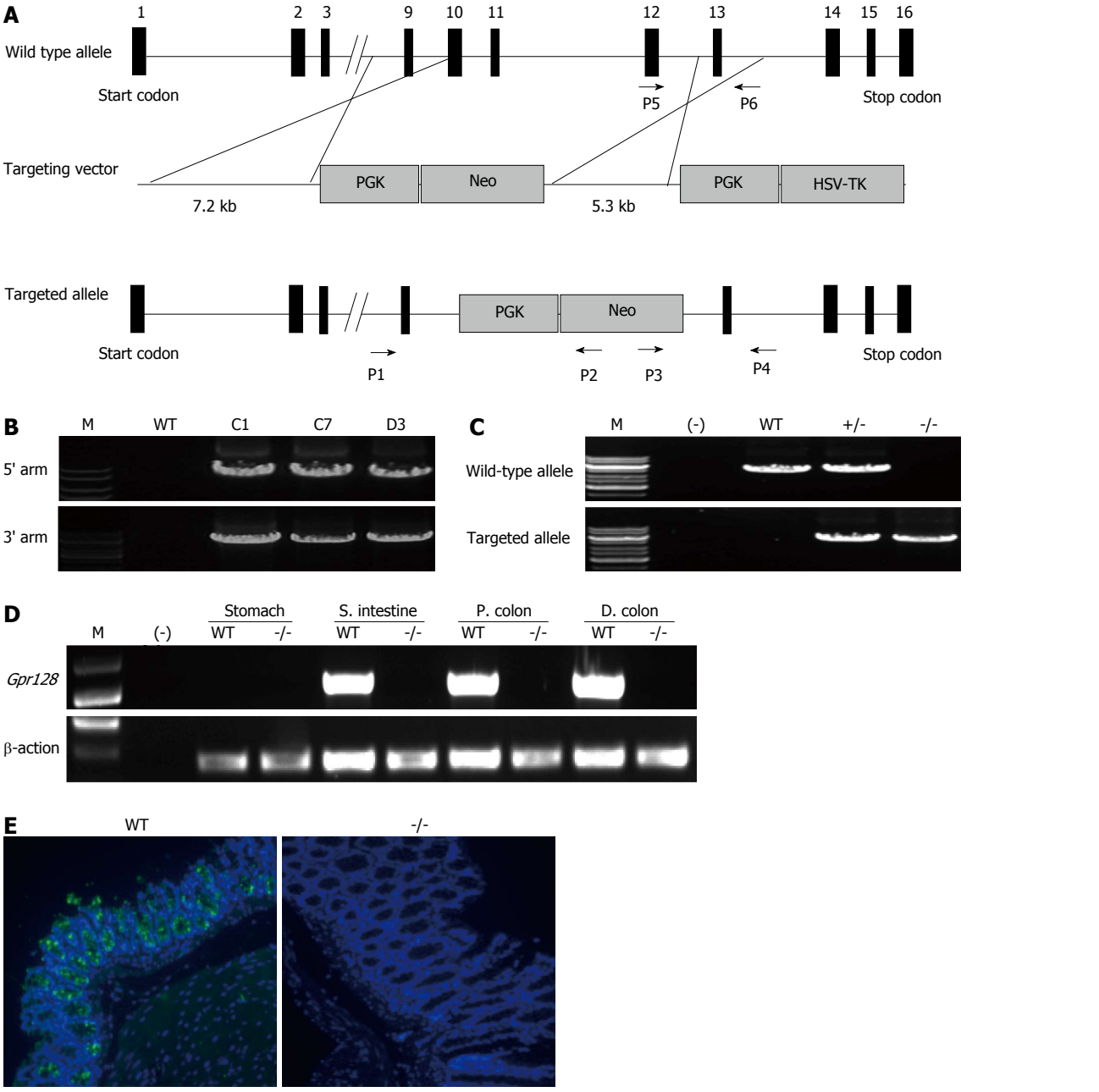

Figure 1 Targeted deletion of *Gpr128* in mice. A: Gene targeting strategy. Numbered boxes represent *Gpr128* coding exons. The start codon and stop codon are indicated as a star and pound sign, respectively. The targeting vector contains a 7.1-kb 5' arm and a 5.3-kb 3' arm. Exons 10, 11 and 12 of the *Gpr128* gene were replaced by a PGK-Neo cassette through homologous recombination. The primer pairs for polymerase chain reaction (PCR) genotyping are indicated by arrows (5' arm: P1, P2; 3' arm: P3, P4); B: PCR screening for targeted embryonic stem (ES) cell clones. Correctly recombined clones show 7.7 and 5.7 kb bands, respectively. Three recombined ES cell clones show the expected bands as detected with primers P1-P4; C: PCR analysis of genomic tail DNA derived from *Gpr128*$^{+/-}$ mouse intercrossing. A 5.4-kb fragment amplified with primers P5 and P6 represents the wild-type (WT) allele. A 5.7-kb band was amplified from the targeted allele with P3 and P4; D: *Gpr128* expression in gastrointestinal tissue with two different genotypes by semiquantitive reverse transcription-polymerase chain reaction. A specific *Gpr128* fragment, which exists in WT mice, was deleted in *Gpr128*$^{-/-}$ mice. The transcript for β-actin was examined as a control for RNA loading and integrity; E: Expression pattern of *Gpr128* protein in WT and *Gpr128*$^{-/-}$ adult mouse colon revealed by immunofluorescence (original magnification, × 200). M: Marker lane; (-): Negative control without template; S. intestine: Small intestine; P. colon: Proximal colon; D. colon: Distal colon.

less weight than their WT counterparts (data not shown). The decreased weight gain in *Gpr128*$^{-/-}$ mice persisted at 28 and 32 wk (26.69 ± 4.29 and 28.46 ± 4.42 g *vs* 33.15 ± 3.20 and 36.75 ± 4.18 g in *Gpr128*$^{+/+}$ mice, $n = 10$, $P < 0.01$, Figure 3A).

To account for the differences in body weight gain between the *Gpr128*$^{+/+}$ and *Gpr128*$^{-/-}$ mice, various tissues were removed and weighed. There were no differences in the epididymal and uterine fat pads, brown fat, or liver weights between male and female *Gpr128*$^{+/+}$ and *Gpr128*$^{-/-}$ mice (Figure 3D). There were also no differences in heart, spleen, lung, and kidney weights between the *Gpr128*$^{+/+}$ and *Gpr128*$^{-/-}$ mice (Figure 3D).

The cell counts and biochemical parameters of the

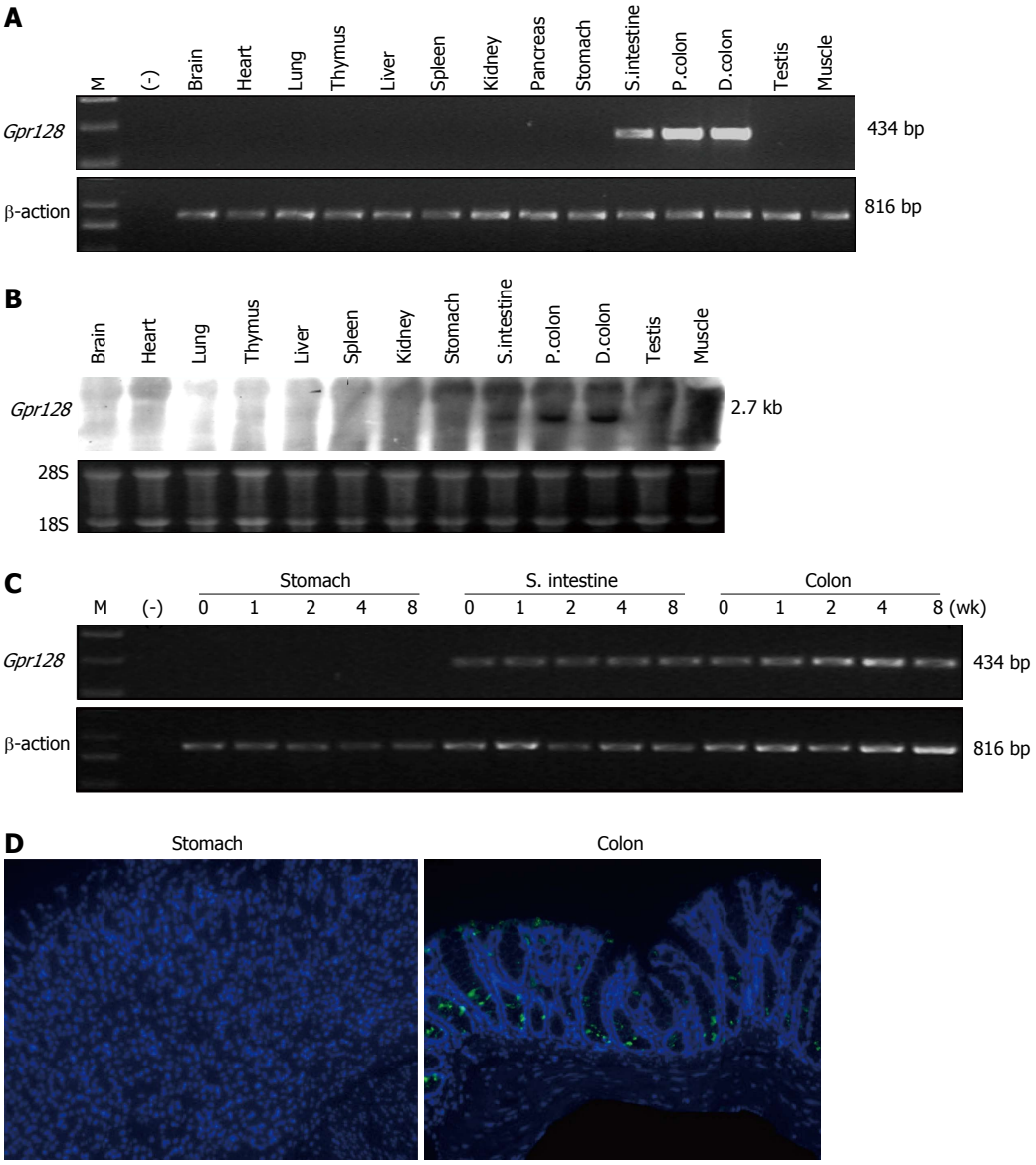

Figure 2 Selective expression of *Gpr128* within the intestine in mice. A: Expression levels of *Gpr128* mRNA. The mRNA levels were examined in major tissues of normal mice using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), and the expression level of β-actin was used as an endogenous control. M: Marker lane; (-): Negative control without template; B: Northern blotting analysis of *Gpr128*. Total RNA from wild type mice was extracted and subjected to Northern blotting analysis using a 715-bp fragment of *Gpr128* cDNA corresponding to exons 1 through 6. The bottom lane shows the 28S and 18S ribosomal RNA as a control; C: Examination of the stage-specific expression of *Gpr128* mRNA. RT-PCR was performed throughout the digestive tract and at various postnatal developmental stages to determine the presence of *Gpr128* mRNA from postnatal day 0 through 8 wk; D: Expression pattern of *Gpr128* protein in the stomach and colon of adult WT mouse revealed by immunofluorescence (original magnification, × 200).

blood of *Gpr128*$^{-/-}$ mice were not different from those of the WT mice (Figure 3E and F). Furthermore, there were no overt differences in the gross morphology or histology (HE staining) of the GI tract between the *Gpr128*$^{-/-}$ and the WT mice (data not shown).

Increased frequency of peristalsis and slow waves of the small intestine in Gpr128$^{-/-}$ *mice*

Using a Trendelenburg model, we analyzed the peristalsis and the slow waves of the small intestine (jejunum) in WT and *Gpr128*$^{-/-}$ mice (Figure 4A). The amplitudes of peristaltic movement at resting intraluminal pressures of 0, 1, 2 and 3 cmH_2O were not different between WT and *Gpr128*$^{-/-}$ mice (data not shown). The frequency of peristaltic contraction was increased in *Gpr128*$^{-/-}$ mice since 8 wk when the resting intraluminal pressure increased. The frequency of peristalsis was higher in *Gpr128*$^{-/-}$ mice than in WT mice when the resting intraluminal pressure was 3 cmH_2O (6.6 ± 2.3 peristalsis/15 min in *Gpr128*$^{-/-}$ intestine *vs* 2.6 ± 1.7 peristalsis/15 min in WT intestine, $n = 5$, $P < 0.05$, Figure 4B). At the age of 32 wk, the frequency of peristalsis was higher in *Gpr128*$^{-/-}$ mice than in WT mice when the resting intraluminal pressure was 2 or 3 cmH_2O (8.3 ± 3.0 and 7.4 ± 3.1 peristalsis/15 min in *Gpr128*$^{-/-}$ intestine *vs* 4.6 ± 2.3 and 3.1 ± 0.8 peristalsis/15 min in WT intestine, $n = 8$, 2 cmH_2O: $P < 0.05$, 3 cmH_2O: $P < 0.01$, Figure 4C) and the frequency of slow waves was also higher in *Gpr128*$^{-/-}$ intestine compared

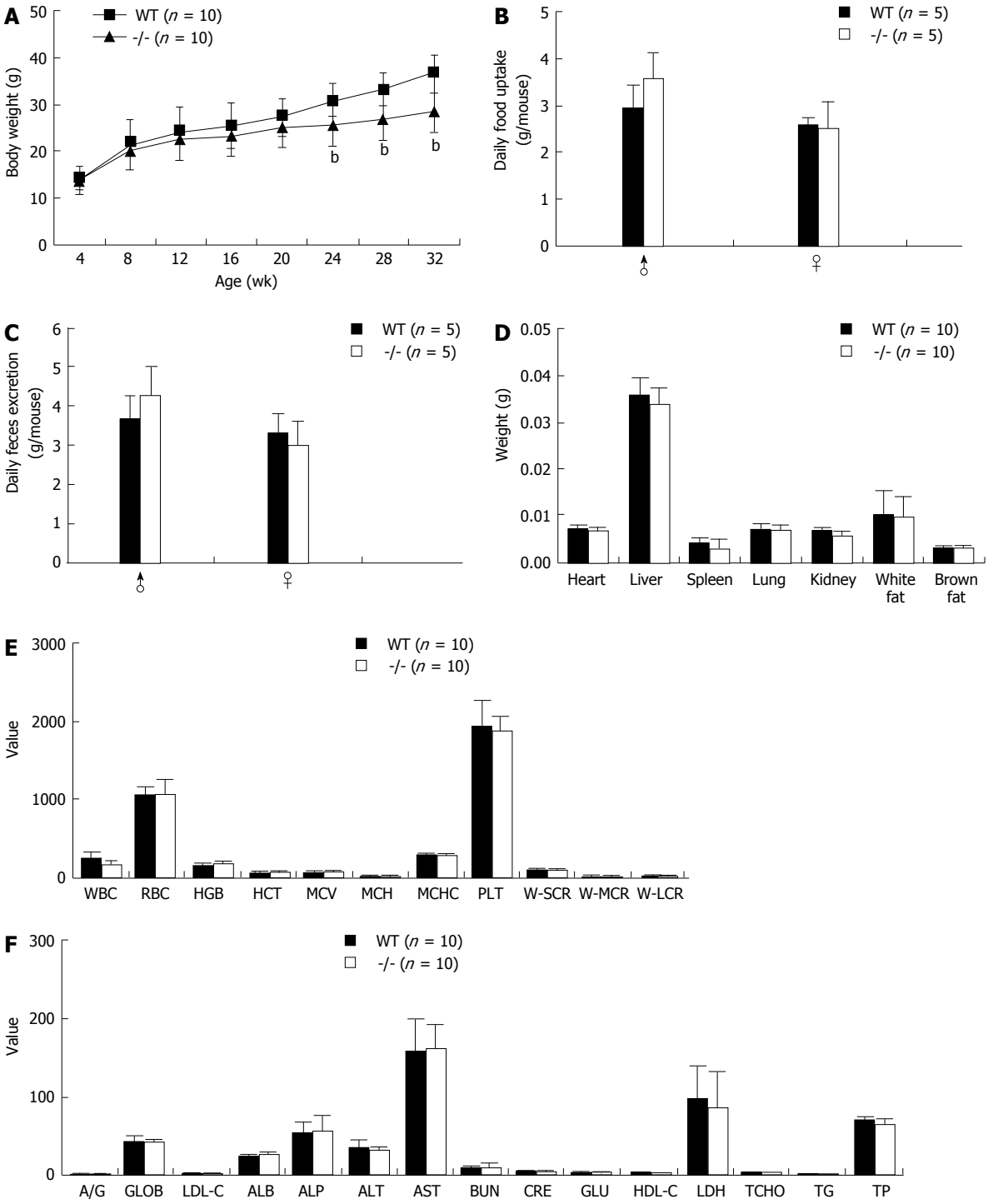

Figure 3 Deletion of Gpr128 results in reduced body weight gain in mice. A: An analysis of the body weight of mice of different genotypes shows that Gpr128-/- mice have a reduced body weight (*P* values of weeks 24, 28 and 32 are 0.0065, 0.0010 and 0.0003); B: Daily food intake of 16-wk-old mice of different genotypes (*P* > 0.05); C: Daily fecal excretion of 16-wk-old mice of different genotypes (*P* > 0.05); D: Organs isolated from 32-wk-old animals weighed and correlated to body weight (*P* > 0.05); E: Blood routine test of 32-wk-old animals using an automated hematology analyzer (*P* > 0.05; WBC: White blood cells; RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelet; W-SCR: White-small cell rate; W-MCR: White-middle cell rate; W-LCR: White-large cell rate); F: Biochemical parameters of 32-wk-old animals using an automated chemistry analyzer (*P* > 0.05; A/G: Albumin/globulin; GLOB: Globulin; LDL-C: Low-density lipoprotein cholesterol; ALB: albumin; ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Urea nitrogen; CRE: Creatinine; GLU: Glucose; HDL-C: High-density lipoprotein cholesterol; LDH: Lactate dehydrogenase; TCHO: Total cholesterol; TG: Triglyceride; TP: Total protein). All values are mean ± SD (*n* = 10, $^{b}P < 0.01$ *vs* wild-type group).

with WT intestine (30.6 ± 4.2, 31.4 ± 3.9, and 31.9 ± 4.5/min and 35.8 ± 4.3, 36.4 ± 4.2, and 37.1 ± 4.8/min in normal and *Gpr128*$^{-/-}$ mice, respectively, $n = 8$, $P < 0.05$, Figure 4D).

DISCUSSION

Here, we describe the first genetic analysis of *Gpr128* function in a mammalian model. A targeted mutation of

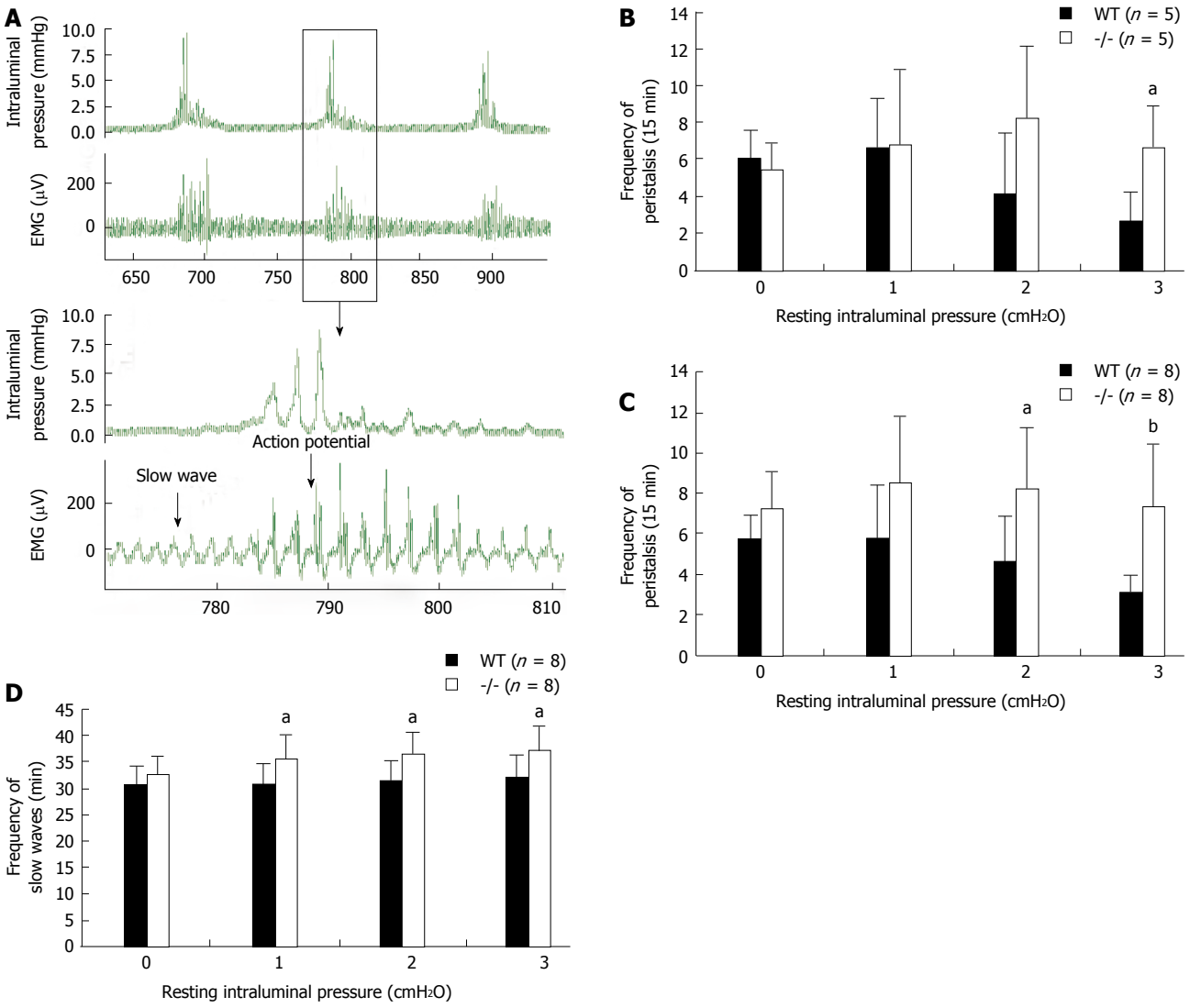

Figure 4 ***Gpr128*** **deficiency leads to increased frequency of intestinal contraction.** A: The raw traces of intraluminal pressure of a jejunum segment of *Gpr128*$^{-/-}$ mice and the simultaneously recorded extracellular electrical potential from the gut wall. The lower panel of A shows an expanded view of the recording within the square of the upper panel; B: Frequency of peristalsis in wild-type (WT) and *Gpr128*$^{-/-}$ mice of 8 wk. The frequency of peristalsis was increased in *Gpr128*$^{-/-}$ mice at a resting intraluminal pressure of 3 cmH$_2$O (n = 5, P = 0.0137); C: Frequency of peristalsis in WT and *Gpr128*$^{-/-}$ mice of 32 wk. The frequency of peristalsis was increased in *Gpr128*$^{-/-}$ mice at resting intraluminal pressures of 2 and 3 cmH$_2$O (n = 8, 2 cmH$_2$O: P = 0.0166, 3 cmH$_2$O: P = 0.0020); D: Frequency of slow waves in WT and *Gpr128*$^{-/-}$ mice of 32 wk. The frequency of slow waves was increased in *Gpr128*$^{-/-}$ mice at resting intraluminal pressures of 1, 2 and 3 cmH$_2$O (n = 8, 1 cmH$_2$O: P = 0.0303, 2 cmH$_2$O: P = 0.0271, and 3 cmH$_2$O: P = 0.0402). All values are mean ± SD ($^aP < 0.05$, $^bP < 0.01$ *vs* wild-type group).

GPR128 causes a deletion of part of the 7TM region (Figure 1A) and is presumably a null allele. Residual WT transcripts could not be detected in the intestines of mutant mice (Figure 1D and E).

GPR128 is an orphan GPCR, the physiological function of which is unknown. To explore the role of *Gpr128*, we first examined its expression profile in different tissues. We found that *Gpr128* mRNA expression is exclusively confined to the small intestine and colon. Through immunofluorescence staining, Gpr128 immunoreactivity was detected in the mucosa of the intestine and was found to be restricted to epithelial cells.

The cell count and biochemical parameters of *Gpr128*$^{-/-}$ mice were not different from those of their WT counterparts, indicating that *Gpr128* is not essential for the maintenance of homeostasis.

A major finding in the *Gpr128*$^{-/-}$ mice was the lower body weight gain compared with the WT littermates by 24 wk of age when the animals were maintained on a standard laboratory rodent chow diet. Additionally, there were no significant differences in the weights of epididymal or uterine fat pads, brown fat, or the liver between WT and *Gpr128*$^{-/-}$ mice. These data suggest that the observed weight difference between the mice was not due to reduced adiposity in the *Gpr128* knockout mice.

A number of factors may potentially participate in the regulation of energy balance and weight gain, including gastric emptying[25], gastrointestinal motility[26] as well as gastrointestinal peptides such as ghrelin and cholecystokinin. The release of these two hormones is known to be regulated by ingestion and their action may in turn regulate gastrointestinal function and food intake[29,30].

However, given that *Gpr128*$^{-/-}$ and WT mice consumed equivalent amounts of chow, the excretion of feces was similar in the two groups and *Gpr128* was confined to the intestinal tissue, we tested the potential differences in intestinal motility between *Gpr128*$^{-/-}$ and WT mice. The frequency of peristaltic movement and slow waves were found to be increased in *Gpr128*$^{-/-}$ intestine compared with WT intestine. Despite similar levels of chow consumption, *Gpr41*$^{-/-}$ mice colonized with the model fermentative community are significantly leaner and lighter than their WT littermates because their increased intestinal motility reduces the time required to harvest energy from the diet[31]. Whether the increase in gut motility accounts for the lower weight gain in *Gpr128*$^{-/-}$ mice awaits further investigation. Because peristalsis is known to be regulated by the enteric nerve plexus[32], whereas the slow waves are known to originate from the interstitial cells of Cajal[33], further studies should be conducted to examine their development and function in *Gpr128*$^{-/-}$ mice. Given the epithelial localization of Gpr128 within the gut, it will also be important to explore its role in the regulation of intestinal secretion and absorption.

In summary, the present study shows that *Gpr128* is expressed exclusively in the small and large intestine, and *Gpr128* deficiency resulted in a decrease in body weight gain and an increase in intestinal motility. The potential for *Gpr128* as a novel therapeutic target for obesity and nutritional disorders is worth exploring.

COMMENTS

Background

The Adhesion family is the second largest subfamily of G-protein-coupled receptors (GPCR) which is one of the largest superfamilies of cell-surface receptors. Family members are characterized by the dual presence of a secretin-like seven-transmembrane domain and a long cell adhesion-like N-terminus that typically contains one functional GPCR proteolytic site domain domain; however, the function of most of these receptors is still not understood.

Research frontiers

An orphan receptor of the Adhesion-GPCR *GPR128* was identified during BLASTP searches of the Celera database in 2003. The tissue distribution of *GPR128* derived from the EST data shows specific pattern in humans and mice. The physiological function of *GPR128* in mammals is still unknown.

Innovations and breakthroughs

In this study, the authors generated a targeted deletion of *Gpr128* mouse model to explore the biological function of *Gpr128*. Furthermore, they found that *Gpr128* is exclusively expressed in mouse intestinal tissue. Finally, we showed that the targeted deletion of the orphan adhesion-GPCR *Gpr128* resulted in reduced body weight gain and increased intestinal contraction frequency in mice.

Applications

The present findings regarding the activities of *Gpr128* in mouse intestinal cells showed for the first time that *Gpr128* is a regulator of host energy balance and may help explain the biological functions of *Gpr128* in the intestine. Future studies are needed to identify the ligands of *Gpr128* which are often the key to determining the functional role, and to determine the mechanism by which *Gpr128* regulates intestinal contraction frequency. *Gpr128* may be a potential drug target and may be useful for the development of novel therapies for obesity and nutritional disorders.

Terminology

GPCRs constitute one of the largest protein families in humans. GPCRs receive extracellular signals and transmit them into cells *via* an intracellular signaling pathway that employs different G-proteins. The GPCR family has attracted significant attention from researchers due to its important role in drug discovery.

Peer review

After the generation of a *Gpr128* gene knockout mouse model and the investigation of its phenotypes and the biological function of *Gpr128*, the authors found that the deletion of *Gpr128* in mice resulted in weight loss and increased intestinal contraction frequency. The authors attempted to demonstrate the relationship between weight loss and intestinal motility. Overall, this study fits nicely within the scope of the journal. The data are generally clean and could potentially uncover the physiological roles of *Gpr128*, which is of value to the field.

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BRIEF ARTICLE

Long-term follow-up of ulcerative colitis patients treated on the basis of their cytomegalovirus antigen status

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Abstract

AIM: To clarify the impact of cytomegalovirus (CMV) activation and antiviral therapy based on CMV antigen status on the long-term clinical course of ulcerative colitis (UC) patients.

METHODS: UC patients with flare-up were divided into CMV-positive and -negative groups according to the CMV antigenemia assay. The main treatment strategy provided for the patients in the CMV-positive group comprised a dose reduction of corticosteroids and administration of ganciclovir.

RESULTS: The median number of days to initial remission was significantly greater for the patients in the CMV-positive group (21 d *vs* 16 d, $P = 0.009$). However, the relapse rate after remission and colectomy rate during more than 30 mo of observation did not differ between the two groups. Multivariate analysis revealed that administration of ganciclovir was the only independent factor for avoiding colectomy in patients of the CMV-positive group.

CONCLUSION: CMV antigen status did not significantly affect the long-term prognosis in UC patients under treatment with appropriate antiviral therapy.

Key words: Ulcerative colitis; Cytomegalovirus; Cytomegalovirus antigenemia assay

Core tip: Cytomegalovirus (CMV) reactivation has a deleterious effect in patients with ulcerative colitis (UC). Although antiviral therapy for CMV antigen-positive UC patients may be effective in the short-term, the long-term prognosis of UC patients with CMV treated by antiviral agents remains unknown. Our study revealed that positive CMV antigen status is likely to prolong time to remission in the treatment of flare-up of UC; however, long-term prognosis, including colectomy rate, was not affected by CMV antigen status treated with antiviral agents. Ganciclovir use is an independent factor for avoidance of colectomy in CMV antigen-positive UC patients.

Inokuchi T, Kato J, Hiraoka S, Suzuki H, Nakarai A, Hirakawa T, Akita M, Takahashi S, Harada K, Okada H, Yamamoto K. Long-term follow-up of ulcerative colitis patients treated on the basis of their cytomegalovirus antigen status. *World J Gastroenterol* 2014; 20(2): 509-517 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/509.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.509

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission and clinical relapses, the affected mucosa spreading from the rectum to the proximal large intestine, diffusely, circumferentially and continuously. The symptoms include bloody diarrhea, fever, tachycardia, anemia, and colicky abdominal pain[1]. In this decade, anti-tumor necrosis factor-α (TNF-α) antibodies, calcineurin inhibitors and apheresis, in addition to amino-salicylates and corticosteroids, have been used widely for the treatment of UC with relapse. The clinical course of UC patients has improved, but 20%-30% of patients with total colitis still come to colectomy[2-4]. Although the mortality of UC patients is not significantly higher than standardized mortality ratios of non-IBD populations, the cause of UC remains uncertain and there is still room for further research[5-7].

Cytomegalovirus (CMV) reactivation is sometimes associated with relapse of UC. A number of reports have debated the pathogenicity of CMV in UC patients and there are contrasting hypotheses that this is a pathogenic virus exacerbating the clinical course of IBD patients and that it is "an innocent bystander" and does not contribute to increased morbidity and mortality of UC patients[5-14]. As the influence of CMV infection on the UC patient remains uncertain, the clinical management of UC patients with CMV reactivation has not yet been standardized. In this context, some studies reported that antiviral therapy decreased the colectomy rate and mortality of UC patients with CMV reactivation[8,9]. Meanwhile, another reported that there were no significant differences in the rates of remission and colectomy between UC patients with and without CMV[10]. Moreover, it is problematic that there are so few studies indicating the long-term prognosis of UC patients complicated with CMV.

Another important problem regarding CMV infection of UC patients is the diagnostic modality. There are several diagnostic techniques for CMV "infection", including endoscopy, histology, serology, viral culture, CMV antigenemia assay and CMV DNA testing, which have varying sensitivity and specificity. At this point, immunohistochemistry (IHC) or CMV DNA testing of intestinal mucosa tissue is recommended for diagnosis of CMV "disease" in IBD patients, according to several guidelines[15-17]. However, these require colonoscopy and the former may have too low sensitivity and the latter too high sensitivity for the indication of antiviral therapy[8,16,18]. The CMV antigenemia assay has been widely used for pre-emptive therapy in hematopoietic stem cell transplantation[19-22]. In the field of UC, in contrast, relatively few studies evaluated the clinical significance of the CMV antigenemia assay, *e.g.*, for decision of administration of antiviral therapy[8,10]. However, the CMV antigenemia assay has advantages in that it is relatively inexpensive and facilitates monitoring of CMV activity continuously without endoscopy. Moreover, the assay may have less sample bias than examinations using tissues from the colon.

We have examined the CMV status in relapse of UC patients using the CMV antigenemia assay and treatment strategies were determined based on the CMV antigen status. The mainstay of our strategy for the patients found to be CMV antigen positive comprised ganciclovir administration and dose reduction of corticosteroids. The patients treated according to the strategy were followed-up at our institute for a relatively long period and the disease course of those patients may reveal the impact of CMV reactivation and antiviral therapy on the long-term prognosis of UC patients with CMV.

In this study, therefore, we investigated the long-term disease course of UC patients diagnosed as CMV-positive and -negative using the CMV antigenemia assay. The aim of this study was to clarify the impact of CMV antigen and antiviral therapy on the UC patients' prognosis: remission rate, relapse rate and colectomy rate.

MATERIALS AND METHODS

Patients

We retrospectively analyzed UC patients who were treated for their first-attack or relapse of disease at Okayama University Hospital from April 2004 to November 2011. Inclusion criteria were disease activity of Lichtiger's clinical activity index (CAI) 7 or more and having results of CMV antigen status evaluated by CMV antigenemia assay, and 121 patients met the criteria[23]. Of these patients, 3 who underwent emergency colectomy prior to the outcome of the CMV antigenemia assay were excluded. The remaining 118 patients were treated according to the strategy based on CMV antigen status, as shown below, and clinical, operative, pathological and treatment data were obtained from the patients' medical records.

CMV evaluation

CMV antigen status was evaluated using the CMV-pp65 antigenemia assay (SRL, Tokyo, Japan). The test is based on immunocytochemical detection of CMV immediate early antigens in blood leukocytes. The results were expressed as the number of CMV-pp65-positive cells per 5×10^4 leukocytes and reported on the day following blood submission to the laboratory, although this took a few days at weekend. A positive result from the CMV antigenemia assay was defined as detection of one or more CMV-pp65-positive cells. Patients were divided into two groups according to CMV antigen status: CMV-positive group and CMV-negative group. CMV antigenemia assay was performed when treatment was started in relapse of UC patients and disease condition did not become better despite administration of immunosuppressive therapy. CMV antigenemia was usually measured once two weeks in patients with positive CMV antigen on the initial assay. Those of whom disease condition worsened after starting ganciclovir received the assay once a week.

If general condition permitted, we performed colonoscopy around the time of the CMV antigenemia assay

Table 1 Characteristics of the study population (n = 118)

Characteristics	CMV Ag$^+$ (n = 40)	CMV Ag$^-$ (n = 78)	P value
Gender			0.009
Male	26	31	
Female	14	47	
Age(yr), median (range)	45 (14-79)	36 (12-87)	0.10
Duration of disease (yr), median (range)	1.5 (0.1-28)	4.6 (0-38)	0.23
Disease activity			0.19[1]
Mild	2	8	
Moderate	15	35	
Severe	23	35	
Lichtiger's CAI, median (range)	11 (7-19)	13 (7-21)	0.15
Extent of disease			0.31
Total colitis	33	58	
Left-sided	7	20	
Dose of prednisolone (mg/d), median (range)	35 (0-80)	20 (0-100)	0.0003
Follow-up period (yr), median (range)	3.2 (0.1-9.3)	2.8 (0.1-9.7)	0.66

[1]Overall P value. By χ^2 test, Fisher's exact test, and Mann-Whitney U test. CMV Ag: Cytomegalovirus antigenemia assay; CAI: Clinical activity index.

and took biopsies for hematoxylin and eosin staining to search for CMV inclusion bodies and for IHC staining with a monoclonal antibody against CMV by means of an automated preparation and processing system, Ventana® (Roche Diagnostics, Tokyo, Japan).

Treatment strategy

In our hospital, UC patients were treated based on the guidelines published by the British Society of Gastroenterology, the European Crohn's and Colitis Organization, and the American College of Gastroenterology[15,17,24]. In general, a sufficient dose of corticosteroids, 0.5-1 mg/kg per day of prednisolone for patients with moderate activity and 1-1.5 mg/kg per day for patients with severe activity, was administered with a target period set at two weeks and then gradually reduced by 5-10 mg/d at weekly intervals until a daily dose of 20 mg was reached. In patients with a steroid-refractory or -dependent course, another treatment, such as apheresis, a calcineurin inhibitor or anti-TNF-α antibody, was started according to their disease severity and treatment history. In this study, anti-TNF-α antibody was used less frequently because infliximab for UC was approved in Japan in June 2010. The effectiveness of additional treatments was also evaluated within about two weeks. Antiviral therapy with ganciclovir was not generally administered for patients in the CMV-negative group. However, antiviral therapy was administered for some patients who underwent acute exacerbation with endoscopic findings suggestive of CMV infection such as large, deep, or longitudinal ulcers[25].

When patients were found to be CMV antigen positive, ganciclovir administration with reduction of the dose of corticosteroids was considered to be the first choice. However, the antiviral agent was not provided for cases with mild disease activity or for cases for which conventional treatment without ganciclovir appeared to be effective. As the next step, steroid-refractory patients received apheresis, anti-TNF-α antibody, or a calcineurin inhibitor after diminishment of CMV antigen.

Evaluation of disease course

Remission, relapse, and colectomy rates were compared between the CMV-positive and -negative groups. The severity was expressed according to Truelove and Witt's criteria[26]. State of relapse was defined as CAI score 7 or more[23]. The definition of remission meets the CAI score 4 or less without any abdominal pains and bloody stools. Short-term clinical courses were evaluated according to patient status within 8 wk of examination of CMV antigen status, while long-term clinical courses were determined at the last visit before March 2012 or the time of colectomy.

Ethical considerations

This retrospective analysis was approved by the institutional review board of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and all the patients and subjects were included after obtaining their written informed consent. There were no conflicts of interest or sponsors of this study.

Statistical analysis

Patient characteristics were compared using the χ^2 test, Fisher's exact test and Mann-Whitney U test. Analyses of remission, relapse and colectomy rates were carried out using the method of Kaplan and Meier. Statistical comparison was carried out by Log-rank test. Univariate and multivariate analysis using a Cox proportional hazard model were also conducted. P value < 0.05 was considered statistically significant. All statistical analyses were performed using JMP ver.9 software (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

The clinical characteristics of analyzed patients are summarized in Table 1. A total of 118 UC patients with known CMV antigen status were treated during the study period; 40 were included in the CMV-positive group and 78 were in the CMV-negative group. CMV antigen was detected more frequently in male patients (P = 0.009). The dose of corticosteroids at the beginning of the treatment was significantly higher for the patients in the CMV-positive group than those in the CMV-negative group (35 mg/d of prednisolone *vs* 20 mg/d, P = 0.0003). CMV status of all the CMV-positive patients except those who underwent colectomy in a short term became negative, regardless of ganciclovir administration or not. The average period between the start of therapy and measurement of CMV antigenemia assay was 10.8 ± 13.4 d.

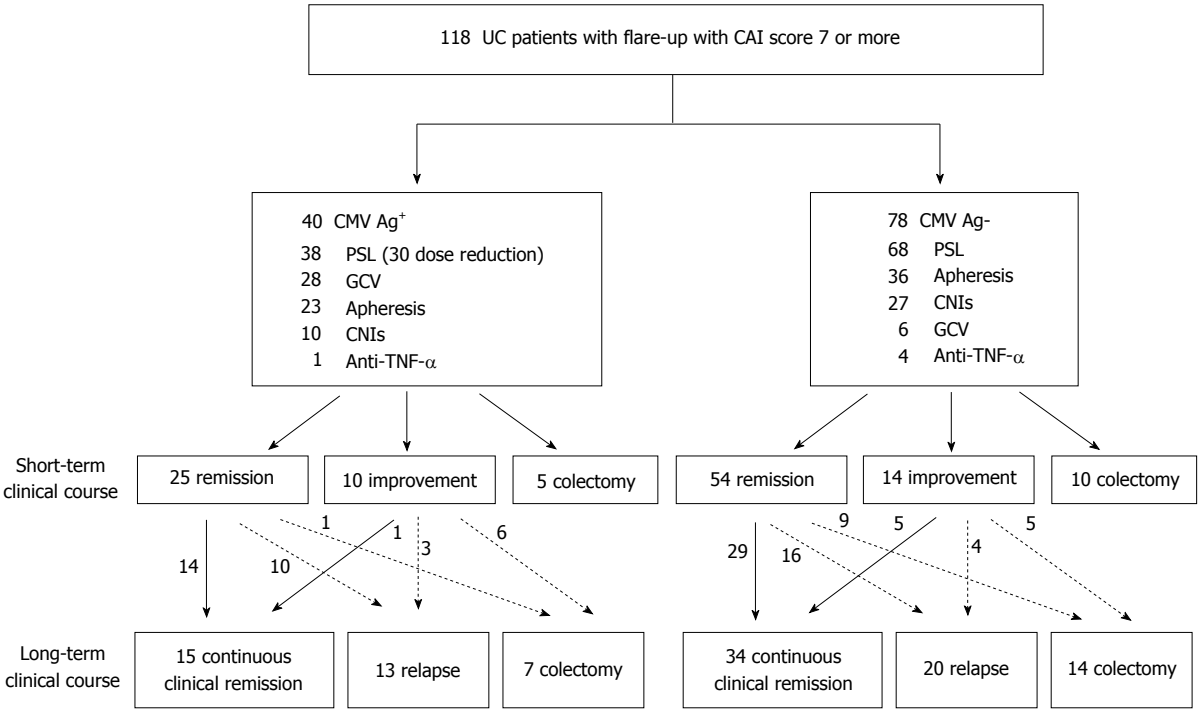

Figure 1 Clinical course of ulcerative colitis patients treated according to the status of cytomegalovirus antigen. A flow chart of the clinical courses of the 118 patients according to the strategy based on cytomegalovirus (CMV) antigen status is shown. UC: Ulcerative colitis; CMV Ag: Cytomegalovirus antigenemia assay; PSL: Prednisolone; CAI: Clinical activity index; GCV: Ganciclovir; CNIs: Calcineurin inhibitors; TNF-α: Tumor necrosis factor-α.

The correlation between CMV antigen status and IHC for CMV in the colonic mucosa was examined using 49 patients who underwent colonoscopy around the time of the CMV antigenemia assay. Of the 23 patients in the CMV-positive group, 9 (39.1%) were positive for IHC. On the other hand, 25 (96.2%) of the 26 patients in the CMV-negative group were negative for IHC. The results of the CMV antigenemia assay were closely correlated with IHC of inflamed colon mucosa for CMV ($P = 0.003$, Fisher's exact test). Taking IHC as the gold standard, positive CMV antigen status predicted positive IHC with 90% sensitivity and 64% specificity.

Initial treatment for patients

Figure 1 is a flow chart of the clinical courses of the 118 patients treated according to the strategy based on CMV antigen status. Of the 38 patients in the CMV-positive group who had received corticosteroids, 30 (78.9%) underwent dose reduction of corticosteroids. The remaining 8 patients did not undergo dose reduction of corticosteroids; 3 received colectomy in the early period and 5 showed a marked response to the corticosteroids. Twenty-eight (70%) patients in the CMV-positive group received ganciclovir infusion. On the other hand, 68 (87.2%) of the 78 patients in the CMV-negative group received corticosteroids without any dose reductions. Six (7.7%) patients in the CMV-negative group were administered ganciclovir infusion because CMV reactivation was suspected, based on specific endoscopic findings and clinical refractoriness to the first-line therapy with clinical symptoms worsening. In both groups, apheresis and calcineurin inhibitors were used relatively frequently.

Short-term remission rates according to CMV antigen status

In the CMV-positive group, 25 (62.5%) patients went into remission and 5 (12.5%) received colectomy during the short-term treatment. The remaining 10 (25%) patients improved, but did not fulfill the criteria of remission. Among the CMV-negative group, on the other hand, 54 (69.2%) patients entered remission successfully, 14 (17.9%) improved, and 10 (12.8%) underwent colectomy in the short-term (Figure 1, center part).

Two types of the Kaplan-Meier curves for the rate of remission induction are shown (Figure 2). Figure 2A indicates the remission rate from the starting day of the remission-induction therapy and Figure 2B shows from the day when the CMV antigen status was determined. Both curves show the better clinical course in the CMV-negative group ($P = 0.0006$ and $P = 0.03$, respectively, Log-rank test). The median number of days to remission from the start of the treatment with our strategy was significantly greater for the patients in the CMV-positive group than those in the CMV-negative group (21 d *vs* 16 d, $P = 0.009$, Mann-Whitney U test). In addition, we analyzed remission rate from the starting day of the remission-induction therapy between the two groups; one group was limited to CMV-positive patients administered ganciclovir and another was CMV-negative patients not administered ganciclovir. These curves showed the better clinical course in the CMV-negative group, too ($P = 0.03$, Log-rank test).

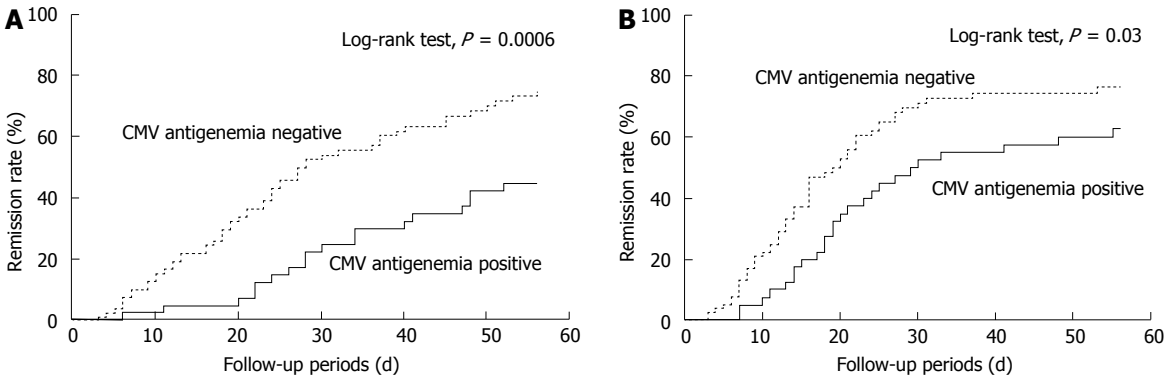

Figure 2 Short-term remission rate according to cytomegalovirus antigen status. Kaplan-Meier curves for the rate of induction of remission. A: The remission rate from the day of starting the remission-induction therapy; B: The remission rate from the day when the status of cytomegalovirus (CMV) antigen was determined. Both curves show the better clinical course in the CMV-negative group (P = 0.0006 and P = 0.03, respectively, Log-rank test). The median number of days to remission from the start of the treatment based on our strategy was significantly greater for patients in the CMV-positive group than for patients in the CMV-negative group (21 d *vs* 16 d, P = 0.009, Mann-Whitney U test).

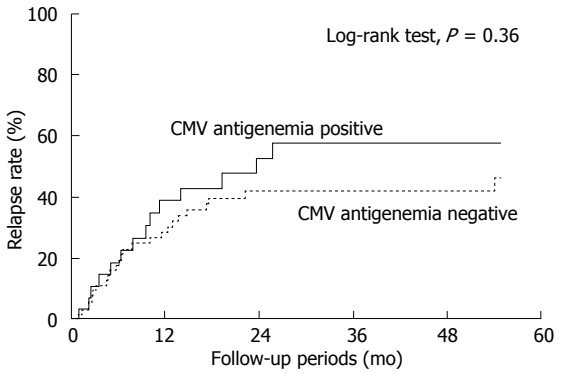

Figure 3 Relapse rate after successful induction of remission according to cytomegalovirus antigen status. The rate of relapse after successful induction of remission with the initial treatment was shown. The rate of relapse after successful induction of remission did not differ between the patients in the cytomegalovirus (CMV)-positive and -negative groups (P = 0.36, Log-rank test).

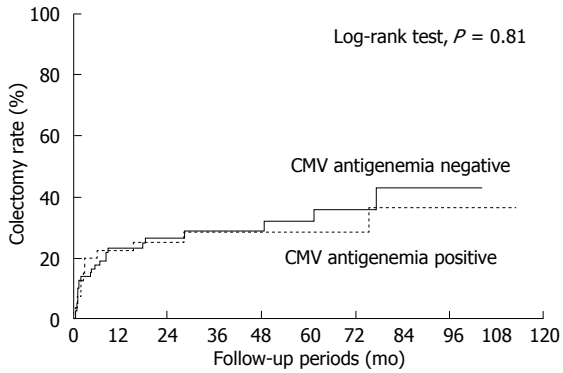

Figure 4 Colectomy rate according to cytomegalovirus antigen status. The cumulative colectomy rate according to cytomegalovirus (CMV) antigen status. Twelve (30%) of the CMV-positive patients and 24 (30.8%) of the CMV-negative patients underwent colectomy during the observation period and no difference in colectomy rate was observed between the two groups (P = 0.81, Log-rank test, median observation period: 31 mo).

Relapse rate after successful remission induction according to CMV antigen status

In the CMV-positive group, 14 (56%) of the 25 patients who had gone into remission within the short-term maintained that status. In contrast, only one (10%) of the 10 patients with improvement in the short-term entered remission afterwards. Meanwhile, in the CMV-negative group, 34 (50%) of 68 patients who avoided colectomy in the short-term could maintain continuous clinical remission (Figure 1, lower part).

The rate of relapse after successful remission induction with the initial treatment did not differ between the patients in the CMV-positive and -negative groups (Figure 3; P = 0.36, Log-rank test). Among 20 patients in the CMV-positive group who underwent relapse or colectomy after the initial induction of remission, 2 became positive for CMV antigen again. These patients were intractable to any therapy, including anti-TNF-α antibodies, and calcineurin inhibitors, and continued to suffer from chronic symptoms during the study period. None of the 34 patients in the CMV-negative group who relapsed or underwent colectomy after the initial remission became positive for CMV antigen.

Colectomy rate according to CMV antigen status

The cumulative colectomy rate according to CMV antigen status is shown in Figure 4. Twelve (30%) in the CMV-positive patients and 24 (30.8%) in the CMV-negative patients underwent colectomy during the observation period and there was no significant difference in the colectomy rate between the two groups (Figure 4; P = 0.81, Log-rank test, median observation period: 31 mo). No independent risk factors for colectomy could be identified among demographic, treatment and disease parameters including CMV antigen status in the analysis of all 118 patients with or without CMV antigen (Table 2). Whereas, for patients in the CMV-positive group, multivariate analysis using parameters including ganciclovir administration, dose reduction of corticosteroids and number of CMV antigen revealed that administration of ganciclovir at the start of therapy was the only factor correlated with avoidance of colectomy (Table 3; OR = 0.04; 95%CI: 0.01-0.50).

Table 2 Multivariate analysis of factors predictive of colectomy in ulcerative colitis patients

Risk factor	Risk ratio	95%CI		*P* value
		Lower	Upper	
Gender (male)	0.97	0.59	1.59	0.89
Age (> 40 yr)	1.13	0.70	1.82	0.61
Duration of disease (> 3 yr)	0.99	0.61	1.61	0.97
Severity (severe)	1.24	0.74	2.08	0.42
Extent of disease (total colitis)	1.21	0.68	2.23	0.51
CMV Ag (+)	0.64	0.36	1.13	0.12
Apheresis use	0.71	0.44	1.17	0.18
CNI use	1.11	0.63	1.92	0.73

CMV Ag: Cytomegalovirus antigenemia assay; CAI: Clinical activity index.

Table 3 Multivariate analysis of factors predictive of colectomy in ulcerative patients with positive cytomegalovirus antigen status

Risk factor	Risk ratio	95%CI		*P* value
		Lower	Upper	
Gender (male)	0.38	0.03	4.24	0.42
Age (> 45 yr)	1.22	0.15	12	0.85
Duration of disease (> 2 yr)	0.98	0.09	9.18	0.98
Severity (severe)	8.37	0.46	751	0.17
Extent of disease (total colitis)	2.43	0.15	118	0.55
Apheresis use	0.87	0.08	9.59	0.91
CNI use	0.54	0.01	10.2	0.69
Ganciclovir use	0.04	0.01	0.5	0.01
Dose reduction of corticosteroids	0.76	0.03	13.1	0.85
Number of CMV Ag[1] (> 3)	3.77	0.53	42.3	0.19

[1]This number means the maximum of CMV Ag detected during the period. CMV Ag: Cytomegalovirus antigenemia assay; CAI: Clinical activity index.

DISCUSSION

We analyzed data from UC patients with flare-up and who were treated according to a strategy based on the CMV antigen status and demonstrated the clinical courses of CMV antigen-positive and -negative patients. In UC patients with flare-up, CMV antigen positive status significantly prolonged the time to remission from the start of treatment but did not affect the relapse or colectomy rate. In addition, ganciclovir administration was a significant factor correlated with avoidance of colectomy in patients with positive CMV antigen status.

It has been suggested that CMV is pathogenic for UC patients and causes clinical condition of UC patients with relapse to become worse and complicate. In fact, several small scale studies indicated that UC patients with CMV infection had higher colectomy rates than those without CMV. Moreover, treatment of CMV infection by ganciclovir can improve the clinical outcome of those patients[8,27-29]. Although a few reports indicated the possibility of CMV being an innocent bystander in the exacerbation of UC[10], CMV can affect some of UC patients deleteriously. However, the long-term effect of CMV infection and antiviral therapy on the prognosis of UC patients has not been elucidated completely. In this context, our results, including the long-term disease course of UC patients with CMV infection, should have a great impact on the clinical practice of UC.

We showed that patients with CMV infection were less likely to enter remission in the short-term. This result is consistent with those of previous reports[9,27]. In contrast, however, no significant difference in colectomy rate was observed between patients with and without CMV, even in the short-term. In this context, the CMV antigenemia assay could reliably detect CMV "disease" which requires antiviral therapy. In our finding, use of ganciclovir is not always correlated with CMV negative conversion in short-term but a predictor of avoidance of colectomy in long-term. These suggest that use of ganciclovir exerts clinical effectiveness in long-term follow-up, *e.g.*, avoiding exacerbation and/or relapse after remission, for a portion of patients with CMV.

In term of diagnosis regarding CMV infection in UC patients, IHC or CMV DNA testing in the intestinal mucosa tissue is recommended as a diagnosis of CMV "disease" in IBD patients according to several guidelines[15-17]. In particular, CMV DNA testing would be a very useful method if the correct cut-off level of CMV DNA load was established[30]. However, CMV DNA testing is expensive and needs colonoscopy with biopsy that has a risk of bleeding or perforation in severely ill UC patients. In addition, there is a sample bias in taking tissues from the mucosa. CMV DNA testing in blood sample may be convenient, but the method and cutoff values remain to be standardized. On the other hand, the CMV antigenemia assay is less expensive and facilitates monitoring of CMV activity continuously without endoscopy. Moreover, there is less sample bias than in taking from colon tissue. We confirmed that the results of the antigenemia assay were consistent with the results of IHC for CMV, although the specificity for IHC was relatively low. In fact, lower specificity may indicate overestimation of CMV disease by the antigenemia assay. Therefore, our strategy may provide unnecessary antiviral therapy for part of patients.

Reduction of the dose of corticosteroids is another aspect of our treatment strategy for UC patients with CMV. There has been no consensus regarding whether immunosuppressive therapies should be continued or discontinued, and whether the dose of corticosteroids should be increased or decreased for UC patients with CMV. Some have reported cases with continuation of the immunomodulating drugs[8,29], while others have discontinued the immunosuppressive therapies[27,28]. Interestingly, all of these studies showed good clinical courses whether immunosuppressive therapies were ongoing or not. In addition, our results indicated that reduction of the dose of corticosteroids and immunosuppressive therapies were not significant factors in multivariate analysis for avoiding colectomy both in the short and long-term. This suggests that status of immunosuppressive thera-

pies, including the dose of corticosteroids, may not affect the clinical course of UC patients with CMV greatly, at least under antiviral therapy.

The highlight of this study is determination of the long-term prognosis of UC patients with CMV infection, including relapse rate and colectomy rate, which have rarely been reported. Unexpectedly, no significant difference was observed in overall long-term prognosis between patients with and without CMV. Kaplan-Meier curves for colectomy were almost parallel, both in the short-term and in the long-term, between those patients. These results suggest that UC patients with positive CMV antigen should be treated with antiviral therapy and that our treatment strategy may be appropriate for UC patients with CMV with regard to long-term prognosis.

However, there may be two types of rebuttal to our results. First, antiviral therapy may not always be required for UC patients with CMV. Although the efficacy of antiviral agents in CMV disease has been recognized widely[15,31,32], a previous report demonstrated that UC patients with CMV infection who were treated without the use of antiviral agents showed similar rates of remission and colectomy to UC patients without CMV[10]. However, the report consisted of 25 patients with CMV (4 underwent colectomy) and 23 patients without CMV (1 underwent colectomy) with an observation period of 8 wk, and such small number of patients with short-term observation may not be sufficient to verify the appropriateness of a treatment strategy without antiviral therapy. For accurate validation of the use of antiviral agents, long-term follow-up data of patients who were treated according to a strategy without antiviral therapy would be expected. However, such a treatment strategy may not be feasible under the guidelines which recommended antiviral therapy for at least a proportion of patients with CMV.

The second problem is the appropriateness of the antigenemia assay for detecting the presence of CMV infection and administration of ganciclovir. As described above, a positive result from the antigenemia assay may overestimate the necessity of administration of antiviral therapy, because specificity for IHC was relatively low. In this regard, Roblin *et al*[30] indicated 250 copies/mg of CMV DNA in the colonic tissue as the cutoff for antiviral therapy, based on 42 hospitalized UC patients. However, few studies indicated a correlation between CMV antigen status and CMV DNA in colonic tissues, perhaps because of sample bias and the lack of validation of the technology of DNA testing, and therefore it is not known what is best as the cutoff for antiviral therapy. Determination of the optimal test modality and cutoff values for CMV disease in UC which requires antiviral therapy should be the goal of future studies.

This study has a limitation mainly because of the retrospective design. First of all, the treatment strategy was not always followed. Not all of the CMV-positive patients were administered ganciclovir and some of the CMV-negative patients were administered ganciclovir. Dose reduction of corticosteroids was not carried out for CMV-positive patients if corticosteroids exerted marked efficacy. However, from the aspects of ethical and moral standpoints, it would be difficult not to administer antiviral agents to severe and refractory patients with CMV infection on the basis of the current evidence. In this regard, our retrospective analysis for the long-term prognosis of UC patients with CMV may be almost optimal for verifying the treatment strategy for those patients. There is also a limitation about patients' selections; dose of corticosteroids at the beginning of the treatment was significantly lower in the CMV-negative group. The patients examined CMV status and enrolled in this study were suspected involvement with CMV for their refractoriness and endoscopic findings. In severer case which was suspected involvement with CMV reactivation, we would avoid using large dose of PSL not to reactivate CMV. As a result, we chose second-line therapy such as CNIs, apheresis and not dose up of corticosteroids, especially in CMV-negative group, CAI of which was severer than positive group. In addition, CMV antigen was detected more frequently in male. However, I tried to stratify the results by gender and dose of corticosteroids, respectively, and we confirm that each result did not show a significant difference.

In conclusion, our treatment strategy, which consisted of dose reduction of corticosteroids and antiviral therapy, appeared to be appropriate for the treatment of UC patients with CMV antigen in view of long-term prognosis. More work is needed for the standardization of appropriate test modalities and cutoff values for administration of antiviral therapy and the administration policy of immunosuppressive agents for UC patients with CMV.

COMMENTS

Background

Cytomegalovirus (CMV) activation is sometimes associated with exacerbation and refractoriness of ulcerative colitis (UC). While there are several diagnostic modalities for CMV disease, these cannot distinguish strictly CMV "infection", meaning that only the symptoms of CMV "disease" should be treated. Although antiviral therapy has been shown to improve the short-term disease course, the long-term prognosis of UC patients with CMV has rarely been reported.

Research frontiers

The CMV antigenemia assay has advantages in that it is relatively inexpensive and facilitates monitoring of CMV activity continuously, without endoscopy. However, the disease course of a UC patients treated according to CMV antigenemia assay has not been revealed. In this study, the authors clarified the impact of CMV antigen and antiviral therapy on the UC patients' prognosis: remission rate, relapse rate and colectomy rate.

Innovations and breakthroughs

The CMV antigenemia assay has been widely used for pre-emptive therapy in hematopoietic stem cell transplantation. In the field of UC, however, relatively few studies have evaluated the clinical significance of the CMV antigenemia assay, *e.g.*, for decision of administration of antiviral therapy. This is the first study to investigate the long-term prognosis of UC patients treated on the basis of cytomegalovirus antigen status.

Applications

The finding that ganciclovir use is a predictor of avoidance of colectomy suggests that ganciclovir use exerts clinical effectiveness, *e.g.*, avoiding exacerbation and/or relapse after remission, for a subset of patients with CMV.

Terminology

Cytomegalovirus antigenemia: The cytomegalovirus antigenemia assay is based on immunocytochemical detection of CMV immediate early antigens in

blood leukocytes. The results were expressed as the number of CMV-pp65-positive cells per 5 × 10^4 leukocytes.

Peer review

The authors examined the long-term prognosis of UC patients with CMV infection, including relapse rate and colectomy rate, which have rarely been reported. It revealed that positive CMV antigen status was likely to prolong time to remission and long-term prognosis. Colectomy rate was not affected by CMV antigen status under the treatment strategy with antiviral agents. In addition, ganciclovir use is an independent factor for avoidance of colectomy in UC patients positive for the CMV antigen. The results are interesting and may suggest that ganciclovir use exerts clinical effectiveness, *e.g.*, avoiding exacerbation and/or relapse after remission, for a subset of patients with CMV.

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BRIEF ARTICLE

Effectiveness of contrast-enhanced harmonic endoscopic ultrasound for the evaluation of solid pancreatic masses

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Abstract

AIM: To evaluate the usefulness of contrast-enhanced harmonic endoscopic ultrasound (CH-EUS) in differentiating between pancreatic adenocarcinomas and other pancreatic disease.

METHODS: This retrospective cohort study evaluated 90 patients who were seen between November 2010 and May 2013. All these patients had solid pancreatic masses that had a hypoechoic appearance on EUS. All patients underwent CH-EUS to evaluate this diagnostic method's usefulness. The mass lesions observed on CH-EUS were classified into three categories based on their echo intensity: hypoenhanced, isoenhanced, and hyperenhanced lesions. We adjusted the sensitivity and the specificity of each category for detecting malignancies. We also estimated the accuracy of CH-EUS by comparing it to a pathological diagnosis.

RESULTS: Of the 90 patients, 62 had a pancreatic adenocarcinoma. Fifty-seven out of 62 pancreatic adenocarcinomas showed a hypoenhanced pattern on CH-EUS. The sensitivity was 92%, the specificity 68% and the accuracy approximately 82%. The area under the curve of the receiver operating characteristic analysis for CH-EUS was 0.799. There is a significant association between the hypoenhanced pattern on CH-EUS and pancreatic duct adenocarcinoma (χ^2 = 35.264, P < 0.001). In pathological examinations, the number of specimens for EUS-fine needle aspiration (EUS-FNA) was considered insufficient for diagnosis in three patients, and in two patients, the results were reported to be negative for malignancy. Pancreatic masses in all five patients revealed a hypoenhanced pattern with CH-EUS. Three patients were diagnosed with pancreatic adenocarcinoma based on the pathology results of a biopsy, and the remaining two patients were clinically diagnosed with malignancy.

CONCLUSION: CH-EUS is useful for distinguishing between pancreatic adenocarcinoma and other pancreatic disease. When a pancreatic mass shows a hypoenhanced pattern on CH-EUS but involves either insufficient samples or negative results with EUS-FNA, clinicians might consider performing another pathologic diagnosis on the basis of an EUS-FNA sample or a biopsy.

Key words: Pancreas neoplasm; Endoscopic ultrasound; SonoVue; Contrast media; Microbubbles

Core tip: This is a retrospective study of 90 patients with a pancreatic hypoechoic masses. We found that contrast-enhanced harmonic endoscopic ultrasound (CH-EUS) can be used for distinguishing between pancreatic adenocarcinoma (n = 62) and other pancreatic disease based on the hypoenhanced pattern on CH-EUS. When considering pancreatic adenocarcinomas as a tumor with a hypoenhanced pattern in CH-EUS and/or showing a positive result in EUS-fine needle aspira-

tion, the sensitivity of detecting pancreatic malignancy increased to 100%.

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INTRODUCTION

Endoscopic ultrasonography (EUS) is widely recognized as a diagnostic tool for pancreatic diseases and is superior to other modalities because of its good spatial resolution[1-3]. Tissue sample acquisition through EUS-guided fine-needle aspiration (EUS-FNA) could offer a clearer diagnosis of a pancreatic lesion[4]. However, differentiation between pancreatic tumors and inflammatory tumor-like masses is difficult[5]. Contrast-enhanced harmonic endoscopic ultrasonography (CH-EUS) is a novel technology that reveals not only parenchymal perfusion but also the microvasculature in the pancreas and aids in the differentiation of pancreatic neoplasia from other pancreatic disease[6,7]. Hirroka *et al*[8] described this new method with a preliminary study in 37 patients with pancreatic disease. This study showed that EUS-enhanced imagery is useful for the diagnosis of pancreatic disease. However, CH-EUS assessments suffered from the limitations caused by blooming artifacts, poor spatial resolution, and low sensitivity to slow flow[5,9]. To overcome these limitations, CH-EUS with a second-generation ultrasound contrast agent was recently developed. The second-generation contrast included a microbubble agent that can achieve good-quality perfusion imaging with harmonic EUS for evaluating the microvasculature of target lesions[10,11]. Therefore, we evaluated the efficacy of CH-EUS with a second-generation ultrasound contrast agent for assessing solid masses in the pancreas. We also estimated the role of CH-EUS as an additional diagnostic method for EUS in pancreatic neoplasia, which is difficult to confirm histologically. Furthermore, we tried to compare the sensitivity and the specificity of CH-EUS and EUS-FNA in the diagnosis of pancreatic neoplasia.

MATERIALS AND METHODS

Patients and equipment

We conducted a retrospective cohort study of 90 patients who presented with pancreatic masses and were seen at our facility from November 2010 to May 2013. These masses were heterogeneous and hypoechoic. Patients underwent CH-EUS to ascertain the character of the mass at the INHA University Hospital. A pathological examination was performed using EUS-FNA samples or on biopsy samples when malignancy was suspected. If the pancreatic mass was suspected to be either a benign or malignant neoplasia but was impossible to get a pathologic diagnosis of due to the location of mass or the inability to obtain specimens with EUS-FNA, the clinical course was tracked until a diagnosis could be confirmed. If a patient could not receive a proper pathologic diagnosis and was not tracked further, then the patient was excluded from this study. We also excluded patients if the mass was clearly revealed as a cystic lesion. Examination of the pancreas by conventional EUS and CH-EUS was performed by one endosonographer, Kim HK, who had conducted more than 8000 EUS procedures. An echoendoscope developed for CH-EUS (GF-UE260-AL5; Olympus Medical systems Co Ltd, Tokyo, Japan) was used. Ultrasound images were analyzed using an ALOKA Prosound Alpha10 processor (ALOKA, Tokyo, Japan). All images were analyzed and reviewed by one endosonographer. The extended pure harmonic detection (E-PHD) mode, which synthesizes receiving frequencies of filtered fundamental and second harmonic components with a transmitting frequency of 8 MHZ, was used for CH-EUS. The ultrasound contrast agent, Sonovue (Bracco international BV, Amsterdam, the Netherlands), was used for CH-EUS. This agent is a second-generation ultrasound contrast agent, consisting of microbubbles of sulfur hexafluoride coated with phospholipids.

Study design

Conventional EUS was performed on all patients. When conventional EUS revealed a mass-like lesion, the imaging mode was changed to the E-PHD mode. After changing to the E-PHD mode, a bolus of 2.4 mL of Sonovue was injected at a speed of 1 mL/s through an 18-gauge cannula placed in the antecubital vein. After infusion, the pancreas was imaged in a real-time fashion for a minimum of 90 s for complete observation of the arterial and venous phases. We observed changes of echo intensity in the mass lesions. If the pancreatic mass was not diagnosed with the first administration of Sonovue, a second dose of 2.4 mL Sonovue was administrated. The mass lesions were classified into three categories based on their echo intensity: hypoenhanced, isoenhanced, and hyperenhanced lesions[12]. This classification was based on the intensity of enhancement relative to the surrounding pancreatic tissue. Hypoenhancement is defined as a lower intensity of enhancement relative to the surrounding pancreatic tissue. Isoenhancement is defined as a similar intensity of enhancement relative to the surrounding pancreatic tissue. Hyperenhancement is defined as a higher intensity of enhancement relative to the surrounding pancreatic tissue (Figure 1). We interpreted the lesion following a modified classification of contrast-enhanced harmonic imaging of the pancreas by transabdominal ultrasound[12-14]. Adenocarcinomas usually have a distinct hypovascular appearance and are usually defined as lesions with hypoenhancement. The sensitivity and specificity of CH-EUS in diag-

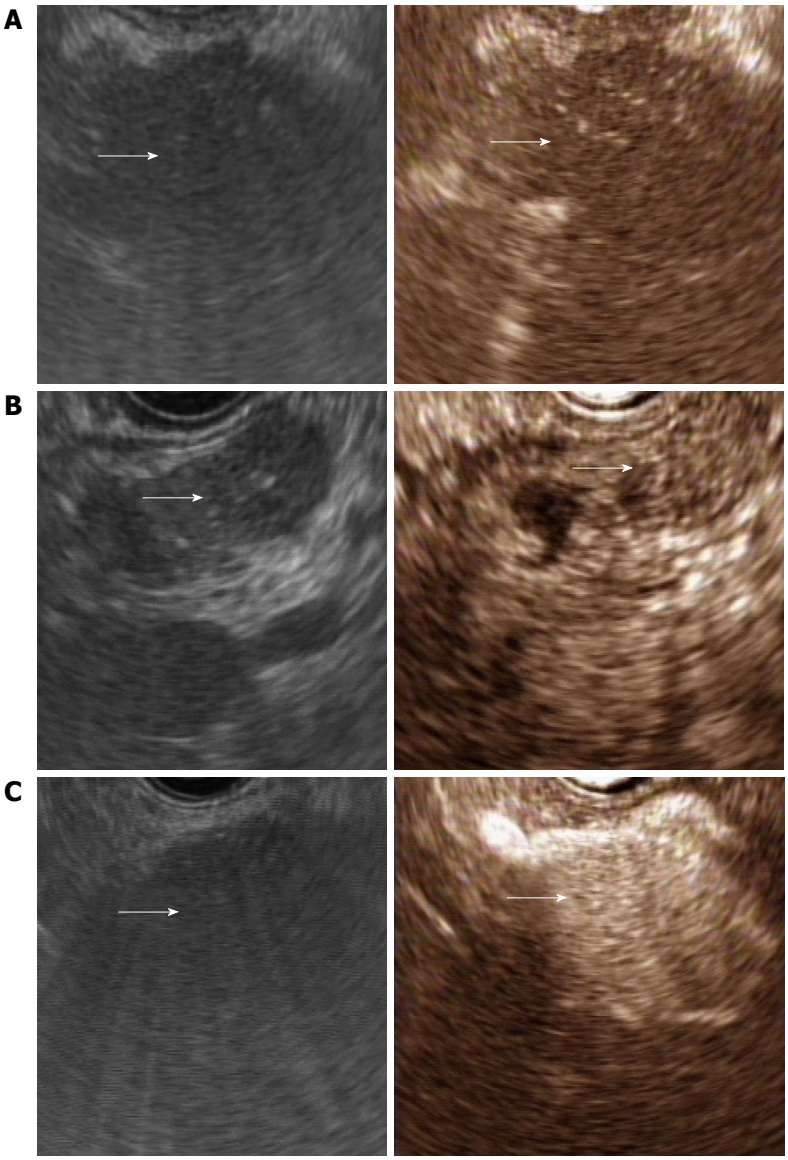

Figure 1 Conventional endoscopic ultrasonography (left) and contrast-enhanced harmonic endoscopic ultrasonography (right). These pictures showed a typical example of a hypoenhancement pattern (A), an isoenhancement pattern (B), and hyperenhancement pattern (C).

nosing pancreatic adenocarcinoma were assessed. We also estimated the accuracy of CH-EUS in comparison with a pathologic diagnosis. To obtain specimens of the pancreatic mass, EUS-FNA was used initially. If specimen acquisition using EUS failed, pathological diagnoses were determined from a biopsy of a metastatic lesion from tissue such as the liver or the ampulla of Vater (hepatopancreatic ampulla). In one patient, a final diagnosis was made by a pathological examination of the patient's abdominal effusion. When we did not get a proper specimen to confirm pancreatic disease, we traced the clinical course of patients until we could confirm the diagnosis of the disease.

Ethics statement

The study protocol and amendments were approved by the Institutional Review Boards at our hospital (IUH-IRB 13-100).

Statistical analysis

All statistical analyses were performed using a statistical software package (SPSS 11.5 for Windows, SPSS, Inc, Chicago, IL, United States). Cross-tabular analyses were performed, accompanied by the chi-square test. Receiver operating characteristic analysis was performed to estimate the diagnostic accuracy of CH-EUS in pancreatic adenocarcinoma. The area under the curve (AUC) was calculated and the 95% confidence intervals (95%CI) were determined.

RESULTS

The sampling frame included 112 patients, and 22 patients who did not have a histological diagnosis were excluded. Sixteen of the 22 patients were lost to follow-ups. The remaining six patients had pancreatic masses accompanied with neoplasia originating at other sites, making it

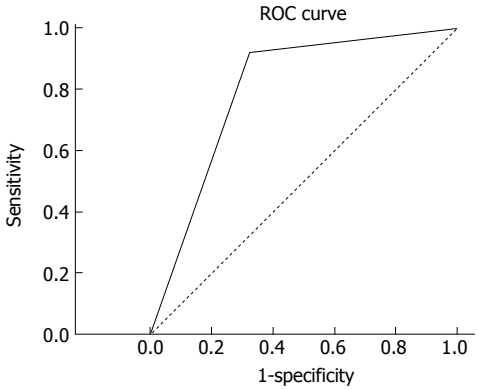

Figure 2 Receiver operating characteristic curve. Hypoenhancement pattern, determined by contrast-enhanced harmonic endoscopic ultrasonography, was calculated to diagnose pancreatic adenocarcinoma with the area under the receiver operating characteristic (ROC) curve of 0.799.

difficult to assess whether the pancreatic mass was metastatic or of pancreatic origin. This left 90 patients with a pancreatic mass who were enrolled. The median age of the study subjects was 63.5 years (range, 19-86 years) and the male:female ratio was about 2:1 (62:28 cases). The final diagnoses based on histological findings with EUS-FNA or surgical resection were pancreatic adenocarcinoma (n = 62), pancreatitis (n = 19), neuroendocrine tumor (n = 3), solid pseudopapillary tumor (n = 1), anaplastic carcinoma (n = 1), diffuse large B cell lymphoma (n = 1), and Castleman's disease (n = 1). The clinically diagnosed malignancies applied to two cases in which EUS-FNA results were unsatisfactory or where insufficient specimens were obtained. Clinically diagnosed malignancy was defined as the pancreatic mass showing suspected malignancy in EUS on the basis of the echogenicity and morphology, or increasing tumor size during the tracking period. Positron emission tomography-computed tomography was performed in these patients and all pancreatic masses yielded positive results using this technique. These patients did not survive longer than 6 months after finding the pancreatic masses.

Of the 62 pancreatic ductal adenocarcinomas, 57 had a hypoenhanced pattern in CH-EUS. Two cases had isoenhanced patterns, and three cases had hyperenhanced patterns. Hypoenhanced patterns in CH-EUS had a high sensitivity (91.93%) for detecting pancreatic adenocarcinomas. The positive predictive value was 86.36%, the negative predictive value 79.16%, the specificity 67.85%, and the accuracy 84.45%. The AUC for pancreatic adenocarcinoma detected by hypoenhanced patterns on CH-EUS was 0.799. There is a significant association between hypoenhanced patterns on CH-EUS and pancreatic duct adenocarcinomas (χ^2 = 35.264, P < 0.001). In contrast, pancreatitis comprised a large proportion of hyperenhanced pancreatic masses in CH-EUS (8/16). Two neuroendocrine tumors and three pancreatic adenocarcinomas were found to have a hyperenhanced pattern (Table 1). Five of the patients with pancreatic neoplasia could not be diagnosed with pancreatic neoplasia by EUS-FNA. Diagnoses could not be made in three of the patients due to insufficient samples. Two patients showed negative results in EUS-FNA. Three patients were diagnosed with a pancreatic adenocarcinoma by surgical resection. Two patients were diagnosed with pancreatic malignancy after a clinical follow-up. Interestingly, all of them had a hypoenhanced pattern on CH-EUS. Fifty-eight out of 90 patients underwent EUS-FNA. The sensitivity of the EUS-FNA in these 58 patients was 90.19% and the specificity 100%. When we assumed that pancreatic adenocarcinomas were the tumors with hypoenhanced patterns on CH-EUS and/or a positive result with EUS-FNA, the sensitivity with which pancreatic adenocarcinomas was diagnosed was 100% and the specificity 85.71% (Figure 2).

DISCUSSION

Pancreatic adenocarcinomas comprise over 90% of pancreatic tumors[15]. Pancreatic adenocarcinomas appear as heterogeneous hypoechoic masses with irregular margins on EUS[16]. The sensitivity of detecting pancreatic adenocarcinomas is 89%-100% in conventional EUS[17]. However, distinguishing pancreatic adenocarcinoma and benign disease such as a focal pancreatitis only on the basis of these EUS findings is difficult because both have similar morphology and echogenicity. In this situation, CH-EUS is useful to characterize pancreatic masses. When microbubbles in contrast agents are hit by an ultrasonic wave, the vibration creates a back-scattered acoustic shadow. The shadow is reproduced as an opacification. Vascular structures are highlighted as hyperechoic[4]. Thus, CH-EUS is able to evaluate the microvasculature of target lesions and parenchymal perfusion and can distinguish pancreatic neoplasia from benign pancreatic diseases[4,18]. According to Napoleon *et al*[19], CH-EUS can diagnose pancreatic adenocarcinoma with a high sensitivity (88%). Kitano *et al*[12] reported a similar result recently. They studied 277 patients with pancreatic solid lesions detected by conventional EUS who underwent CH-EUS for evaluation of the vascularity. CH-EUS depicted hypoenhancement of the masses diagnosed as pancreatic carcinomas with a sensitivity of 95.1% and specificity of 89.0%. Moreover, the microbubbles, which consists of gases other than air, in second-generation ultrasound contrast agents can be oscillated or broken by lower acoustic power[20-22]. Because of this, microvessels and parenchymal perfusion can be visualized to a greater extent than that achieved with CH-EUS using first-generation contrast agents, which have microbubbles of air[14,23,24]. According to a recent study, the sensitivity and specificity of CH-EUS with second-generation contrast agents in detection of pancreatic adenocarcinoma were 94% and 89%, respectively[20]. In the present study, when the pancreatic adenocarcinoma appeared as a mass with hypoenhancement on CH-EUS, the sensitivity was 91.93%, positive predictive value was 86.36%, negative predictive value 79.16%, specificity 67.85%, and accuracy 84.45%. Furthermore, there is a significant association between

Table 1 The vascular patterns of each pancreatic disease in contrast-enhanced harmonic endoscopic ultrasonography

	Total	Adenocarcinoma	Neuroendocrine tumor	Clinically diagnosed malignancy	Pancreatitis	Others
Hypoenhancement	66	57	1	2	6	0
Isoenhancement	8	2	0	0	5	1
Hyperenhancement	16	3	2	0	8	3
Total		62	3	2	19	4

the hypo-enhancement pattern and pancreatic adenocarcinoma. Generally, pancreatic adenocarcinoma is regarded as a pancreatic mass showing a hypoenhancement pattern in CH-EUS. The reason why pancreatic cancer shows a hypoenhancement pattern on CH-EUS is that pancreatic cancer has a hypovascular structure and the mean vascular density of pancreatic adenocarcinoma is low and often inferior to that of the normal pancreatic parenchyma[25]. In addition, on pathological examination, the adenocarcinoma is characterized by the presence of marked desmoplasia, which justifies its hard consistency[26]. Because of these characteristics, the margins and size of the lesion are distinguished well with the surrounding enhanced normal pancreatic tissue[27]. In addition, microbubbles adopted in second-generation contrast agents do not leak out from blood vessels and the pancreatic adenocarcinoma is increasingly demarcated as a hypoenhanced lesion. Fusaroli *et al*[28] also reported that the hypoenhanced pancreatic mass is very sensitive and accurate for the prediction of adenocarcinoma (96% and 82%, respectively). This result was supported by our results. Therefore, we recommend that CH-EUS be performed upon pancreatic masses when it is difficult to distinguish pancreatic adenocarcinoma from other pancreatic diseases. It would be a helpful tool for diagnosis of pancreatic adenocarcinoma.

Although the sensitivity of EUS for pancreatic adenocarcinoma detection is very high, the diagnostic specificity is only 53%[29]. EUS-FNA is useful to overcome this specificity. The sensitivity of EUS-FNA for solid pancreatic masses reaches 95% with a specificity of 100%[30]. However, despite these advantages, negative EUS-FNA findings sometimes do not help determine whether a pancreatic mass requires surgery or follow-up, because of the possibility of false-negative findings.

In the course of Kitano's study[12], there were five cases with false-negative EUS-FNA findings, but all these cases showed hypoenhancement patterns in CH-EUS. Likewise, five pancreatic malignancies in our study were not diagnosed as pathological malignancy with EUS-FNA. Interestingly, all those pancreatic masses showed hypoenhancement patterns using CH-EUS. All these masses were finally diagnosed as malignant through pathology results following surgical resection or clinical follow-up. When considering pancreatic adenocarcinomas as a tumor with a hypoenhancement pattern using CH-EUS and/or a positive result in EUS-FNA, the sensitivity of detecting pancreatic malignancy is increased to 100%.

Therefore, if CH-EUS reveals a hypoenhancement pattern in a pancreatic mass that showed negative or inconclusive results in EUS-FNA diagnostics, we recommend performing a pathological diagnosis again using EUS-FNA. If a second pathological diagnosis is impossible, we recommend observation of the clinical course to avoid missing a malignancy.

There were three cases of neuroendocrine tumors in our study. Two out of the three neuroendocrine tumors had hyperenhancement patterns, and the third had an isoenhancement pattern. Our result supported the finding that neuroendocrine tumors are hypervascular lesions using CH-EUS techniques with a sensitivity of 79%[12]. We surmise that the reason for isoenhancement patterns in neuroendocrine tumors is that the size of the tumor was too large (3.9 cm × 3.5 cm) to be accompanied by necrosis. Therefore, vascular structure was not visible on CH-EUS, and the mass was misinterpreted as an isoenhancement pattern. However, neuroendocrine tumors were too few to analyze in this study. Further studies with more patients are therefore required to investigate the characteristic features of neuroendocrine tumors using CH-EUS, and to estimate the diagnostic abilities of CH-EUS.

Our study has a few limitations. As our study is a retrospective single center study, selection bias is a possible problem. Secondly, the number of enrolled patients was too small to form a definite conclusion. Although our results were similar to those of other recent studies, and our study showed the possibilities of the use of CH-EUS for differential diagnoses in pancreatic disease, a large prospective study is required to reach a definite conclusion. Thirdly, our entire study was performed and reviewed by a single endoscopist. As a result, interpretation errors might be present in our study. In our study, the sensitivity and specificity of CH-EUS for detecting pancreatic adenocarcinoma were lower than those of the other studies[13,19,31]. We believe that these results are related to interpretation errors.

In conclusion, CH-EUS is a useful tool for the detection of pancreatic adenocarcinoma and differentiation between pancreatic malignancies and other pancreatic benign diseases. However, due to its low specificity, CH-EUS cannot be an alternative tool of EUS-FNA, which is used in the definitive diagnosis of pancreatic cancer. We recommend that a pathological diagnosis be performed on the lesion to reveal the hypoenhancement pattern in CH-EUS to avoid missing a malignancy.

COMMENTS

Background

One of the most important and challenging tasks of pancreatic endoscopic ul-

trasound is differentiation between pancreatic tumors and inflammatory tumor-like masses. Contrast-enhanced harmonic endoscopic ultrasonography (CH-EUS) is reported to improve diagnosis of pancreatic cancers. Nevertheless, CH-EUS suffers from limitations such as blooming artifacts, poor spatial resolution, and low sensitivity to slow flow. To overcome these, CH-EUS with a second-generation ultrasound contrast agent was recently developed.

Research frontiers

The microbubbles in the second-generation ultrasound contrast agent have a distinctive structure in comparison with those in first-generation agents. The microbubbles consist of gases other than air. Because of this, the microbubble can be oscillated or broken by lower acoustic power, allowing microvessels and parenchymal perfusion to be visualized to a greater extent than that achieved in CH-EUS with first-generation agents.

Innovations and breakthroughs

In the current study, CH-EUS was a very effective diagnostic tool for detection of pancreatic adenocarcinoma with the 92% sensitivity, 68% specificity, and 82% accuracy. This result is very similar with the results of previous reports. However, our result has a unique significance: it indicates the role of CH-EUS as an additional diagnostic tool for pancreatic adenocarcinoma. Five patients with suspected pancreatic cancer revealed hypoenhancement patterns in CH-EUS, but their diagnosis failed in the pathologic examination results of EUS-fine needle aspiration (EUS-FNA). As a result, three patients were diagnosed with pancreatic adenocarcinoma after surgical resection, and the remaining two patients were clinically diagnosed with malignancy.

Applications

This study suggested that CH-EUS is useful for distinction between pancreatic adenocarcinoma and other pancreatic disease and if the pancreatic mass reveals a hypoenhancement pattern in CH-EUS with negative results or insufficient samples in EUS-FNA, clinicians might consider performing a pathologic diagnosis again with EUS-FNA or surgical resection.

Terminology

Clinically diagnosed malignancy was defined if the pancreatic mass showed suspected malignancy in EUS on the basis of the echogenicity and morphology, or an increasing tumor size during the tracking period. Positron emission tomography-computed tomography was performed and all pancreatic masses in these patients yielded positive results in this examination. These patients did not survive longer than 6 mo after identification of the pancreatic masses.

Peer review

This is a good retrospective study in which the authors analyzed the efficacy of CH-EUS in diagnosis of pancreatic cancer. The result shows CH-EUS has the ability to distinguish pancreatic cancer from other pancreatic benign lesions and suggests that the presence of lesions showing a hypoenhancement pattern in CH-EUS may indicate pancreatic cancer.

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BRIEF ARTICLE

Comparative analysis of radiofrequency ablation and resection for resectable colorectal liver metastases

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Abstract

AIM: To evaluate the therapeutic efficacy of radiofrequency ablation (RFA) for resectable colorectal liver metastases (CRLM) compared with that of resection.

METHODS: Between June 2004 and June 2009, we retrospectively analyzed 29 patients with resectable CRLMs; 17 patients underwent RFA, and 12 underwent hepatic resection. All of the patients were informed about the treatment modalities and were allowed to choose either of them. RFA including an intraoperative approach was performed by a radiologist; otherwise, hepatic resection was performed by a surgeon. Comparative analysis of the two groups was performed, including comparisons of gender, age, and clinical outcomes, such as primary tumor stage and survival rates.

RESULTS: The mean tumor size was significantly larger in the resection group (3.59 cm *vs* 2.02 cm, $P < 0.01$), and the 5-year overall survival (OS) rate for all patients was 44.7%. There was no difference in the 5-year OS rates between the RFA and resection groups (37.8% *vs* 66.7%). Univariate analysis indicated significantly lower 5-year OS rates for patients with a tumor size > 3 cm. The 5-year disease-free survival (DFS) rates were 17.6% and 22.2% in the RFA and resection groups, respectively ($P = 0.119$). Univariate analysis revealed that in cases of male gender, age > 65 years, T stage < Ⅳ, absence of lymphatic metastasis, and tumor size > 3 cm, RFA resulted in significantly inferior 5-year DFS rates compared with surgical resection.

CONCLUSION: Surgical resection revealed superior outcomes in the treatment of resectable CRLMs, particularly in cases with a hepatic tumor size > 3 cm.

Key words: Colorectal neoplasm; Metastasis; Radiofrequency; Hepatectomy; Survival

Core tip: Colorectal liver metastasis is diagnosed in approximately 50% of patients with colorectal cancer. Surgical resection is the optimal treatment strategy. Alternative local treatment modalities can be adapted, and radiofrequency ablation (RFA) is widely accepted. We examined whether RFA is an appropriate alternative method to surgery for resectable colorectal liver metastases. This study retrospectively compared the therapeutic efficacy of RFA and compared it with that of surgical resection in a single institute.

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INTRODUCTION

A 2005 annual report of cancer incidence indicated that colorectal cancer (CRC) is the third most common malignancy worldwide and the second most common (12.5%) in Korea, following gastric cancer. The 5-year survival rate of CRC is reportedly 61%[1,2], and hepatic metastasis develops in approximately 40%-50% of patients with CRC; approximately 50% of diagnosed patients present the synchronous type[3-5]. Although surgical resection is the most effective current treatment for resectable colorectal cancer liver metastases (CRLMs)[6,7], only 10%-15% of such cases are suitable for the procedure[8,9]. Several alternative treatment modalities for unresectable CRLMs have been developed, of which radiofrequency ablation (RFA) is widely accepted as an effective alternative local treatment modality[10].

Surgical hepatic resection is the treatment of choice for resectable CRLMs. Although RFA is an alternative to resection in hepatocellular carcinoma[11,12], there is little information regarding indications for RFA in resectable CRLMs. RFA is performed within a limited number of clinical settings for resectable CRLMs[13,14]. The purpose of the present study was to compare the therapeutic efficacies of RFA and hepatic resection for resectable CRLMs within a single institution.

MATERIALS AND METHODS

In this study, we compared the treatment outcomes of 12 patients who underwent hepatic resection with 17 who underwent RFA for synchronous or metachronous resectable CRLMs between June 2004 and June 2009 at the Department of Surgery, Pusan National University Hospital (Busan, South Korea). The inclusion criteria for this study were as follows: (1) no signs of preoperative extrahepatic metastases; (2) tumor size < 5 cm; and (3) a single metastatic tumor. The exclusion criteria were as follows: (1) simultaneous performance of resection and RFA; (2) resection of hepatic recurrence after RFA; (3) lymph node metastases identified during or after resection; and (4) associated multiple hepatic metastases. The selection of patients for each treatment modality was fully based on the patient's decision.

Diagnosis of CRLM

The diagnosis of hepatic or extrahepatic metastasis was confirmed on the basis of the findings of serum carcinoembryonic antigen (CEA), contrast-enhanced computed tomography (CT) of the abdomen and chest, magnetic resonance imaging (MRI), and ^{18}F-2'-fluoro-2'-deoxyglucose positron emission tomography (FDG-PET). Hepatic metastasis was defined as any newly developed hepatic tumors detected during patient follow-up after curative resection of CRC. A needle biopsy was not routinely performed before RFA but was performed in patients with atypical hepatic mass enhancement.

RFA

RFA for hepatic metastases was performed when patients refused surgical hepatic resection after being informed of the treatment method, complications, and survival rates. RFA was performed percutaneously under local anesthesia or during and simultaneously with CRC resection. RFA was performed using a 200-W generator in the impedance control mode and a monopolar single or clustered internally cooled electrode (Covidien, Boulder, CO, United States). Written informed consent was obtained from all patients before initiating treatment.

Table 1 Types of surgery for colorectal liver metastasis

Type of surgery		*n* (%)
Major resection	Right hemihepatectomy	5 (41.7)
Minor resection	Segmentectomy	6 (50.0)
	Left lateral sectionectomy	1 (8.3)
Total		12

Surgical resection

A major resection was defined as resection of more than three hepatic segments and minor resection as two segments or less. Major and minor resections were performed in five and seven patients, respectively (Table 1). None of the patients received perioperative transfusion, and there was no incidence of postoperative mortality.

Follow-up protocol

Seven days after resection or RFA, contrast-enhanced CT of the abdomen was performed, and serum CEA levels were measured to determine the baseline values. The same evaluations were repeated every four months during the initial two years and every six months thereafter. Endoscopic analysis and FDG-PET were performed annually, and chest CT or MRI was added when tumor recurrence was suspected.

Statistical analysis

Overall survival (OS) and disease-free survival (DFS) rates were analyzed using the Kaplan-Meier method, and the statistical significance of differences in the survival rates was evaluated using the log-rank test. A two-tailed *P*-value < 0.05 was considered statistically significant. The statistical analysis was performed using SPSS statistical software (ver. 12.0; SPSS Inc., Chicago, IL, United States).

RESULTS

Clinicopathological data

Information regarding the patients and pathological results is provided in Tables 2 and 3. The mean tumor diameter in the RFA group (2.02 cm; range, 0.8-4.6 cm) was significantly smaller than that in the resection group (3.59 cm; range, 1.6-4.9 cm). There were no other significant differences between the two groups. Four of the 17 patients in the RFA group and 7 of the 12 in the resection group presented a hepatic tumor > 3 cm in size; no significant difference was evident between the two

Table 2 Summary of patient information

No.	Age (yr)	Sex	Comorbidity	Treatment modality	Location	Timing of metastasis	Recurrence	Results
1	51	M		RFA	Colon	Meta	Yes	S
2	60	M		RFA	Colon	Meta	Yes	D
3	69	M		Resection	Colon	Meta	Yes	D
4	76	M	DM, HT	Resection	Rectum	Meta	Yes	D
5	62	M		RFA	Colon	Syn	No	S
6	61	M	DM	RFA	Rectum	Meta	Yes	D
7	70	F		Resection	Colon	Meta	Yes	S
8	70	M		Resection	Rectum	Meta	No	S
9	71	F		Resection	Rectum	Meta	Yes	S
10	69	F	DM, HT	Resection	Colon	Syn	Yes	S
11	74	F		Resection	Colon	Syn	No	S
12	71	M		Resection	Colon	Meta	Yes	S
13	82	M		Resection	Rectum	Meta	Yes	D
14	58	F		RFA	Colon	Meta	No	S
15	60	M		RFA	Colon	Syn	Yes	D
16	56	F		RFA	Colon	Meta	Yes	S
17	56	M		RFA	Colon	Meta	Yes	S
18	54	F		RFA	Colon	Meta	Yes	D
19	52	F		RFA	Rectum	Meta	No	S
20	60	M		RFA	Colon	Meta	Yes	D
21	55	M		RFA	Rectum	Meta	Yes	D
22	75	M		Resection	Colon	Syn	Yes	D
23	54	F		RFA	Rectum	Meta	Yes	D
24	63	F		RFA	Colon	Syn	Yes	D
25	66	M		Resection	Rectum	Syn	Yes	S
26	56	F		RFA	Rectum	Meta	Yes	D
27	67	M		Resection	Colon	Meta	Yes	D
28	58	M		RFA	Rectum	Syn	No	S
29	71	M		Resection	Colon	Meta	Yes	S

DM: Diabetes mellitus; HT: Hypertension; RFA: Radiofrequency ablation; Meta: Metachronous; Syn: Synchronous; S: Survival; D: Death; M: Male; F: Female.

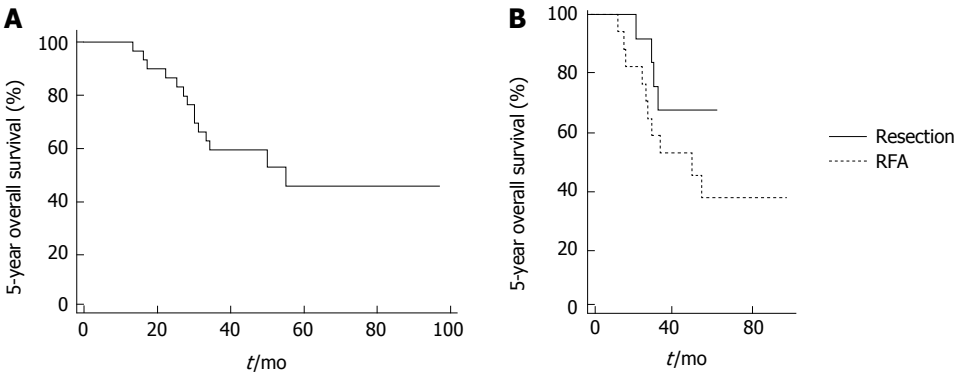

Figure 1 The 5-year overall survival rate. A: For all patients (44.7%); B: In the surgical resection (66.7%) and radiofrequency ablation (RFA) groups (37.8%).

groups (Table 4).

Survival rates

The 5-year OS rate was 44.7% among all patients with CRLMs, 37.8% in the RFA group, and 66.7% in the resection group (P = 0.29; Figure 1). The 5-year OS rate was lower in patients with a hepatic tumor size > 3 cm than in those with a tumor size < 3 cm (Table 5). Moreover, the 5-year DFS rates were 17.6% and 22.2% in the RFA and resection groups, respectively (P = 0.119; Figure 2). The variables associated with lower DFS rates included male gender, age > 65 years, CRC T stage < Ⅳ, absence of lymphatic invasion, and tumor size > 3 cm (Table 6).

DISCUSSION

Surgical resection is the treatment of choice for resectable CRLMs, whereas RFA has been used for unresectable CRLMs as an alternative treatment to improve patient survival[13,14]. While some series have reported RFA equivalent to resection, others have shown RFA to be inferior to resection based on overall survival[15-17]. However, the efficacy of RFA for resectable CRLMs remains controversial. Reuter *et al*[18] reported superior DFS rates in patients with resectable CRLMs following surgical resection than follow-

Table 3 Summary of pathological findings

No.	Metastatic tumor size (cm)	Diff	T	N	M	LNR	LV	PN
1	2.5	Mod	3	2	0	0.23	Pos	Pos
2	1.6	Mod	3	2	0	0.17	Pos	Pos
3	4	Mod	4	2	0	0.37	Pos	Pos
4	4.2	Mod	4	1	0	0.43	Pos	Pos
5	3.2	Mod	3	1	1	0.07	Pos	Neg
6	4.2	Mod	2	2	0	0.25	Pos	Pos
7	4	Mod	3	0	1	0	Pos	Pos
8	2.8	Mod	4	0	0	0	Pos	Pos
9	4.4	Mod	1	2	0	0.06	Neg	Neg
10	4.9	Well	3	1	1	0.03	Pos	Neg
11	2.8	Mod	3	0	0	0	Neg	Pos
12	2.3	Mod	4	1	0	0.08	Neg	Neg
13	3.2	Mod	4	2	1	0.33	Neg	Pos
14	1.2	Mod	3	2	0	0.14	Pos	Pos
15	0.9	Poor	3	2	0	0.12	Pos	Pos
16	1.7	Poor	3	1	0	0.04	Pos	Pos
17	2	Mod	3	1	0	0.03	Neg	Neg
18	1	Mod	3	2	0	0.34	Pos	Pos
19	2.5	Mod	4	0	0	0	Neg	Neg
20	2	Mod	3	1	0	0.14	Pos	Pos
21	1.6	Mod	3	1	0	0.08	Pos	Pos
22	1	Mod	3	1	0	0.04	Pos	Pos
23	3.8	Mod	2	0	0	0	Neg	Neg
24	3.7	Mod	3	0	1	0	Neg	Neg
25	1	Mod	3	0	0	0	Neg	Neg
26	3.6	Mod	4	2	1	0.58	Neg	Pos
27	1.2	Mod	3	0	0	0	Pos	Pos
28	0.8	Mod	4	1	1	0.03	Neg	Neg
29	1.3	Mod	3	0	0	0	Neg	Neg

Diff: Differentiation of primary tumor; T: T stage; N: N stage; M: M stage; LNR: Lymph node ratio; LN: Lymphovascular invasion; PN: Perineural invasion; Mod: Moderate; Poor: Poorly; Pos: Positive; Neg: Negative.

Table 4 Clinicopathological data of colorectal liver metastasis *n* (%)

		RFA (*n* = 17)	Resection (*n* = 12)	*P*-value
Sex	Male	7 (41)	4 (33)	0.49
	Female	10 (59)	8 (67)	
Age (yr)		61.35 ± 8.33	67.50 ± 7.44	0.07
Age (yr)	≤ 65	12 (71)	4 (33)	0.07
	> 65	5 (29)	8 (67)	
Timing of metastasis	Synchronous	5 (29)	3 (25)	1.00
	Metachronous	12 (71)	9 (75)	
Primary site	Colon	10 (59)	8 (67)	0.72
	Rectum	7 (41)	4 (33)	
T stage	I - III	13 (76)	8 (67)	0.68
	IV	4 (24)	4 (33)	
Lymphovascular	Positive	8 (47)	8 (67)	0.45
Invasion	Negative	9 (53)	4 (33)	
Perineural invasion	Positive	10 (59)	7 (58)	1.00
	Negative	7 (41)	5 (42)	
Lymph node metastasis	Positive	11 (65)	9 (75)	0.69
	Negative	6 (35)	3 (25)	
Size of metastasis (cm)		2.02 ± 1.17	3.59 ± 0.81	0.03
Size of metastasis	≤ 3 cm	13 (76)	5 (42)	0.07
	> 3 cm	4 (24)	7 (58)	
Recurrence	Yes	14 (82)	9 (75)	0.67
	No	3 (18)	3 (25)	

RFA: Radiofrequency ablation.

ing RFA. By contrast, Mulier *et al*[19] reported no significant difference in OS between RFA and surgical resection for local control of CRLMs. Furthermore, in a recent study, Kanas *et al*[20] reported a 5-year OS rate of 30%-40% in

Table 5 Univariate analysis of 5-year overall survival

		RFA (n = 17)		Resection (n = 12)		*P*-value
		3-yr	5-yr	3-yr	5-yr	
Sex	Female	42.9%	42.9%	100%	100%	0.083%
	Male	60.0%	30.0%	50.0%	50.0%	0.925%
Age (yr)	≤ 65	58.3%	38.9%	50.0%	50.0%	0.848%
	> 65	40.0%	40.0%	75.0%	75.0%	0.187%
Primary	1-3	50.0%	33.3%	75.0%	75.0%	0.194%
T stage	4	66.7%	66.7%	50.0%	50.0%	0.847%
Timing of	Synchronous	33.3%	33.3%	100%	100%	0.093%
Metastasis	Metachronous	63.6%	42.4%	55.6%	55.6%	0.975%
Preoperative chemotherapy	No	50.0%	50.0	66.7	66.7	0.410
	Yes	54.5%	36.4%	66.7%	66.7%	0.495%
Primary site	Colon	50.0%	37.5%	75.0%	75.0%	0.202%
	Rectum	57.1%	38.1%	50.0%	50.0%	0.992%
Metastatic	Negative Positive	50.0%	50.0%	100%	100%	0.175%
Lymph node		54.5%	32.7%	55.6%	55.6%	0.502%
Lymphovascular invasion	Negative	55.6%	55.6%	100%	100%	0.140%
	Positive	50.0%	25.0%	50.0%	50.0%	0.638%
Perineural invasion	Negative Positive	71.4%	71.4%	100%	100%	0.214%
		40.0%	20.0%	42.9%	42.9%	0.515%
Primary	≤ 3 cm	69.2%	49.50%	80.0%	80.0%	0.464%
Tumor size	> 3 cm	0.0%	0.0%	57.1%	57.1%	0.005%
Postoperative chemotherapy	No	20.0%	20.0%	0.0%	0.0%	0.929%
	Yes	66.7%	47.6%	80.0%	80.0%	0.341%

RFA: Radiofrequency ablation.

Table 6 Univariate analysis of 5-year disease-free survival

		RFA (n = 17)		Resection (n = 12)		*P*-value
		3-yr	5-yr	3-yr	5-yr	
Sex	Female	28.6%	28.6%	50.0%	50.0%	0.350
	Male	10.0%	10.0%	50.0%	33.3%	0.039
Age (yr)	≤ 65	25.0%	25.0%	50.0%	25.0%	0.449
	> 65	0.0%	0.0%	46.9%	46.9%	0.012
Primary	I -Ⅲ	7.1%	7.1%	50.0%	25.0%	0.023
T Stage	Ⅳ	66.7%	66.7%	50.0%	50.0%	0.702
Timing of	Synchronous	16.7%	16.7%	66.7%	66.7%	0.201
Metastasis	Metachronous	18.2%	18.2%	41.7%	20.8%	0.172
Preoperative chemotherapy	No	16.7%	16.7%	53.3%	53.3%	0.112
	Yes	18.2%	18.2%	33.3%	0.0%	0.617
Primary site	Colon	10.0%	10.0%	50.0%	25.0%	0.048
	Rectum	28.6%	28.6%	37.5%	37.5%	0.385
Metastatic	Negative	16.7%	16.7%	100.0%	100.0%	0.037
Lymph node	Positive	18.2%	18.2%	29.6%	14.8%	0.325
Lymphovascular invasion	Negative	22.2%	22.2%	50.0%	50.0%	0.137
	Positive	12.5%	12.5%	50.0%	33.3%	0.197
Perineural invasion	Negative	28.6%	28.6%	40.0%	40.0%	0.423
	Positive	10.0%	10.0%	57.1%	28.6%	0.074
Primary	≤ 3 cm	23.1%	23.1%	60.0%	30.0%	0.204
Tumor size	> 3 cm	0.0%	0.0%	38.1%	38.1%	0.013
Postoperative chemotherapy	No	0.0%	0.0%	50.0%	50.0%	0.151
	Yes	25.0%	25.0%	50.0%	33.3%	0.235

RFA: Radiofrequency ablation.

patients with resectable CRLMs. Moreover, they observed that the survival rate in the resection group was favorable and reported that statistical significance could be expected using a larger patient population, even in the actual 5-year OS rate in the RFA group and in the nonactual survival in the resection group. Our 5-year OS rates were 66.7% in the resection group and 37.8% in the RFA group (actuarial 5-year survival rates), which is comparable to those reported in other published studies. In patients with hepatic tumors < 3 cm, the 5-year OS rate was 80.0% in the RFA group and 49.5% in the resection group (P = 0.46). In patients with a hepatic tumor size > 3 cm, the 5-year OS rates were 0% in the RFA group and 57.1% in the resection group (P = 0.005). In addition, the DFS rate in the resection group was superior to that in the RFA group.

To date, there exist some controversies regarding the

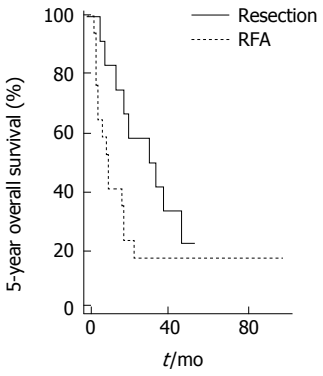

Figure 2 Disease-free survival rates in the surgical resection (22.2%) and radiofrequency ablation groups (17.6%). RFA: Radiofrequency ablation.

contribution of clinicopathological factors to survival following surgery for resectable CRLMs[21]. Surgical resection in CRLM is considered the treatment of choice for local tumor control rather than systemic therapy. RFA, which has the advantages of minimal invasiveness and sparing the liver parenchyma, might be favorable for the local control of CRLMs, which requires adjuvant chemotherapy as well[19]. However, less definitive evidence exists regarding the risk of intrahepatic or hematogenous metastases after RFA for patients with CRLMs. Fourteen patients who underwent RFA in our study experienced recurrences of multiple liver metastases and peritoneal carcinomatosis, and two patients developed metastases in the lung and spleen. The local recurrence rates after RFA are reportedly 2%-40%[22-25], and Abitabile *et al*[26] reported that local recurrence rates reached 8.8% overall and 1.6% for CRLMs < 3 cm in diameter. In the present study, one patient developed tumor recurrence following RFA and was excluded; the patient was followed up for 37 mo without recurrence after consecutive hepatic resection.

The statistical analysis in the present study identified the following risk factors for poor DFS in the RFA group compared with the resection group: male gender, age > 65 years, lower T stage, colon cancer, and absence of lymph node metastasis. These findings might be the result of the omission of intensive adjuvant chemotherapy in patients with less-advanced CRC stages.

Some limitations to the present study include its retrospective design and the small number of included cases. However, to our knowledge, this is the first report regarding the actuarial 5-year survival rate after RFA, which was 37.8% in patients with resectable CRLMs. Surgical resection is believed to be superior to RFA for resectable CRLMs; nevertheless, RFA displayed some interesting advantages to justify its adoption in patients with resectable CRLMs. Although a randomized controlled study of RFA is warranted, more strict indication criteria are needed before adopting RFA as a replacement for surgical resection in resectable CRLMs.

COMMENTS

Background

Hepatic metastasis develops in approximately 40%-50% of patients with colorectal cancer, and approximately 50% of diagnosed patients present the synchronous type. Surgical resection is the most effective current treatment for colorectal cancer liver metastasis (CRLM), and several alternative treatment modalities for CRLM have been developed.

Research frontiers

Radiofrequency ablation (RFA) is widely accepted as an effective alternative local treatment modality for CRLM. However, there is little information regarding indications for RFA in resectable CRLMs.

Innovations and breakthroughs

The authors retrospectively reviewed 29 patients with resectable CRLMs, who were treated with either RFA or surgical resection. This comparison was performed to determine whether RFA can be an effective alternative treatment for resectable CRLMs.

Applications

The authors suggest that RFA can be an alternative treatment for resectable CRLMs, primarily in patients with hepatic tumors < 3 cm in size.

Terminology

RFA is a procedure in which tissue ablation is performed using the heat generated by a high-frequency alternating current.

Peer review

This article examined the usefulness of RFA in patients with resectable CRLMs. In this study, the authors found that RFA can be useful in patients with CRLMs < 3 cm in diameter.

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BRIEF ARTICLE

Incidence trends and predictors for cost and average lengths of stay in colorectal cancer surgery

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Abstract

AIM: To evaluate the changing trends and outcomes of colorectal cancer (CRC) surgery performed at a large single institution in Taiwan.

METHODS: This study retrospectively analyzed 778 patients who received colorectal cancer surgery at E-Da Hospital in Taiwan from 2004 to 2009. These patients were from health examination, inpatient or emergency settings. The following attributes were analyzed in patients who had undergone CRC surgical procedures: gender, age, source, surgical type, tumor number, tumor size, number of lymph node metastasis, pathologic differentiation, chemotherapy, distant metastases, tumor site, tumor stage, average hospitalization cost and average lengths of stay (ALOS). The odds ratio and 95% confidence intervals were calculated to assess the relative rate of change. Regression models were employed to predict average hospitalization cost and ALOS.

RESULTS: The study sample included 458 (58.87%) males and 320 (41.13%) females with a mean age of 64.53 years (standard deviation, 12.33 years; range, 28-86 years). The principal patient source came from inpatient and emergency room (96.02%). The principal tumor sites were noted at the sigmoid colon (35.73%) and rectum (30.46%). Most patients exhibited a tumor stage of 2 (37.28%) or 3 (34.19%). The number of new CRC surgeries performed per 100000 persons was 12.21 in 2004 and gradually increased to 17.89 in 2009, representing a change of 46.52%. During the same period, the average hospitalization cost and ALOS decreased from $5303 to $4062 and from 19.7 to 14.4 d, respectively. The following factors were associated with considerably decreased hospital resource utilization: age, source, surgical type, tumor size, tumor site, and tumor stage.

CONCLUSION: These results can be generalized to patient populations elsewhere in Taiwan and to other countries with similar patient profiles.

Key words: Colorectal cancer; Average hospitalization cost; Average lengths of stay; Incidence trend; Colorectal cancer surgery

Core tip: We evaluated the trend of colorectal cancer surgery and compared hospitalization cost and length of stay with those in other countries. Age, source, surgical type, tumor size, tumor site, and tumor stage were associated with decreased hospital resource utilization. To efficiently allocate of medical resources, these factors must be managed.

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in the world[1]. In a recent data analysis from cancer registries participating in the Surveillance, Epidemiology, and End Results program, 148810 new cases of CRC with 49960 deaths were estimated for the United States in 2008[2]. In Europe, CRC was the second most common form of cancers and cause of death from cancer in 2006[3]. By contrast, incidence rates tend to be lower in Africa and Asia, which is attributed to differences in diet and lifestyle. Moreover, the medical expenses associated with CRC cannot be ignored[4,5]. The estimated medical expense for a patient with CRC in the United States of America per year is $38577, which is seven times higher than that for a patient without CRC ($5126)[6].

The effectiveness of CRC surgery in relieving pain and improving physical function has been well documented[7,8]. In addition to improved surgical techniques, the excellent performance of new materials and designs has substantially increased the demand for CRC surgery. The growing population of elderly patients is yet another factor[9]. Because the system provides insurance coverage for expensive and frequently used medical items, the financial burden of CRC surgery should not be overlooked.

Although the volume of CRC surgical procedures is increasing annually, the incidence rates and hospital resource utilization for these procedures have not been documented in a Taiwan study. Thus, this study explored the changing trends and risk factors of these outcomes for CRC surgery.

MATERIALS AND METHODS

E-Da Hospital is a 1200-bed hospital, a large medical institution in Taiwan, and provides secondary and tertiary medical care for approximately one million people. A retrospective review of all patients who underwent CRC surgery from 2004 to 2009 was performed. If patients had intraoperative perforations, concurrent malignancy, or a psychological or linguistic impairment, they were excluded. All malignancies were confirmed upon histological evaluation. The study analyzed 778 CRC surgical procedures. The study protocol was approved by the Institutional Review Board of E-Da Hospital.

The following attributes were analyzed in patients who had undergone CRC surgical procedures in Taiwan: gender, age, source, surgical type, tumor number, tumor size, number of lymph node metastasis, pathologic differentiation, chemotherapy, distant metastases, tumor site, tumor stage, average hospitalization cost and average lengths of stay (ALOS). The age categories were $\leqslant$ 30, 31-40, 41-50, 51-60, 61-70, 71-80 and $\geqslant$ 81 years old. The patient source came from health examination, inpatient and emergency room settings. The surgical types were grouped as low anterior resection, high anterior resection, abdominoperineal resection, right hemicolectomy, left hemicolectomy, super low anterior resection and endoscopic polypectomy. Pathologic differentiation was classified as well, moderate or poor. The patients were grouped by tumor site as follows: ascending colon, transverse colon, descending colon, sigmoid colon and rectum. Tumor stage was categorized as 1, 2, 3 or 4. All colorectal cancers were staged according to the guidelines of the American Joint Committee of Cancer.

Continuous variables were tested for statistical significance by one-way analysis of variance (ANOVA), and categorical variables were tested by χ^2 analysis. Temporal trends were assessed by the Cochrane-Armitage trend test. The study period was divided into three equal time intervals (P1: 2004-2005; P2: 2006-2007; and P3: 2008-2009). The odds ratio and 95% confidence interval were determined to assess the relative change for each factor when using P1 as the reference group when compared to P3. We define the incidence rate as the number of new cases of colorectal cancer surgery from health examination, inpatient or emergency room divided by the total number of cases from those settings[10].

Regarding treatment costs, the standard administrative claims data required by the Bureau of National Health Insurance (BNHI) included the following fees: operating room, radiology, physical therapy, hospital room, anesthetist, pharmacy, laboratory, special materials, surgeon, and others. To reflect changes in real dollar value, the cost data were adjusted by the consumer price index for each year from 2004-2009 (93.70, 95.86, 96.43, 98.17, 101.63 and 100.00). The hospital treatment costs were then converted from Taiwan dollars to United States dollars at an exchange rate of 31.5:1, which was the average exchange rate during 2004-2009. The hospital treatment costs at different hospital levels were also adjusted for differences in BNHI reimbursements. The multiple regression models used to predict average hospitalization cost and ALOS included both patient and clinical attributes.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, United States). All of the tests were two-sided, and *P* values less than 0.05 were considered statistically significant.

RESULTS

The study sample included 458 (58.87%) males and 320 (41.13%) females with a mean age of 64.53 years (standard deviation, 12.33 years; range, 28-86 years). The principal patient source came from inpatient and emergency rooms (96.02%). The principal surgical types for the study population were low anterior resection, right hemicolectomy, super low anterior resection, left hemicolectomy, high anterior resection, abdominoperineal resection, and endoscopic polypectomy at the following frequencies: 25.32%, 23.78%, 17.74%, 12.20%, 10.54%, 6.56%, and 3.86%,

Table 1 Patient characteristics (n = 778)

Variable	n (%)
Gender	
Female	320 (41.13)
Male	458 (58.87)
Age (yr)	64.53 ± 12.33
≤ 30	9 (1.16)
31-40	23 (2.96)
41-50	62 (7.97)
51-60	187 (24.04)
61-70	218 (28.02)
71-80	219 (28.15)
≥ 81	60 (7.71)
Source	
Health examination	31 (3.98)
Inpatient or emergency room	747 (96.02)
Surgical type	
Low anterior resection	197 (25.32)
High anterior resection	82 (10.54)
Abdominoperineal resection	51 (6.56)
Right hemicolectomy	185 (23.78)
Left hemicolectomy	95 (12.2)
Super low anterior resection	138 (17.74)
Endoscopic polypectomy	30 (3.86)
Tumor number	1.05 ± 0.29
Tumor size	5.06 ± 2.14
No. of lymph node metastases	3.56 ± 5.15
Pathologic differentiation	
Well	86 (11.05)
Moderate	638 (82.01)
Poor	54 (6.94)
Chemotherapy	
No	368 (47.30)
Yes	410 (52.70)
Distant metastasis	
No	727 (93.44)
Yes	51 (6.56)
Tumor site	
Ascending colon	145 (18.64)
Transverse colon	67 (8.61)
Descending colon	51 (6.56)
Sigmoid colon	278 (35.73)
Rectum	237 (30.46)
Tumor stage	
1	144 (18.51)
2	290 (37.28)
3	266 (34.19)
4	78 (10.03)
Treatment cost (dollars)	128543.94 ± 85360.59
Average length of stay (d)	15.40 ± 8.12

respectively. On average, the number of tumors, size of the tumor, and number of lymph node metastases were (mean ± SD) 1.05 ± 0.29, 5.06 ± 2.14, and 3.56 ± 5.15, respectively. Pathologic differentiation included the following classifications: moderate (82.01%), well (11.05%), and poor (6.94%). In the study, 52.70% cases received chemotherapy after colorectal cancer surgery, and distant metastases were not found in 93.44% cases when these patients were included in the study. The principal tumor site was described in the sigmoid colon (35.73%) and rectum (30.46%). Most patients exhibited a tumor stage of 2 (37.28%) or 3 (34.19%). Additionally, the average treatment costs were US$4285 ± 2845.4, and the ALOS was 15.40 ± 8.12 d. The detailed patient characteristics are shown in Table 1.

Table 2 The incidence rate of colorectal cancer surgery

Year	Patients (n)	Surgeries (n)	Incidence rate ($1/10^5$)
2004	245734	30	12.21
2005	621243	99	15.94
2006	727286	158	21.72
2007	782659	136	17.38
2008	825907	199	24.09
2009	877791	157	17.89

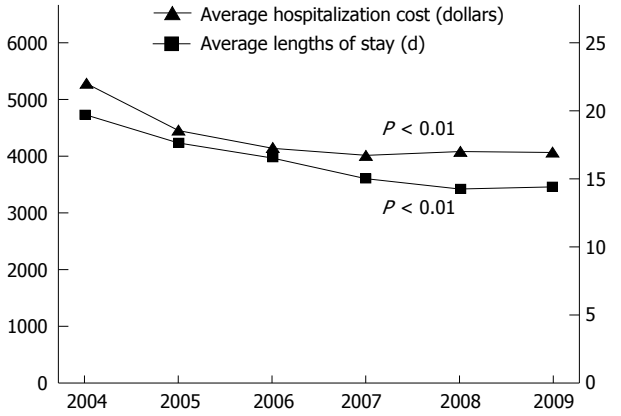

Figure 1 The trend analyses of average hospitalization cost and average lengths of stay in colorectal cancer surgery patients during the study period.

The incidence rate of CRC surgery in 2004 was 12.21 per 100000 persons, and the rate gradually increased to 17.89 in 2009, which represented a change of 46.52% (Table 2). A significant decreased trends analysis was also observed in average hospitalization cost and ALOS in CRC surgery patients during the study period ($P < 0.01$) (Figure 1).

Table 3 shows the increasing volume of CRC surgical procedures and the changes in patient demographic and clinical characteristics. Approximately 40% of all CRC surgery patients treated from P1 to P3 were female, and the number of female patients significantly increased between P1 and P3 (OR = 1.13, 95%CI: 1.01-1.25). Conversely, the number of male patients significantly decreased. The number of CRC surgery patients younger than 30 years old significantly decreased between P1 and P3 (OR = 0.46, 95%CI: 0.33-0.60). The number of CRC surgery patients aged 51 to 60 years old significantly increased from P1 to P3 (OR = 1.78, 95%CI: 1.58-1.97) but the number of CRC surgery patients aged 61 to 70 years old significantly decreased (OR = 0.71, 95%CI: 0.56-0.87). The number of CRC surgery patients older than 81 years old significantly increased between P1 and P3 (OR = 1.22, 95%CI: 1.06-1.37). The number of CRC surgery patients from health examinations significantly increased (OR = 5.78, 95%CI: 5.48-6.08). In terms of surgical type, the data revealed a statistically significant decrease in the number of patients with low anterior resection and super low anterior resection between P1 and P3 (OR = 0.02, 95%CI: 0.00-0.04 and OR =

Table 3 Logistic regression model for colorectal cancer surgery during periods 1-3[1]

Variables		P1 (n = 118)	P2 (n = 273)	P3 (n = 387)	OR (95%CI)
Gender	Female	35.59	44.69	40.31	1.13 (1.01-1.25)
	Male	64.41	55.31	59.69	0.93 (0.83-1.06)
Age (yr)	≤ 30	1.69	1.47	0.78	0.46 (0.33-0.60)
	31-40	2.54	3.30	2.84	1.12 (0.96-1.28)
	41-50	5.93	10.62	6.72	1.13 (0.99-1.27)
	51-60	16.10	20.88	28.68	1.78 (1.58-1.97)
	61-70	35.59	28.94	25.06	0.71 (0.56-0.87)
	71-80	31.36	27.47	27.65	0.88 (0.73-1.03)
	≥ 81	6.78	7.33	8.27	1.22 (1.06-1.37)
Source	Health examination	0.85	4.03	4.91	5.78 (5.48-6.08)
	Inpatient or emergency room	99.15	95.97	95.09	0.96 (0.74-1.18)
Surgical type	Low anterior resection	35.59	25.64	0.62	0.02 (0.00-0.04)
	High anterior resection	3.39	2.2	18.60	5.49 (5.19-5.82)
	Abdominoperineal resection	2.54	4.03	9.56	3.76 (3.54-3.99)
	Right hemicolectomy	20.34	25.64	23.51	1.16 (1.00-1.36)
	Left hemicolectomy	14.41	12.09	11.63	0.81 (0.61-1.05)
	Super low anterior resection	21.19	24.18	12.14	0.57 (0.38-0.76)
	Endoscopic polypectomy	2.54	6.23	2.58	0.31 (0.05-1.77)
Pathologic differentiation	Well	14.41	9.89	10.85	0.75 (0.51-0.92)
	Moderate	77.97	82.78	82.69	1.06 (0.82-1.27)
	Poor	7.63	7.33	6.46	0.85 (0.59-1.05)
Chemotherapy	No	52.54	38.1	52.20	0.99 (0.70-1.37)
	Yes	47.46	61.9	47.80	1.01 (0.80-1.28)
Distant metastasis	No	83.05	93.41	96.64	1.16 (1.05-1.31)
	Yes	16.95	6.59	3.36	0.20 (0.02-0.40)
Tumor site	Ascending colon	18.64	19.05	18.35	0.98 (0.75-1.19)
	Transverse colon	10.17	7.69	8.79	0.86 (0.70-1.06)
	Descending colon	6.78	6.96	6.20	0.91 (0.79-1.14)
	Sigmoid colon	22.88	35.16	40.05	1.75 (1.43-2.23)
	Rectum	41.53	31.14	26.61	0.64 (0.42-0.93)
Tumor stage	1	15.25	16.12	21.19	1.39 (1.12-1.74)
	2	38.98	35.16	38.24	0.98 (0.78-1.19)
	3	38.98	32.60	33.85	0.87 (0.56-1.14)
	4	6.78	6.12	6.72	0.99 (0.80-1.29)

[1]Period 3 (P3) *vs* period 1 (P1) (reference group). P1: 2004-2005; P2: 2006-2007; P3: 2008-2009.

0.57, 95%CI: 0.38-0.76, respectively) but a statistically significant increase in the number of patients with high anterior resection, abdominoperineal resection and right hemicolectomy between P1 and P3 (OR = 5.49, 95%CI: 5.19-5.82; OR = 3.76, 95%CI: 3.54-3.99; and OR = 1.16, 95%CI: 1.00-1.36, respectively). The number of CRC surgery patients who exhibited well-differentiated tumors significantly decreased (OR = 0.75, 95%CI: 0.51-0.92). The number of CRC surgery patients without distant metastases significantly increased from P1 to P3 (OR = 1.16, 95%CI: 1.05-1.31) while the number of CRC surgery patients with distant metastases significantly decreased (OR = 0.20, 95%CI: 0.02-0.40). The number of CRC surgery patients with tumors at the sigmoid colon significantly increased from P1 to P3 (OR = 1.75, 95%CI: 1.43-2.23) while the number of CRC surgery patients with tumors at the rectum significantly decreased (OR = 0.64, 95%CI: 0.42-0.93). The number of CRC surgery patients with stage 1 tumors significantly increased from P1 to P3 (OR = 1.39, 95%CI: 1.12-1.74).

Table 4 shows the multiple regression models used to evaluate the predictors for hospital resource utilization. After controlling for time, the statistically significant predictors for hospital resource utilization among CRC surgery patients were age, source, surgical type, tumor size, tumor site, and tumor stage ($P < 0.05$).

DISCUSSION

This large survey study is the first to examine how patient and clinical attributes reflect changing trends in the incidence of CRC surgery and the first to identify factors that predict average hospitalization costs and ALOS for the procedure. This study showed a gradual increase in the incidence of new CRC surgeries during the study period and, during the same period, a demographic decrease in hospital resource utilization, which is consistent with other series studies. The following factors were associated with the considerably decreased hospital resource utilization of CRC surgery: age, source, surgical type, tumor size, tumor site, distant metastasis, and tumor stage.

Old age is a risk factor for the occurrence of CRC[2,11,12]. However, according to the results of this study, we found a trend of increasing incidence of CRC in patients aged 51-60 years old from the first to third time period. In the first time period, the percentage of 51-60 years old patients with CRC is only 16.10%, but this incidence increases to 20.88% and 28.68% in the second and third

Table 4 Impact factors for predicting the average hospitalization cost and average lengths of stay for colorectal cancer surgery[1]

Variables		Average hospitalization cost[2]			Average lengths of stay[3]		
		Coefficient	Standard coefficient	*P* value	Coefficient	Standard coefficient	*P* value
Gender	Female	0.01	0.02	0.344	0.02	0.05	0.140
Age		0.01	0.08	< 0.001	0.01	0.10	0.010
Source	Inpatient or emergency room	-0.17	-0.11	< 0.001	0.10	0.10	0.010
Surgical type	High anterior resection	-0.03	-0.03	0.168	-0.06	-0.11	0.009
	Abdominoperineal resection	0.03	0.03	0.213	0.05	0.07	0.090
	Right hemicolectomy	-0.01	-0.01	0.891	0.01	0.02	0.816
	Left hemicolectomy	-0.01	-0.01	0.625	-0.01	-0.02	0.707
	Super low anterior resection	0.02	0.02	0.478	0.01	0.02	0.687
	Endoscopic polypectomy	-1.16	-0.75	< 0.001	-0.69	-0.14	< 0.001
Tumor number		0.03	0.03	0.110	-0.01	-0.01	0.805
Tumor size		0.01	0.06	0.010	0.01	0.07	0.062
No. of lymph node metastases		0.01	-0.01	0.568	0.01	0.01	0.997
Pathologic differentiation	Moderate	0.04	0.05	0.104	0.04	0.07	0.126
	Poor	0.05	0.04	0.139	0.04	0.05	0.289
Chemotherapy	Yes	-0.01	0.02	0.345	-0.02	-0.05	0.239
Distant metastasis	Yes	0.01	0.01	0.903	0.04	0.06	0.117
Tumor site	Transverse colon	0.01	0.01	0.726	0.09	0.14	0.003
	Descending colon	0.02	0.02	0.557	0.04	0.06	0.287
	Sigmoid colon	0.01	0.02	0.730	0.06	0.15	0.149
	Rectum	0.03	0.04	0.514	0.04	0.11	0.314
Tumor stage	2	0.04	0.06	0.076	0.03	0.07	0.198
	3	0.05	0.08	0.019	0.03	0.08	0.177
	4	0.17	0.17	< 0.001	0.09	0.15	0.002

[1]Reference groups: gender (male), source (health examination), surgical type (low anterior resection), pathologic differentiation (well), chemotherapy (no), distant metastasis (no), tumor site (ascending colon), tumor stage (1); [2]R^2 = 0.68 and adjusted R^2=0.67; [3]R^2 = 0.42 and adjusted R^2 = 0.39.

time periods. The increasing incidence of CRC is obvious and substantial in early old age from 51-60 years of age. From the first to third time period, the other difference is the increased rate of CRC cases from health examinations. In the first time period, the percentage of CRC cases from health examinations is less then 1%, but this incidence increases to nearly 5% in the third time period. We also noted that the percentage of state 1 tumor cases increases from the first to third time period. The incidence of colorectal cancer from 2004 to 2009 is between 12.21 per 100000 per year and 24.09 per 100000 per year in this study. In another study, the median unadjusted incidence of colorectal cancer from 1989 to 2008 was 6.17 per 100000 per year in South Asians compared with 71.70 per 100000 per year in non-South Asians (77.79% white British)[13].

In the results from this study, we note that CRC cases at an older age and with stage 4 tumors increase the average hospitalization cost and average lengths of stay. However, if the CRC cases receive endoscopic polypectomy, the average hospitalization cost and average lengths of stay decrease. Most of the CRC cases who receive endoscopic polypectomy are from the health examinations, demonstrating both cost and health benefits for the patients who receive health examinations. However, in our study, surgical types including high anterior resection, abdominoperineal resection, right hemicolectomy, left hemicolectomy and super low anterior resection were done by open approach. Other studies show have documented that the laparoscopic surgery is safe, feasible and less traumatic, with an oncological adequacy comparable to the open approach, a shorter compromise of immunological homeostasis and faster recovery[14-18]. Furthermore, another meta-analysis study showed the robotic surgery for rectal cancer is superior to laparoscopic surgery for treatment of rectal cancer[19,20].

A previous study showed that colorectal cancer that originates from a different site may provide additional prognostic information[21]. In our study, the trend analysis showed an increasing percentage of sigmoid colon cancer, from 22.88% at time period 1 to 40.05% at time period 3, and the multiple regression mode showed that tumors at transverse colon were a significant factor leading to increased ALOS.

According to the result of this study, we note that CRC patients with or without distal metastases have no statistically significant difference between the average hospitalization cost and ALOS. An increasing tumor size elevates the average hospitalization cost but dose not significantly affect ALOS. These findings may be due to the limited case numbers, which affected out ability to demonstrate the effect of distal metastases and tumor size on ALOS for CRC patients.

A study in Brazil showed the increasing hospital admission rates and economic burden for colorectal cancer from 1996 to 2008[22]. Our results reveal an approximate four times greater average hospital cost for patients who received colorectal cancer surgery in Taiwan compared to Brazil, but the average hospitalization costs and length of stay decrease each year in Taiwan. The median length of stay was 14 d for elective admissions in Ireland from 2002 to 2008[23]. Our study shows a similar length of stay

of 15.40 d. Compared to the conditions in the United States and Europe (between March and November 2003 in the United Kingdom, France, Germany, Italy, and Spain, and between September 2003 and October 2004 in the United States), the ALOS for a colonic operation is shortest in the United States at 7.8 d, while the ALOS for a colonic operation in countries in Europe, including France, Italy, United Kingdom, Spain and Germany, is 12.8-16.5 d[24]. The ALOS is similar between Taiwan and Europe. In Taiwan, the pre-operative preparation, such as radiology examination, biochemical laboratory examination, electrocardiogram and colon preparation, are arranged and performed after admission, so the average length of stay is longer in Taiwan than in the United States. Another reason that may contribute to the longer average length of stay in Taiwan is the health insurance. Public citizens in Taiwan have well-care health insurance, and they also have a higher frequency of out-patient department visits and a longer average length of stay. Furthermore, we used the regression model to observe trends and effective predictors for the average hospitalization costs and ALOS in patients who received colorectal cancer surgery.

The average hospitalization cost and ALOS decrease each year in the study. This finding may be related to the increase in stage 1 CRC that was found in a younger group of patients (51-60 years old) by general screening using a stool occult blood test and a colonoscopy at a health examination. Compared to patients with stage 4 CRC, the patients with stage 1 CRC not only have a decreased average hospitalization cost but also ALOS. The study indirectly show the benefit of the general screening using a stool occult blood test for the older patients and the value of colonoscopy at health examinations. However, the effectiveness of colonoscopy is diminished by operator-dependent factors. Therefore, quality assurance programs should be implemented in all colonoscopy practices[25].

Our results should be interpreted in the context of certain limitations. For example, the existing data set used for the study contained only 6 years of data from one institution. Thus, it is possible that some of the patients in our analysis who apparently did not undergo CRC surgery had, in fact, received CRC surgery in the 6 years prior to the study's time frame. Second, our analysis was not able to capture data on tests performed outside of the study hospital if they were not paid for by the study hospital. This limitation may have resulted in an underestimation of the adequacy of average hospitalization cost. Another potential limitation of our analysis is its dependence on claims data and the lack of data for readmission. A different study previously showed that enhanced recovery after surgery protocols may decrease the length of stay in a hospital. However, that study also noted that a reduced length of stay at a hospital stay was associated with a high rate of readmission[26]. Finally, the analysis did not examine outcome data such as patient-reported quality of life and indirect costs incurred after discharge. However, given the robust magnitude of the effects and the statistical significance of the effects in this study, these limitations are unlikely to compromise the results.

In conclusion, this analysis of CRC data from a large scale survey in Taiwan evaluated changing trends and risk factors of hospital resource utilization. The data improve the understanding of medical resource allocation for CRC surgery and may help to formulate public health policies for optimizing hospital resource utilization for related diseases. Government officials and health care providers should recognize that hospital resource utilization of CRC surgery may depend on both patient and clinical attributes. These results can be generalized to CRC surgery patient populations elsewhere in Taiwan and to similar populations in other countries.

COMMENTS

Background

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in the world. Although the volume of CRC surgical procedures is increasing annually, the incidence and hospital resource utilization for these procedures have not been documented in a Taiwan study. Thus, this study explored the changing trends and risk factors of these outcomes for CRC surgery.

Research frontiers

These results can be generalized to patient populations elsewhere in Taiwan and to other countries with similar patient profiles.

Innovations and breakthroughs

This is a large retrospective study focused on the relationship between hospital costs, hospital stay and patients characteristics. It is beneficial to analyze changes in these factors over time.

Applications

To efficiently allocate of medical resources, these factors must be carefully managed. Moreover, government officials and health care providers should understand that these outcomes depend on both patient and hospital attributes.

Peer review

This research is a large sample, retrospective study and presents an interesting investigation of trend analysis and predictors for costs and hospital stay lengths after CRC surgery in the Taiwan region. The results showed that age, source, surgical type, tumor size, tumor site, and tumor stage were associated with decreased hospital resource utilization, and these findings may help to formulate public health policies in optimizing hospital resource utilization.

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BRIEF ARTICLE

Hyperamylasemia is associated with increased intestinal permeability in patients undergoing diagnostic oral double-balloon enteroscopy

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Abstract

AIM: To investigate the correlations between serum amylase levels, intestinal permeability (IP), and pancreatic injury and to explore the mechanisms responsible for hyperamylasemia in double-balloon enteroscopy (DBE).

METHODS: A prospective study was conducted in 20 patients who underwent DBE from August 1, 2008 to February 28, 2009. Serum amylase was examined 0, 2, 6 and 24 h post-DBE, C-reactive protein and lipase were examined at 24 h, and urine lactulose, mannitol, and trypsinogen-Ⅱ (TRY-Ⅱ) levels were measured at 6 h. Lactulose/mannitol ratio indicated IP, and TRY-Ⅱ indicated pancreatic injuries. Procedure duration and enteroscope insertion length were recorded.

RESULTS: Twelve patients underwent oral DBE (M:F, 5:7; mean age 50.42 ± 11.11 years) and 8 underwent anal DBE (M:F, 5:3; mean age 44.75 ± 12.66 years). They all showed significantly increased post-DBE serum amylase. Amylase and lipase levels were higher in the oral DBE group ($P < 0.05$). Hyperamylasemia was diagnosed in 9 (75.0%) patients undergoing oral DBE. Only patients receiving oral DBE showed increased post-procedure IP, which correlated with increased serum amylase ($r = 0.611$, $P = 0.035$) and procedure duration ($r = 0.668$, $P = 0.018$). Adverse events included one oral case with pancreatic injury (elevated TRY-Ⅱ) and two cases of abdominal discomfort in each group. Pancreatitis was not reported.

CONCLUSION: Hyperamylasemia correlates with increased IP and clinically undetectable pancreatic injuries. DBE could cause intestinal mucosa damage, which may result in IP elevation and increased amylase absorption, necessitating improvements and standardization of DBE methods.

Key words: Double-balloon enteroscopy; Hyperamylasemia; Intestinal permeability; Pancreatitis; Mechanism

Core tip: Hyperamylasemia, or increased serum amylase, and acute pancreatitis following double-balloon enteroscopy (DBE) have been reported in patients receiving diagnostic DBE, particularly oral DBE.

Feng N, Dai J, Lu H, Li XB, Gao YJ, Ge ZZ. Hyperamylasemia is associated with increased intestinal permeability in patients undergoing diagnostic oral double-balloon enteroscopy. *World J Gastroenterol* 2014; 20(2): 539-545 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/539.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.539

INTRODUCTION

Double-balloon enteroscopy (DBE) is a relatively safe digestive endoscopic technique for diagnosis and treatment of small bowel diseases. DBE can be performed either anally or orally and has been widely used in clinical practice for over 5 years[1]. DBE postoperative complication rate is about 4% in the Unites States[1] and has been reported to be as low as 1.4% worldwide[2]. Therapeutic endoscopy has been associated with severe complications (bleeding, perforation, and concomitant tissue necrosis), but these complications are rarely observed in diagnostic procedures[2,3]. However, hyperamylasemia and acute pancreatitis were reported in DBE patients undergoing diagnostic procedures, especially when performed orally[4-6]. Because both hyperamylasemia and acute pancreatitis are threatening conditions, a better understanding of these disorders is required to design effective preventative strategies.

DBE-induced acute pancreatitis was first reported by Groenen *et al*[4], and was later mainly associated with oral DBE[2,5,6]. Moreover, hyperamylasemia after oral DBE was also reported by Kopácová *et al*[7], who demonstrated that hyperamylasemia was much more frequent than acute pancreatitis. Damage to the intestine caused by local strain and friction in the small bowel during DBE may be central to the development of hyperamylasemia[8]. Thus, shorter BDE time, fewer passes, and cautious insertion may be useful in reducing small bowel injury[8]. On the other hand, some studies reported no association between small bowel damage and hyperamylasemia in DBE patients[9].

Damage to the bowel can be indicated by altered intestinal permeability (IP)[10]. Injuries to the intestinal mucosal barrier may change the inter-epithelial structure and increase permeability, increasing lactulose absorption, but without influence on mannitol absorption via the cell membrane. Therefore, the lactulose/mannitol (L/M) ratio can reflect the change in IP[10]. Intestinal barrier permeability defects caused by DBE may have other effects, such as altered biochemical parameters and increased bacterial translocation[11].

The present study was conducted to determine the relationships between serum amylase levels, IP (indicated by the L/M ratio), and pancreatic injury [indicated by urinary trypsinogen-Ⅱ (TRY-Ⅱ) levels] in patients undergoing oral or anal DBE. This study was designed to comprehensively investigate pancreatic injury and systemic abnormalities associated with DBE, and to clarify the mechanisms responsible for DBE-induced hyperamylasemia.

MATERIALS AND METHODS

Study design

A prospective study was conducted in consecutive DBE patients treated at the Endoscopy Center of the Shanghai Jiaotong University School of Medicine from 1st August 2008 to 28th February 2009. The study protocol was approved by the Institutional Ethics Committee of the Shanghai Jiaotong University School of Medicine. All procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Patients

Inclusion criteria were: (1) patients aged 17 to 70 years; and (2) patients who underwent diagnostic DBE within the study period. Exclusion criteria were: (1) patients who underwent any other recent endoscopic treatments for diagnostic or therapeutic purposes; (2) patients who were diagnosed with dysfunction of the liver, gallbladder, pancreas or kidneys; and (3) patients who exhibited current or previous enterostenosis, tumors, or inflammatory bowel disease. Based on these criteria, of 60 patients who were initially recruited and received the double-sugar solution, 40 were excluded after DBE.

Clinical data collection

Clinical data was recorded for each patient, including abdominal ultrasonographic findings and blood biochemical results. For each patient, levels of alanine transaminase, aspartate transaminase, total and direct bilirubin, γ-glutamyl transpeptidase, alkaline phosphatase, blood urea nitrogen and creatinine were recorded.

Definition of hyperamylasemia and pancreatitis

Hyperamylasemia was defined as increased serum amylase levels above the upper limit of normal (> 100 U/L) or 3 × higher than baseline (preoperative) levels[8]. Pancreatitis was defined in accordance with the consensus definitions for major complications of endoscopic retrograde cholangiopancreatography[12]. Briefly, acute pancreatitis was defined as occurrence of clinical signs that included typical epigastric pain with radiation to the back and/or nausea, and vomiting combined with amylase/lipase levels ⩾ 3 × the upper limit of normal, requiring unplanned admission or prolongation of planned admission.

Pre-procedure assessments

Indications for DBE were recorded. All patients were asked to drink 60 mL of double-sugar solution containing 10 g of mannitol and 5 g of lactulose after an overnight fast, anytime within the 3-d period prior to DBE, and pre-procedure urine samples were collected approximately 6 h after drinking, as previously described[10]. Right before the DBE procedure, baseline serum amylase was examined (which was marked as "0 h" below).

Table 1 Demographic, clinical, and procedural information for included patients undergoing oral or anal double-balloon enteroscopy (*n* = 20) *n* (%)

	Oral DBE (*n* = 12)	Anal DBE (*n* = 8)
Age (yr), mean ± SD	50.42 ± 11.11	44.75 ± 12.66
Gender		
Male	5 (41.7)	5 (62.5)
Female	7 (58.3)	3 (37.5)
Indications		
Abdominal pain	5 (41.7)	3 (37.5)
Abdominal distension	4 (33.3)	1 (12.5)
Anemia	3 (25.0)	1 (12.5)
Intermittent diarrhea	0 (0.0)	3 (37.5)
Procedure, mean ± SD		
Duration (min)	71.92 ± 20.19	65.63 ± 17.80
Length of insertion (m)	3.55 ± 1.42	2.8 ± 1.09
Adverse events		
Abdominal discomfort/distention	2 (16.7)	2 (25.0)
Pancreatic injury (elevated urinary trypsinogen-Ⅱ)	1 (8.3)	0

DBE: Double-balloon enteroscopy.

DBE procedures and assessments during procedures

Oral and anal DBE procedures were performed by a single experienced endoscopist using an EN-450P5 DBE (Fujinon, Saitama, Japan). All patients received gas and intravenous anesthesia during the DBE procedures. For oral DBE, the endoscopist initiated inflation of the overtube balloon after the enteroscope had passed the ligament of Treitz, thus avoiding direct compression or injury to the duodenal papilla. For anal DBE, the enteroscope was inserted as far as possible beyond the ileocecal valve. After examinations were completed, patients in both groups were injected with 60 mL of double-sugar solution through the enteroscope into the duodenal bulb as the enteroscope was removed. We recorded procedure duration and the length of enteroscope insertion relative to the ligament of Treitz or ileocecal valve for both groups of patients. Therefore, when the enteroscope reached its deepest level, we pulled the endoscope, and the insertion length was calculated as: [reference point on the endoscope - (60 cm, oral or 65 cm, anal)] × 8.

Post-procedure assessments

After DBE, serum amylase levels were examined at 2, 6 and 24 h (normal range: 40-100 U/L). Serum C-reactive protein (CRP) (normal range: < 10.0 mg/L) and lipase (normal range: < 40 U/L) were examined 24 h after DBE. Post-procedure urine samples were collected 6 h after injection of double-sugar solution during DBE. Urine lactulose, mannitol, and TRY-Ⅱ levels were measured. The L/M ratio was calculated to determine IP and TRY-Ⅱ was used as an indicator of pancreatic injuries. After final samples were taken, asymptomatic patients were discharged, and patients with confirmed or suspected acute pancreatitis were admitted to the hospital.

Safety assessments

After DBE, patients were assessed by routine clinical examinations at approximately 30 min intervals, and epigastric abdominal pain, distension, nausea, or vomiting was recorded. Adverse events (AEs) were reported, including abdominal signs and symptoms.

Statistical analysis

Continuous data are reported as mean ± SD and ranges. Statistical analysis was conducted using SAS 5.1 (SAS Institute, Cary, NY, United States). Continuous variables were compared using unpaired or paired *t* tests or, alternatively, using the Wilcoxon rank-sum test. Categorical variables were compared using the Chi-squared test. Correlation analysis was performed by linear regression test. A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

Patient demographic characteristics and indications

Of a total of 60 consecutive DBE patients, 40 (66.7%) were excluded due to liver, gallbladder, and/or pancreas diseases (*n* = 7); renal dysfunction (*n* = 6); enterostenosis (*n* = 6), tumors (*n* = 2); or inflammatory bowel disease (*n* = 19). The remaining 20 (33.3%) patients (M:F, 10:10; mean age 48.15 ± 11.77 years, ranging 26-70 years) were included in the study, including 12 undergoing the oral procedure (M:F, 5:7; mean age 50.42 ± 11.11 years, ranging 26-70 years) and 8 undergoing the anal procedure (M:F, 5:3; mean age 44.75 ± 12.66 years, ranging 26-60 years). No significant differences were observed in demographic characteristics between the two groups of patients. Indications for DBE included unexplained abdominal pain (*n* = 8), abdominal distension (*n* = 5), iron deficiency anemia (*n* = 4), and intermittent diarrhea with negative esophagogastroduodenoscopy and colonoscopy (*n* = 3) (Table 1).

DBE procedure duration and insertion length

Mean procedure duration was 71.92 ± 20.19 min for patients in the oral DBE group and 65.63 ± 17.80 min for those in the anal DBE group. Insertion length was 3.55 ± 1.42 m beyond the ligament of Treitz for patients in the oral DBE group, and 2.80 ± 1.09 m beyond the ileocecal valve for those in the anal DBE group (Table 1).

Hyperamylasemia occurrence and serum amylase levels

Nine (75.0%) patients undergoing oral DBE were diagnosed with hyperamylasemia, including 9 at 2 h, 7 at 6 h, and 6 at 24 h. By 6 h after DBE, 2 of these cases exhibited normalized amylase levels, and no new cases of hyperamylasemia were subsequently reported. Although post-procedure amylase levels were also significantly elevated in patients undergoing anal DBE at 2 h ($P = 0.013$) and 6 h ($P < 0.001$), no patients in the anal DBE group were diagnosed with hyperamylasemia.

In addition, compared with baseline values, serum amylase levels at 2 h after DBE were increased by 141.0% in the oral DBE group, but only by 19.0% in the anal DBE group. Patients in the oral DBE group exhibited

Table 2 Serum and urine biochemical markers in patients undergoing oral or anal double-balloon enteroscopy (*n* = 20)

	Oral DBE (*n* = 12)	Anal DBE (*n* = 8)
Serum amylase (U/L)		
0 h	63.08 ± 25.13	53.88 ± 17.35
2 h	150.92 ± 87.98[a]	63.50 ± 19.82[a]
6 h	160.83 ± 99.61[a]	66.25 ± 20.89[a]
24 h	90.75 ± 41.98[a]	57.38 ± 15.81
Serum amylase ratio (*vs* at 0 h)		
2 h	2.41 ± 1.08[c]	1.19 ± 0.15
6 h	2.54 ± 1.08[c]	1.24 ± 0.11
24 h	1.51 ± 0.53[c]	1.10 ± 0.24
Serum C-reaction protein (mg/L) at 24 h	7.46 ± 6.50[1]	6.91 ± 8.95
Urine lactulose (mg/L)		
0 h	0.571 ± 0.134	0.481 ± 0.096
6 h	0.691 ± 0.139	0.294 ± 0.081
Urine mannitol (mg/L)		
0 h	7.881 ± 2.664	7.726 ± 2.247
6 h	8.059 ± 2.131	4.308 ± 1.802
Urine lactulose/mannitol ratio		
0 h	0.083 ± 0.048[2]	0.067 ± 0.023
6 h	0.095 ± 0.049[a3]	0.075 ± 0.028[a]

[a]$P < 0.05$ *vs* 0 h value; [c]$P < 0.05$ *vs* anal double-balloon enteroscopy (DBE) group; [1]$P = 0.3191$ *vs* anal DBE group (Wilcoxon test); [2]$P = 0.3941$ *vs* anal DBE group; [3]$P = 0.3296$ *vs* anal DBE group.

amylase levels of 63.08 ± 25.13, 150.92 ± 87.98, 160.83 ± 99.61 and 90.75 ± 41.98 U/L at 0, 2, 6 and 24 h, respectively, after DBE, while patients in the oral DBE group exhibited amylase levels of 53.88 ± 17.35, 63.50 ± 19.82, 66.25 ± 20.89 and 57.38 ± 15.81 U/L at 0, 2, 6 and 24 h, respectively (Table 2). Notably, no correlations were observed between serum amylase levels and enteroscope insertion length.

Serum lipase

Patients undergoing oral DBE and those undergoing anal DBE exhibited serum lipase levels of 37.92 ± 26.64 and 19.61 ± 8.96 U/L, respectively, 24 h after DBE. Serum lipase levels at 24 h after DBE were significantly increased in 5 (41.7%) patients receiving the oral DBE procedure, but no increased lipase levels were observed in the anal DBE group. Furthermore, serum lipase levels were significantly higher in the oral DBE group than in anal DBE group ($P = 0.008$). Furthermore, serum lipase at 24 h post-DBE appeared to be related to serum amylase levels at 2 h post-procedure, though this correlation was not statistically significant ($r = 0.514$, $P = 0.08$).

CRP levels

CRP was normal in 10 (83.3%) patients receiving oral DBE and 7 (87.5%) patients receiving anal DBE. Thus, only 2 (16.7%) patients receiving oral DBE and 1 (12.5%) patient receiving anal DBE had high CRP levels.

Urine TRY-II levels

Elevated urine TRY-Ⅱ was only observed in a 49-year-old male patient receiving oral DBE, who exhibited a TRY-Ⅱ value of 30.59 μg/L. In this case, the duration of DBE procedure was 40 min and the insertion length was 2.8 m, with serum amylase peaking 2 h after the procedure at 312 U/L. Notably, no abdominal symptoms were observed in this patient, and serum lipase and IP were normal. Mean TRY-Ⅱ level for other 19 patients was 2.6 ± 0.1 μg/L.

Urine L/M ratio and IP

Post-DBE urine L/M ratios were elevated in both groups of patients, but the elevation was more significant in patients receiving oral DBE. In the oral group, urine mannitol levels were slightly increased, but lactulose levels were much more dramatically increased after the procedure. Although the absolute values of urine mannitol and lactulose concentrations in the anal group appeared to be decreased, their ratios increased compared with preoperative levels ($P < 0.05$) (Table 2). Increased IP indicated by urine L/M ratios in patients receiving oral DBE positively correlated with procedure duration ($r = 0.668$, $P = 0.017$) (Figure 1A). Moreover, L/M ratios and 6 h/0 h serum amylase ratios were significantly correlated in patients receiving oral DBE ($r = 0.611$, $P = 0.035$) (Figure 1B). Furthermore, in the oral group, IP was increased by a mean of 15%. In the anal group, although the absolute values of mannitol and lactulose levels after DBE decreased compared with the pre-DBE levels, the L/M ratio was increased by about 11% on average, compared with the preoperative value.

Adverse events

The procedures were successful in all patients. Four (20%) patients, including 2 (16.7%) in the oral DBE group and 2 (25.0%) in the anal DBE group, complained of abdominal discomfort. By 6 h after the procedure, abdominal distension disappeared in all affected patients and all of them were able to walk normally, leading to prompt discharge. Pancreatic injuries were observed only in 1 patient receiving oral DBE (elevated urinary TRY-Ⅱ), and no pancreatitis cases were reported. No other acute abdominal symptoms, pancreatitis, or other serious AEs were reported.

DISCUSSION

In the present study, post-DBE serum amylase levels were increased in patients treated by diagnostic oral or anal DBE. This increase, along with the increase in lipase, was more significant in patients receiving oral DBE. Furthermore, a significant number of patients receiving oral DBE developed hyperamylasemia, and increased post-DBE IP correlated with increased serum amylase and procedure duration. Notably, no pancreatitis and only a single case of pancreatic injury were reported in the present study. These findings may have mechanistic implications for the development of DBE-induced hyperamylasemia in patients treated by oral DBE, suggesting that increased serum amylase and, hypothetically, increased

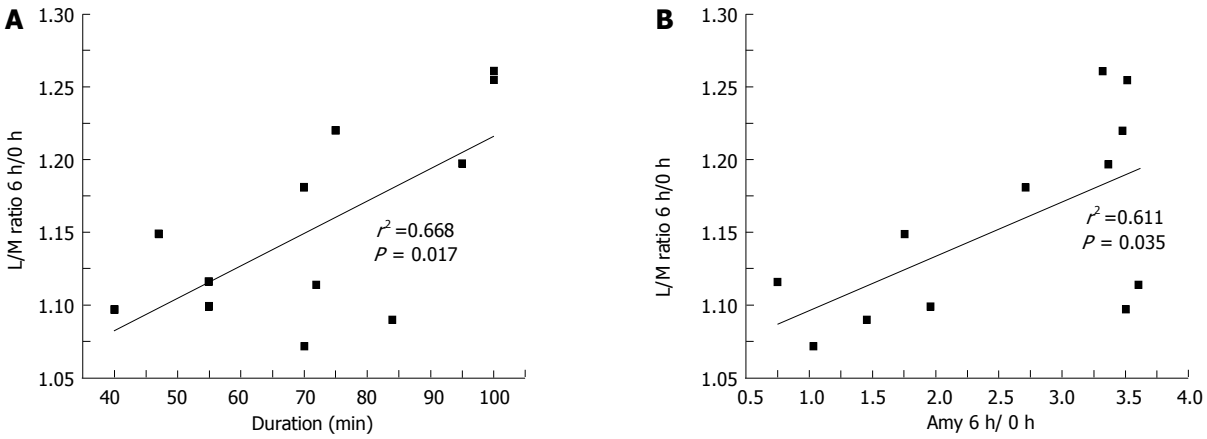

Figure 1 Correlations between double-balloon enteroscopy duration and lactulose/mannitol ratio 6 h/0 h (A) and between serum amylase 6 h/0 h and lactulose/mannitol ratio 6 h/0 h (B) in patients undergoing oral double-balloon enteroscopy. Analysis was performed using Pearson's correlation tests (n = 12). L/M: Lactulose/mannitol.

amylase absorption due to DBE-induced IP elevation, may play a role in causing virtually undetectable pancreas injury leading to hyperamylasemia.

An international, multicenter retrospective study reported that the incidence of acute pancreatitis in 2362 patients who underwent DBE was 0.3% (n = 7)[2], demonstrating an extremely low rate of pancreatitis that may explain the lack of pancreatitis in the present study. Honda *et al*[13] reported that 46% of patients exhibited increased serum amylase 3 h after diagnostic oral DBE but, in the present study all patients had increased serum amylase by 6 h, which is likely due to the later sampling. Similarly, Kopácová *et al*[7] reported that post-DBE serum amylase levels were increased in 58% of patients, of whom 9 (25.7%) were diagnosed with hyperamylasemia, 1 (11%) with mild acute pancreatitis, and 5 (55.6%) with abdominal discomfort, which is very similar to results of the present study.

It has also been suggested that the development of hyperamylasemia and acute pancreatitis following DBE may be related, though associated acute pancreatitis generally appears mild and may represent only minor pathological changes in the pancreas[7]. Furthermore, hyperamylasemia tends to resolve shortly after DBE[7]. These findings indicate that measurement of serum amylase 2 h after DBE may be sufficient for clinical monitoring of abnormal serum amylase level and diagnosis of hyperamylasemia. Furthermore, Sugiyama *et al*[14] explored the risk factors associated with hyperamylasemia, and showed that the bile duct size, history of pancreatitis, and young age may play a role in the development of hyperamylasemia, though no relationship between hyperamylasemia and pancreatitis was determined. Some studies also demonstrated that the insertion technique can increase the risk of hyperamylasemia[8]. Indeed, the endoscopist may exert forces on the small intestine to correctly position the enteroscope, thus exerting pressure and friction on the intestinal wall that may mechanically and functionally damage intestinal mucosa and potentially raise the risk of infection and inflammation[15-18].

In the present study, the L/M ratios, indicating altered IP, were increased in both groups, though the extent was significantly greater in patients receiving oral DBE. These findings are consistent with those of previous studies, though the present study did not explore the clinical implications of these alterations, including increased bacterial proliferation and infection risk[10]. However, the present study demonstrated that IP was increased by about 15% and the L/M ratio by about 11%. Based on these findings, we postulate that damage to the small intestinal mucosa and intestinal mucosal barrier may lead to increased IP, though this hypothesis and its potential clinical implications requires further study. Thus, L/M ratio may be a clinically useful prognostic indicator in DBE patients.

Furthermore, the present findings demonstrated a correlation between IP and procedure duration in patients receiving oral DBE, which may indicate that prolonged procedures increase IP. As a result, high serum amylase levels may be attributable to increased intestinal absorption, but this hypothesis requires further verification. Notably, Aktas *et al*[8] and Pata *et al*[19] both reported that enteroscope length and procedure duration were correlated with serum amylase levels, and that balloon inflation time was an independent predictive factor of post-operative pancreatitis after oral DBE. Thus, it is reasonable to consider that longer and more extensive enteroscopy may be associated with greater damage to the intestinal mucosa, promoting changes in IP and the subsequent increase in serum amylase levels.

In the present study, some patients showed signs of pancreatic injury, indicated by elevated TRY-Ⅱ levels, which is consistent with previous reports[20-23]. Notably, Kemppainen *et al*[24] detected TRY-Ⅱ 6 h post-DBE and showed the high sensitivity and specificity (81% and 90%, respectively) of TRY-Ⅱ-based diagnosis of pancreatic injury. Some pancreatic injuries may be too mild to be considered as acute pancreatitis, but elevated TRY-Ⅱ may indicate that minor DBE-induced pancreatic injury may be a critical factor in the development of complica-

tions after DBE. Thus, more gentle insertion techniques, shortening of balloon time and reducing the number of passes were tried to decrease DBE complications[8,19,24].

Though our results are highly consistent with previous studies, it is important to note that the relatively small sample size and lack of long-term follow-up may limit the applicability of these findings. Longitudinal studies conducted in more diverse patient populations will be required to confirm these findings and to fully explore the mechanism and clinical implications associated with hyperamylasemia in patients undergoing diagnostic DBE.

In conclusion, increased serum amylase levels, or hyperamylasemia, were found to be significantly more common in patients undergoing oral DBE. In these patients, hyperamylasemia was correlated with increased IP, and increased serum amylase levels may be attributable to increased amylase absorption. Furthermore, TRY-II assessment demonstrated that, though mild in nature, pancreatic injuries that did not develop into full acute pancreatitis were sometimes detectable in DBE patients, particularly those treated by oral DBE. Based on these findings and contemporary literature, we suggest improvements (more gentle insertion techniques, shortening of balloon time and reducing the number of passes) and standardization of DBE to limit small intestine damage and subsequent increase in IP, thus reducing the occurrence of hyperamylasemia in patients undergoing diagnostic DBE.

COMMENTS

Background

Hyperamylasemia (increased serum amylase) and acute pancreatitis following double-balloon enteroscopy (DBE) have been reported in patients undergoing diagnostic DBE, particularly oral DBE.

Research frontiers

Hyperamylasemia and acute pancreatitis were reported in DBE patients undergoing diagnostic procedures, especially when performed orally. Because both hyperamylasemia and acute pancreatitis are threatening conditions, a better understanding of these disorders is required to design effective preventative strategies.

Innovations and breakthroughs

Hyperamylasemia correlates with increased intestinal permeability (IP) and clinically undetectable pancreatic injuries. DBE could cause intestinal mucosa damage, which may result in IP elevation and increased amylase absorption, necessitating improvements and standardization of DBE methods.

Peer review

This is a significant study which focused on the correlations between serum amylase level, IP and DBE. Though DBE-associated acute pancreatitis is rare, hyperamylasemia is common but its mechanism remains unknown. This is the first study to prove the hyperamylasemia was due to enhanced IP rather than the injury to pancreas.

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BRIEF ARTICLE

Consecutive laparoscopic gallbladder and spleen resections in cirrhotic patients

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Abstract

AIM: To evaluate the feasibility, safety, and effectiveness of consecutive laparoscopic cholecystectomy (LC) plus splenectomy (LS) in liver cirrhosis patients.

METHODS: From 2003 to 2013, 17 (group 1) patients with liver cirrhosis complicated by hypersplenism and symptomatic gallstones were treated with combined LC and LS, while 58 (group 2) patients with liver cirrhosis and hypersplenism received LS alone. An additional 14 (group 3) patients who received traditional open procedures during the same period were included as controls. Data were retrospectively collected and reviewed in regard to demographic characteristics and preoperative, intraoperative and postoperative features. Differences between the three groups were assessed by statistical analysis.

RESULTS: The three groups showed no significant differences in the demographic characteristics or preoperative status. However, the patients treated with LC and LS required significantly longer operative time, shorter postoperative stay as well as shorter time of return to the first oral intake, and suffered less intraoperative blood loss as well as fewer postoperative surgical infections than the patients treated with traditional open procedures (group 1 *vs* group 3, $P < 0.05$ for all). The patients treated with LC and LS showed no significant differences in the intraoperative and postoperative variables from those treated with LS alone (group 1 *vs* group 2). All patients showed significant improvements in the haematological responses (preoperative period *vs* postoperative period, $P < 0.05$ for all). None of the patients treated with LC and LS presented with any gallstone-associated symptoms following discharge, while the patients treated with the traditional open procedures expressed complaints of discomfort related to their surgical incisions.

CONCLUSION: Consecutive LC and LS is an appropriate treatment option for liver cirrhosis patients with gallstones and hypersplenism, especially for those with Child-Pugh A and B.

Key words: Laparoscopic cholecystectomy; Laparoscopic splenectomy; Liver cirrhosis; Hypersplenism; Open surgery

Core tip: Cholelithiasis occurs more frequently in liver cirrhosis patients with hypersplenism than in the general population. The recent significant advances in minimally invasive surgery have aroused surgeons' interest in performing combined laparoscopic procedures. Here, we report our experience with concomitant laparoscop-

ic cholecystectomy and splenectomy in liver cirrhosis patients. This is the first reported case series of its kind and will provide useful clinical information to physicians facing the challenge of treating cirrhotic patients who are in need of surgical attention to address coexisting abdominal diseases.

Wang MJ, Li JL, Zhou J, Wu Z, Peng B. Consecutive laparoscopic gallbladder and spleen resections in cirrhotic patients. *World J Gastroenterol* 2014; 20(2): 546-554 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/546.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.546

INTRODUCTION

The incidence of gallbladder stones in liver cirrhosis patients is two-fold higher than the estimates for the general population[1,2]. Underlying risk factors for this co-morbidity are associated with dysfunction of gallbladder motility and emptying, hypersplenism, increased haemolysis, and enhanced circulating oestrogen levels[1,3]. Considering the significant intraoperative blood loss that accompanies portal hypertension, liver cirrhosis was classified as a contraindication to laparoscopic cholecystectomy (LC) in the early 1990s[4].

Since the first study of LC in patients with liver cirrhosis was reported in 1993[5], extensive clinical data pertaining to this topic have been included in the publicly available literature; in general, these data have demonstrated that LC can be performed safely in liver cirrhosis patients[6-9]. On the other hand, hypersplenism resulting in thrombocytopenia and leukopenia is a common complication observed in patients with liver cirrhosis complicated by portal hypertension[10]. Splenectomy should be considered for these patients, to avoid fatal bleeding. Several recent studies have demonstrated the feasibility, safety, and effectiveness of laparoscopic splenectomy (LS) in patients with hypersplenism secondary to liver cirrhosis, and the results of short- and long-term outcomes attained with this procedure are encouraging[11-13].

To help maximize the safety of consecutive gallbladder and spleen resections using the conventional open procedures, a wide upper abdominal incision is required. Technical advances in laparoscopic surgery have prompted surgeons to develop combination approaches for simultaneous treatment of coexisting abdominal diseases[14-17]. However, few studies have systematically assessed the risks and benefits of LC plus LS in treating liver cirrhosis patients, which aroused our interest in performing the study described herein.

MATERIALS AND METHODS

Patients

Between 2003 and 2013, the procedure of concomitant cholecystectomy and splenectomy was performed on 51 liver cirrhosis patients with hypersplenism and/or portal hypertension as well as gallbladder stones at the Department of Hepato-Biliary-Pancreatic Surgery of the West China Hospital of Sichuan University. Among them, 19 patients received the laparoscopic procedures while the remaining 32 were treated with the open procedures. Twenty of the total patients who underwent other surgeries (radiofrequency ablation for hepatocarcinoma, herniorrhaphy for hernia, liver resection for hepatocarcinoma, or biliary duct exploration) were excluded from analysis. Therefore, 17 of the patients who received LC and LS were included as group 1, while 14 of the patients who received open cholecystectomy and splenectomy were included as group 3. An additional 58 liver cirrhosis patients with hypersplenism and/or portal hypertension who had received LS only between 2003 and 2013 were included as group 2. The inclusion and exclusion criteria are detailed in Figure 1. Written informed consent was obtained from all study participants, and the study was approved by the Ethics Committee of Sichuan University.

Diagnosis and indications

Diagnosis was made according to the patient's clinical history (hepatitis B or C, schistosome infection, and alcohol abuse), results from blood tests (thrombocytopenia, leukopenia or/and anaemia), computed tomography (CT) scans, B-ultrasound examinations (enlarged spleen, abnormal hepatic shape, size and edges with/without gastroesophageal varices, and gallstones) and postoperative pathological examinations (stages on a four-point Metavir scoring scale[18]: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis). Indications for splenectomy were as follows: (1) severe thrombocytopenia due to hypersplenism with a platelet count < 30×10^9/L and/or a white blood cell count < 3×10^9/L; and (2) being prone to oesophageal variceal haemorrhage as a result of severe portal hypertension. Cholecystectomy was taken into consideration when the diagnosis of symptomatic cholelithiasis was established on the basis of clinical presentations and confirmed by CT scans or B-ultrasound examinations.

Perioperative surveillance

Craniocaudal splenic length was measured preoperatively using CT scanning. B-ultrasound examinations were selectively applied in addition to the CT scan to detect gallbladder stones. The Child-Pugh class and the American Society of Anaesthesiologists (ASA) classification systems were applied respectively for evaluating the liver reserve function and the anaesthetic risk preoperatively. Laboratory data, including peripheral blood counts (haemoglobin, leukocytes, platelets, and prothrombin time), total bilirubin (TBil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin (ALB) concentrations were obtained before surgery and on postoperative days (POD) 1, 3, and 5. Seven days of postopera-

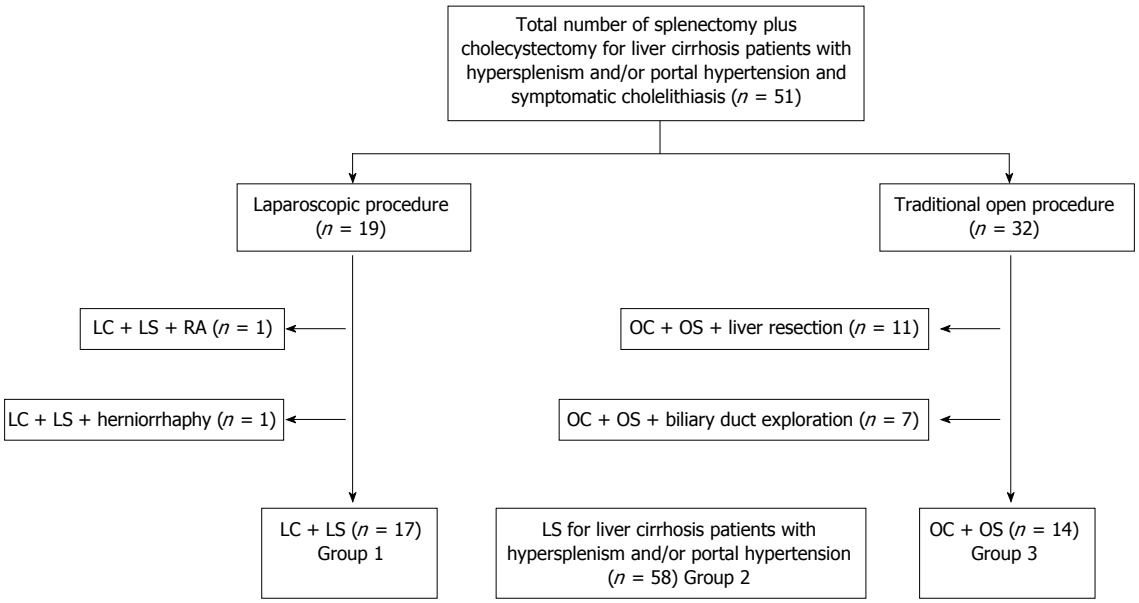

Figure 1 Inclusion and exclusion criteria for study participation. LC: Laparoscopic cholecystectomy; LS: Laparoscopic splenectomy; OC: Open cholecystectomy; OS: Open splenectomy; RA: Radiofrequency ablation.

tive surveillance were performed for portal or splenic vein thrombosis (PSVT), biliary and pancreatic leakage (all by B-ultrasound; following detection of PSVT with B-ultrasound, CT scanning was performed to make a definite diagnosis), and fluid drainage. After discharge, patients were recommended to undergo B-ultrasound or CT scanning once a month for the following 3 mo to monitor PSVT.

Surgical techniques

All of the operations were performed by a single team with a chief physician who is expert at LS[19]. First, the LC procedure was carried out in a three-trocar manner with the patient in the supine position and with the operation table tilted to the left. After establishment of pneumoperitoneum with a pressure of 14 mmHg CO_2, the first 10 mm trocar for laparoscopy was inserted in the lower umbilicus. A 12 mm subxiphoid trocar and a 5 mm right subcostal margin trocar were then added for resections of the gallbladder and a 1 cm × 1 cm piece of hepatic tissue. Subsequently, the patients were placed in a right semi-decubitus position with the left side elevated 45-60 ° and with the operating table slightly tilted to the reverse Trendelenburg position. Two other trocars were then placed at the left mid-clavicular line below the inferior margin of the spleen (12 mm), and at the left axillary line below the inferior pole of the spleen (5 mm). Splenic attachments and ligaments were dissected using an ultrasonic dissector or the LigaSure™ Vessel Sealing System (Covidien, Mansfield, MS, United States) in the order of splenogastric ligament, splenic flexure attachment, splenorenal ligament, and splenophrenic ligament. After the spleen was released from the attachments and ligaments, an endoscopic linear vascular stapler was applied to transect the splenic hilar pedicles. The mobilized spleen was placed into a retrieval bag, mechanically morcellated with forceps, and retracted from the 12 mm trocar site. A suction drain was placed in the splenic fossa, while another one was placed at the site of Calot's triangle.

For the traditional open procedure, left and right subcostal incisions were required. The details of the open splenectomy and the single LS were described previously[11]; the open cholecystectomy was performed in the anterograde or retrograde manner.

Statistical analysis

Patients' complete medical records were retrospectively collected and reviewed, including demographic characteristics and information related to the preoperative (ASA classification, Child-Pugh class, splenic size, complete blood count, liver function index, and aetiology of cirrhosis), intraoperative (conversion rate, operative time, transfusion rate, estimated blood loss, and additional operations) and postoperative (postoperative stay, time to the first oral intake, histologic fibrosis stage, complete blood count, liver function index, and short-term complications) periods.

Continuous numerical variables are expressed as mean ± SD or median and interquartile, while categorical data are presented as number of cases and percentage. All statistical analyses were carried out using the SPSS statistical software package (version 16.0; SPSS Inc., Chicago, IL, United States) and included the Student's *t*, nonparametric Mann-Whitney *U*, χ^2, and Fisher's exact tests, with rejection level for the null hypothesis set at a *P*-value of < 0.05.

RESULTS

As presented in Table 1, comparisons of group 1 with

Table 1 Demographic characteristics and preoperative information

Variable	Group 1	Group 2[1]	Group 3[2]
Cases	17	58	14
Sex (M/F)	10/7	26/32	8/6
Age (yr)	55.9 ± 12.9	49.7 ± 14.5	50.8 ± 7.6
ASA classification			
Ⅱ	1	13	0
Ⅲ	15	42	13
Ⅳ	1	3	1
Child-Pugh class			
A	7	37	10
B	10	19	4
C	0	2	0
Splenic size (cm)	24.8 ± 6.8	22.1 ± 4.9	24.6 ± 3.3
Laboratory data			
HGB (g/L)	96.0 (86.0-128.0)	111.5 (91.5-125.0)	116.0 (93.0-133.5)
Platelet (× 10^9/L)	34.0 (27.0-38.5)	37.0 (27.0-44.5)	37.5 (32.0-51.0)
WBC (× 10^9/L)	2.0 (1.7-3.2)	2.2 (1.7-3.0)	2.6 (1.7-3.5)
Total bilirubin (mmol/L)	24.2 (14.3-39.6)	21.0 (16.0-25.3)	16.3 (15.5-19.1)
AST (IU/L)	50.0 (24.5-83.0)	31.5 (22.0-45.8)	38.5 (30.3-47.0)
ALT (IU/L)	34.0 (25.0-74.0)	33.5 (25.8-50.3)	33.0 (25.0-42.5)
ALB (g/L)	37.5 (34.2-39.5)	38.3 (34.8-40.8)	39.6 (36.6-42.2)
Prothrombin time (s)	13.2 (12.5-15.1)	12.9 (11.2-14.9)	13.8 (12.3-14.7)
Diagnoses			
Posthepatitic cirrhosis	17	51	11
Alcoholic cirrhosis	0	4	1
Schistosomiasis cirrhosis	0	3	2

Data are expressed as absolute median (range) or mean ± SD. No significant differences were detected for [1]group 1 *vs* group 2 or [2]group 1 *vs* group 3. ASA: American Society of Anaesthesiologists; HGB: Haemoglobin; WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin.

groups 2 and 3 revealed no significant differences in terms of demographic characteristics (sex and age), distributions of ASA classification and Child-Pugh class, as well as diagnoses, splenic size, and preoperative laboratory data. The entire study population consisted of 89 patients (44 males and 45 females; mean age: 52.7 ± 13.8 years), 17 of whom were intended to receive LC plus LS. Preoperative evaluations of anaesthetic risk and liver reserve function showed that there were 14 (15.7%), 70 (78.7%) and 5 (5.6%) patients classified as ASA II, III and IV, while 54 (60.7%), 33 (37.1%) and 2 (2.2%) patients fell into to Child-Pugh A, B and C categories. Chronic hepatitis B or C (79 patients, 88.8%) was the leading cause of liver cirrhosis, while excessive alcohol consumption (5 patients, 5.6%) and schistosomiasis (5 patients, 5.6%) also contributed to liver damage in the study population.

Table 2 details the intraoperative information. The concomitant LC and LS procedure was successfully performed in 16 patients in group 1, with a conversion rate of 5.9%. The single case in group 1 that had to be converted to the open procedure was mainly due to bleeding from the splenic hilar vessels when transecting the splenic pedicel with the endoscopic linear vascular stapler in the narrow intra-abdominal space. For the same reason, the conversion rate in group 2, comparable to that in group 1, was 5.2%, suggesting that LC did not increase the rate when performing the laparoscopic combined surgery. The operative time, transfusion rate, and estimated blood loss were similar between groups 1 and 2. Compared with group 3, patients in group 1 required longer operative time (221.2 ± 44.8 min *vs* 166.4 ± 64.5 min, $P = 0.009$) and suffered less intraoperative blood loss (225.9 ± 195.7 mL *vs* 421.4 ± 234.3 mL, $P = 0.017$), while the transfusion rate was comparable between these two groups. Liver biopsy was performed in all patients for postoperative pathological examinations.

Table 2 Intraoperative information

Variable	Group 1	Group 2	Group 3	P^1
Conversion rate	1 (5.9)	3 (5.2)	ND	ND
Operative time (min)	221.2 ± 44.8	218.0 ± 58.4	166.4 ± 64.5	0.009
Transfusion rate	3 (17.6%)	8 (13.8%)	5 (35.7%)	NS
EBL (mL)	225.9 ± 195.7	170.2 ± 130.2	421.4 ± 234.3	0.017
Additional operation				
Liver biopsy	17	58	14	ND

Data are expressed as *n* (%) or mean ± SD. [1]Group 1 *vs* group 3. EBL: Estimated blood loss; NS: No significance; ND: Analysis not done.

Table 3 Postoperative information

Variable	Group 1	Group 2	Group 3	P^1
Postoperative stay (d, mean ± SD)	8.7 ± 2.6	7.8 ± 1.8	11.3 ± 2.9	0.013
Oral intake (d, mean ± SD)	2.8 ± 1.4	2.2 ± 0.9	4.3 ± 1.1	0.004
Histologic fibrosis stage				NS
F3	5	11	4	ND
F4	12	45	9	ND
Short-term complications				
CBD injury	0	ND	0	ND
Bile leakage	0	ND	0	ND
Splenic fossa collection	1	1	0	ND
Pleural effusion	1	4	2	ND
Pulmonary infection	1	3	3	ND
Incision infection	0	0	2	ND
Pancreatic leakage	2	8	1	ND
Portal or splenic vein thrombosis	2	4	1	ND
Postoperative bleeding	1	2	0	ND
Total	8	22	9	ND

[1]Group 1 *vs* group 3. CBD: Common bile duct; NS: No significance; ND: analysis not done.

The postoperative information is presented in Table 3. Comparisons of groups 1 and 2 showed no differences in terms of postoperative stay, and time to the first oral intake. Comparing group 1 with group 3, the length of postoperative stay was shorter (8.7 ± 2.6 d *vs* 11.3 ± 2.9 d, $P = 0.013$) as was the time of return to the first oral intake (2.8 ± 1.4 d *vs* 4.3 ± 1.1 d, $P = 0.004$). Pathological results of liver biopsies taken during splenectomy indicated that most of the histological fibrosis stages belonged

Table 4 Dynamic changes of haematological and liver function parameters perioperatively

Parameter	Preoperative	POD 1	POD 3	POD 5
HGB (g/L)				
Group 1	96.0 (86.0-128.0)	102.0 (86.0-119.5)	108.0 (89.5-118)	105.0 (88.5-115.5)
Group 2	111.5 (91.5-125.0)	107.0 (92.8-119.0)	102.0 (90.8-118.0)	105.5 (93.8-120.3)
Group 3	116.0 (93.0-133.5)	101.0 (91.3-126.3)	105.5 (88.3-127.3)	111.5 (89.8-123.5)
Platelet ($\times 10^9$/L)				
Group 1	34.0 (27.0-38.5)	63.0 (39.5-81.0)[a]	105.0 (50.0-175.5)[a]	184.0 (127.5-303.0)[a]
Group 2	37.0 (27.0-44.5)	63.0 (42.0-79.0)[a]	115.0 (74.3-160.5)[a]	192.0 (130.8-307.3)[a]
Group 3	37.5 (32.0-51.0)	54.0 (41.8-78.8)[a]	118.0 (59.8-138.5)[a]	242.5 (110.0-285.3)[a]
WBC ($\times 10^9$/L)				
Group 1	2.0 (1.7-3.2)	11.1 (8.1-14.9)[a]	10.5 (8.1-12.5)[a]	9.1 (7.3-10.6)[a]
Group 2	2.2 (1.7-2.9)	11.1 (8.9-13.3)[a]	10.4 (8.4-13.8)[a]	8.6 (6.7-10.4)[a]
Group 3	2.6 (1.7-3.5)	13.3 (10.2-14.9)[a]	12.1 (8.9-15.3)[a]	9.2 (7.6-11.1)[a]
TBil (mmol/L)				
Group 1	24.2 (14.3-39.6)	27.8 (14.5-34.6)	21.3 (17.3-35.5)	24.0 (17.3-30.3)
Group 2	21.0 (16.0-25.3)	21.8 (14.5-31.0)	20.1 (13.5-26.7)	19.5 (12.8-28.0)
Group 3	16.3 (15.5-19.1)	15.1 (12.0-20.0)	24.3 (15.0-31.7)	19.4 (13.5-26.7)
ALT (IU/L)				
Group 1	34.0 (25.0-74.0)	43.0 (28.5-99.0)	40.0 (31.5-57.5)	42.0 (26.0-58.0)
Group 2	33.5 (25.8-50.3)	32.5 (27.8-53.3)	33.5 (24.8-54.0)	31.5 (21.8-43.0)
Group 3	33.0 (25.0-42.5)	37.5 (26.8-75.8)	34.0 (27.5-57.0)	32.0 (20.2-54.5)
AST (IU/L)				
Group 1	50.0 (24.5-83.0)	52.0 (42.5-91.0)	43.0 (26.5-54.0)	34.0 (28.0-58.5)
Group 2	31.5 (22.0-45.8)	44.5 (34.8-64.0)	38.5 (28.0-53.0)	33.5 (26.0-49.0)
Group 3	38.5 (30.3-47.0)	47.0 (41.8-83.0)	40.5 (33.5-71.5)	38.5 (24.5-55.0)
ALB (g/L)				
Group 1	37.5 (34.2-39.5)	29.0 (26.8-32.5)[a]	31.6 (28.9-32.9)[a]	32.4 (30.5-35.5)[a]
Group 2	38.3 (34.8-40.8)	31.5 (28.0-34.7)[a]	32.3 (29.5-35.1)[a]	34.2 (31.9-38.0)[a]
Group 3	39.6 (36.6-42.2)	27.8 (25.9-33.2)[a]	35.3 (31.9-36.5)[a]	34.5 (30.0-38.1)[a]

[a]$P < 0.05$ *vs* preoperative status within the group. HGB: Haemoglobin; WBC: White blood cell; TBil: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; POD: Postoperative days.

to F3 (numerous septa without cirrhosis) or F4 (cirrhosis), and no significant differences regarding the stage distribution among the three groups were detected.

In regard to short-term surgical complications, given that one patient may suffer more than one complication, the events were recorded numerically instead of as a complication rate. Fortunately, no one suffered common bile duct injury or bile leakage. The detailed data of other complications are presented in Table 3. Although the complication rate was not calculated, a trend was observed towards more pulmonary and incision infections occurring in the patients who received open surgery. All of the complications were resolved by conservative treatments, except the two cases of splenic fossa collections and the five cases (one in group 1, three in group 2, and one in group 3) of the seven pleural effusions which required punctured drainage guided by B-ultrasound, and the two patients in group 2 who suffered postoperative bleeding that required emergency laparotomy and blood transfusions.

Table 4 shows the dynamic changes of haematological and liver function parameters from the perioperative period. No obvious perioperative changes were observed for haemoglobin, AST, ALT and TBil levels. The platelet counts, however, gradually ascended to the normal range on POD 5 for all groups. The changes of the leukocyte counts rapidly ascended at first and then gradually descended to the normal range. A similar trend was observed for ALB, but the levels reached on POD 5 were slightly lower than the normal range for all groups.

None of the patients in group 1 presented any gallstone-associated symptoms following discharge, while the patients in group 3 expressed complaints of discomfort related to their surgical incisions.

DISCUSSION

LC application was restricted by several contraindications, including liver cirrhosis, according to the consensus statement issued by the National Institutes of Health (NIH) in 1993[20]. Since the first report of LC was published by Yerdel *et al*[5] in 1993, the literature pertaining to this procedure as a treatment in liver cirrhosis patients has grown substantially. In general, the studies have indicated that early liver cirrhosis patients with symptomatic gallstones could undergo LC safely, without any significant increase in morbidity and mortality; moreover, performing LC in patients with advanced cirrhosis should be seriously considered[3,7,21-24]. Additionally, the past decade has seen substantial progress in the understanding of the risks and benefits of LS in patients with hypersplenism secondary to liver cirrhosis, with studies providing encouraging surgical outcomes of this mini-invasive procedure[11,25]. As surgeons' experience in applying LS increased, so did the interest in using this procedure in a consecutive approach to cope with the challenging conditions of coexisting ab-

Table 5 Reports in the literature of laparoscopic cholecystectomy plus laparoscopic splenectomy

Ref.	Year	*n*	Main diagnosis	Surgical sequence	Conversion rate	Morbidity
Trías *et al*[26]	1994	1	Hereditary spherocytosis	LC → LS	NR	NR
Patton *et al*[15]	1997	1	Hereditary spherocytosis	LC → LS	NR	Atelectasis
Caprotti *et al*[31]	1999	7	Hereditary spherocytosis	LC → LS	NR	NR
Brink *et al*[32]	2003	1	Autoimmune haemolytic anaemia	LC → LS	NR	PSVT
Rossi *et al*[34]	2007	1	Congenital haemolytic anaemia	NR	NR	PSVT
Choi *et al*[33]	2007	2	Hereditary spherocytosis	LC → LS	NR	NR
Sasaki *et al*[14]	2010	9	Hereditary spherocytosis (*n* = 4) Splenic artery aneurysm (*n* = 2) Hypersplenism with liver cirrhosis (*n* = 2) Evans syndrome (*n* = 1)	LS → LC	NR	PSVT
Nobili *et al*[16]	2011	30	Hereditary spherocytosis (*n* = 22) Thalassemia (*n* = 4) Idiopathic thrombocytopenic purpura (*n* = 3) Sickle cell disease (*n* = 1)	LC → LS	1 (3.3%)	Hemoperitoneum

PSVT: Portal or splenic vein thrombosis; NR: Not reported: LC: Laparoscopic cholecystectomy; LS: Laparoscopic splenectomy.

dominal diseases.

The first study regarding concomitant LC and LS was reported in 1994[26]. Since then only a few additional studies have been reported in the literature (Table 5), and almost all have enrolled only a few patients with benign haematological disorders. The current study analysed 17 liver cirrhosis patients with gallstones who received LC plus LS and compared the surgical outcomes between laparoscopic and open procedures. Similar to the other studies that have compared these two procedures, the conclusion was drawn that patients benefited from the mini-invasive surgery in terms of shorter postoperative stay, less intraoperative blood loss, faster return to oral intake, better cosmetic outcome, and fewer surgical infections.

Cirrhotic patients are often treated with operative interventions when they suffer from concomitant late stage gallbladder disease, which had led to severe chronic cholecystitis. Consequently, in addition to the risks associated with liver cirrhosis itself, the woody and friable gallbladder tissue has represented a challenge to surgeons during LC. Bearing in mind that the periumbilical collaterals and the falciform ligament might be sources of bleeding in these patients[27-29], the trocar for laparoscopy is recommended to be placed in the lower umbilicus. Furthermore, according to our clinical experience, ultrasonic shears are recommended for division to avoid bleeding from dilated tortuous veins around the gallbladder. Nonetheless, it is dangerous and difficult to resect the gallbladder from the cirrhotic liver bed, which is easily injured and bleeds profusely. In this case, laparoscopic subtotal cholecystectomy (LSC), with the posterior wall of the gallbladder left on the cirrhotic liver bed, may be a better treatment choice[9,27,28,30]. The major disadvantage of LSC is the high rate (38.1%-94.1%) of bile leakage from the closed stump[27]. None of the patients in the current study received LSC or suffered bile leakage, which may be attributed to appropriate patient selection and sufficient preoperative assessment.

In the current study, the LS procedure was performed consecutively to the LC procedure during the same operative period. While some studies support performing the procedures in this order, others do not[14-16,26,31-34]; the main advantage of performing the LC procedure first is that it has a relatively low conversion rate. Indeed, in our case series one patient required conversion during the LS procedure and a left subcostal incision was required. In addition, four patients (one in group 1 and three in group 2) had to be converted to the open procedures, due to bleeding from the splenic pedicel during LS. The overall conversion rate of 5.3% is in line with that reported previously[35]. It has also been reported that preoperative splenic artery embolization (SAE) could reduce operative time, intraoperative blood loss, and the need for transfusion during LS[36,37]; however, because of serious complications, such as severe pain, pancreatitis, atelectasis and microcoil migration, the application of this approach before LS remains controversial[38,39]. None of the patients in the current study received preoperative SAE.

A variety of studies have focused on the development of PSVT after splenectomy, but the true incidence remains uncertain[40]. This complication is described as an uncommon but fatal occurrence and has been reported to result from some combination of hypercoagulability, platelet activation, disturbance and activation of the endothelium, and altered lipid profiles[41]. It is difficult to make early clinical diagnoses of PSVT because of the non-specific symptom profile, which includes fever, abdominal pain and vomiting[42-44], and potential asymptomatic profile[45]. Encouragingly, hidden PSVT may be detected by Doppler ultrasound or CT scans. The first report of PSVT after LC plus LS was published in 2003[32], and in the current study seven patients (7.9%) suffered postoperative PSVT. The rate of PSVT was higher in group 1 (11.8%) than in group 2 (6.9%) and group 3 (7.1%), but the small sample size precluded our ability to determine whether concomitant LC and LS was a significant risk factor for this complication. In view of the potentially deadly outcome of PSVT, B-ultrasound was performed to monitor the development of throm-

bosis until POD 7, and B-ultrasound or CT scanning was performed once a month for 3 mo after discharge. Once a PSVT diagnosis was established, early anticoagulation therapy was initiated.

During laparoscopic procedures, a 12-14 mmHg CO_2 pneumoperitoneum is required for the establishment of a sufficient operative field[46]. This method has been reported to lead to ischemia-reperfusion injury to the liver[47], which may further damage the already impaired liver function. Some studies have demonstrated that pneumoperitoneum can cause postoperative increases in AST, ALT, bilirubin and prothrombin time[48-50], but these results have not been consistent[11,51]. In the current study, a 14 mmHg CO_2 pneumoperitoneum did not appear to result in significant postoperative changes in AST, ALT, or bilirubin; the reasons for this phenomenon are unknown. Therefore, different pressures of pneumoperitoneum should be used when performing laparoscopic surgeries to assess their impacts on liver function in the future studies.

Admittedly, our results could be affected by several potential biases, because this study was neither blinded nor randomized and the sample size was small. However, the preoperative information among the three groups was comparable (Table 1), which could balance the potential biases to some extent. A large-volume, prospective, and randomized study is necessary to confirm our findings.

In summary, based on this comparative study, consecutive LC and LS is not only feasible, safe and effective, but also a superior choice for liver cirrhosis patients with gallstones and hypersplenism, especially for those patients with Child-Pugh A and B.

COMMENTS

Background

Laparoscopic cholecystectomy (LC) was once contraindicated for liver cirrhosis patients due to well-developed collateral vessels and increased risk of bleeding, and the same was true for laparoscopic splenectomy (LS). However, the technical improvements in laparoscopic technique and accumulation of surgeons' experience have led clinicians to question this concept. An increasing number of studies have demonstrated the efficacy and safety of laparoscopic surgery for treating gallstones or hypersplenism in the patient population with liver cirrhosis. On the other hand, the incidence of gallbladder stones in liver cirrhosis patients has been estimated as two-fold higher than that in the general population. It is necessary to determine the efficacy and safety of consecutive resections of the gallbladder and spleen by laparoscopy in liver cirrhosis patients.

Research frontiers

To date, few comparable studies of LS plus LC in treating liver cirrhosis patients with gallstones and hypersplenism/portal hypertension have been reported. In the era of minimally invasive surgery, patients prefer to receive laparoscopic operations if possible. In the current study, the authors demonstrated the feasibility, safety, and effectiveness of consecutive LC and LS in treating liver cirrhosis patients with gallstones and hypersplenism.

Innovations and breakthroughs

This is the first report of laparoscopic treatment of hypersplenism/portal hypertension combined with gallstones in liver cirrhotic patients. The results represent good news to liver cirrhosis patients who are in need of surgical attention to address coexisting abdominal diseases.

Applications

This is the first case series of LC plus LS treatment in liver cirrhosis patients and will add value to the surgical literature. This study revealed that LC plus LS may be a better treatment option for liver cirrhosis patients; however, long-term results need to be confirmed in a future study.

Terminology

Hypersplenism is a related hematologic syndrome leading to thrombocytopenia, neutropenia, or both, and frequently occurs in patients with liver cirrhosis due to portal hypertension. Higher incidence of gallstones have been reported in the population of patients with liver cirrhosis, and their development is associated with dysfunction of gallbladder motility and emptying, hypersplenism, increased haemolysis, and enhanced circulating oestrogen levels.

Peer review

This is an interesting novel comparative analysis of the perioperative outcomes of consecutive laparoscopic and open surgeries in treating liver cirrhosis patients with gallstones and hypersplenism.

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BRIEF ARTICLE

Total splenic artery embolization for splenic artery aneurysms in patients with normal spleen

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Abstract

AIM: To evaluate total embolization of the main splenic artery in patients with splenic artery aneurysms (SAAs) and normal spleen.

METHODS: Thirty-five consecutive patients with SAAs were referred for treatment with coil embolization. Patients were classified into two groups: coil embolization of the main splenic artery with complete occlusion of the artery and aneurysms (group A, n = 16), and coil embolization of the aneurysmal sac with patency of the splenic artery (group B, n = 19). Data on white blood cell (WBC) and platelet counts, liver function, and complications were collected on days 7 and 30, and subsequently at a 6-mo interval postoperatively. Abdominal computed tomography was routinely performed to calculate the splenic volume before and 1 mo after the procedure, and subsequently every 6 mo during follow-up.

RESULTS: Coil embolization of the SAAs was technically successful in all 35 patients, with no procedure-related complications. The post-embolization syndrome, including abdominal pain, fever and vomiting, occurred in six patients (37.5%) in group A and three patients in group B (15.8%). There were no significant differences in WBC and platelet counts between preoperatively and at each follow-up point after the procedures. There were also no significant differences in average WBC and platelet counts between the two groups at each follow-up point. There were significant differences in splenic volume in group A between preoperatively and at each follow-up point, and there were also significant differences in splenic volume between the two groups at each follow-up point.

CONCLUSION: Total embolization of the main splenic artery was a safe and feasible procedure for patients with SAAs and normal spleen.

Key words: Splenic artery aneurysms; Spleen; Coil embolization; Splenic artery

Core tip: Total embolization of the splenic artery sometime must be performed to achieve complete occlusion of large or multiple aneurysms. Thirty-five patients were classified into two groups: coil embolization of the splenic artery with complete occlusion of the artery and aneurysms, and coil embolization of the aneurysmal sac with patency of the splenic artery. Except for shrinkage of splenic volume, no changes in white blood cell and platelet counts or liver function were found in the former group. These results suggest that total embolization of the splenic artery is a safe and feasible procedure for patients with splenic artery aneurysm and normal spleen.

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INTRODUCTION

Splenic artery aneurysms (SAAs) are the second most common abdominal aneurysm with a reported prevalence of 0.8% at arteriography and 0.04%-0.10% at autopsy. SAAs are usually small (< 2 cm in diameter), circular, and located at a bifurcation in a middle or distal segment of the splenic artery. Most SAAs are detected incidentally without symptoms during diagnostic imaging for other indications. Rupture of SAAs is rare, with the rate of potential rupture ranging from 3% to 9.6%[1], but is associated with a high mortality rate (10%-25%) if left untreated[2-4].

Coil embolization is now a widely accepted treatment for SAAs; however, for patients with large or multiple SAAs, total embolization of the splenic artery must be performed to produce complete occlusion of the SAAs. Nowadays, total embolization of the splenic artery has been shown to be a safe and effective method for the treatment of SAAs[1,5-8], but the changes in splenic function and morphology after embolization have rarely been reported, especially in patients with SAAs and normal spleen.

In this study, we retrospectively reviewed patients with SAAs treated by transcatheter embolization in our institution, evaluated the clinical outcomes of total embolization of the main splenic artery for patients with SAAs and normal spleen, and compared the results with coil embolization of the aneurysmal sac with patency of the splenic artery.

MATERIALS AND METHODS

Patients

The protocol was approved by the institutional ethics committee, and all the patients provided written informed consent. From January 2007 to June 2013, 35 consecutive patients with SAAs referred for the treatment with coil embolization were screened for enrollment in this retrospective study, and subsequently underwent computed tomography (CT) follow-up at our hospital. Diagnosis of SAAs was established in all patients by CT with contrast medium injection and multiplanar reconstructions.

Patients were eligible for enrollment if they met the following criteria: (1) definite SAAs of the main splenic arteries with normal spleen; (2) transcatheter coil embolization; and (3) at least one CT and clinical follow-up ⩾ 1 year after initial treatment. Patients were excluded if any of the followings were present: (1) severe cardiopulmonary comorbidity; (2) untreatable coagulopathy; (3) hypersplenism; (4) SAA at the branch of the splenic arteries or splenic parenchyma; or (5) contraindication for contrast medium.

A total of 48 patients with SAAs were enrolled in this study. SAAs were located in the proximal (n = 10), middle (n = 17), or distal (n = 21) segment of the main splenic artery. Thirty-eight patients had asymptomatic lesions that were discovered incidentally, and the remaining 10 patients presented with isolated abdominal pain. Forty-one of the 48 patients had a single aneurysm, and seven had multiple aneurysms. None of the patients experienced rupture of their lesions.

Table 1 Clinical characteristics, outcomes and complications in patients with splenic artery aneurysms n (%)

	Group A (n = 16)	Group B (n = 19)	P value
Age (yr, mean ± SD)	50.81 ± 7.66	48.42 ± 6.37	0.320
Female/male	6/10	8/11	0.782
SAA size (mm)	16.43 ± 3.59	10.14 ± 2.07	< 0.001
SAAs			0.156
Single	12	18	
Multiple	4	1	
Technical success	16 (100)	19 (100)	0.999
Hospital stay (d)	9.25 ± 2.84	8.52 ± 1.74	0.320
Complications			
Post-embolization syndrome	6 (37.5)	3 (15.8)	0.245
Major complications	2 (12.5)	0 (0)	0.202
Splenic abscess	1	0	
Pleural effusion	1	0	
Ascites	0	0	
CT follow-up (mo)	38.69 ± 15.66	32.84 ± 15.02	0.269
Clinical follow-up (mo)	39.94 ± 14.90	35.16 ± 16.01	0.370

SAA: Splenic artery aneurysm; CT: Computed tomography.

Patients were classified into two groups according to two embolization techniques: coil embolization of the main splenic artery with complete occlusion of the artery and aneurysms (group A), and coil embolization of the aneurysmal sac with patency of the splenic artery (group B). Initially, 21 and 27 patients were assigned to groups A and B, respectively. Of these, 13 patients did not meet the inclusion criteria, with CT and clinical follow-up < 1 year in eight patients and loss to follow-up in five patients (3 in group A and 2 in group B). The remaining 35 patients were enrolled. There were 21 men and 14 women with a mean age of 49.51 ± 7.0 years (range, 34-60 years). Demographic features and clinical presentations are summarized in Table 1.

Coil embolization

There are two embolization techniques: endovascular ligation that requires the positioning of the coils on either side of the aneurysm ("sandwich technique") in order to attain complete occlusion[1,5,9,10], and embolization using coils limited to the aneurysmal sac with patency of the splenic artery. In general, the former technique was used for large or multiple SAAs, and the latter for small or single SAA. All embolization procedures were performed by two experienced interventional radiologists on a single plane angiography system under fluoroscopic guidance.

Prior to embolization, selective angiography of the celiac trunk, splenic artery, and superior mesenteric artery was performed in all patients *via* the right femoral artery with a 5-Fr diagnostic catheter. To avoid total splenic infarction, we confirmed patency of the collateral arteries, which were shown to be connected to the hilar splenic

artery from the left gastric artery or from the gastroepiploic artery on a celiac arteriogram.

Coil embolization of the SAAs was performed after confirmation of these connections, and details of the coiling procedures were described previously[1,5,9-11]. Coils and/or gelfoam were used as embolization material either alone or in combination. In general, the embolization coils used in this series were standard 0.089-cm (0.035-in) fibered microcoils (Tornado; Cook, Bloomington, IN, United States). Post-embolization checks were performed with selective splenic, celiac and superior mesenteric artery angiograms to confirm occlusion of the main splenic artery and patency of the collateral arteries. Preoperative antibiotic prophylaxis was used routinely for 3 d. Following embolization, patients were monitored clinically, and antibiotics were administered after the procedure for several days to avoid infectious complications.

Postoperative outcome evaluation

All the patients were followed at our outpatient clinic. Peripheral blood cell parameters, including white blood cell (WBC), red blood cell and platelet counts, were monitored at different time points prior to the procedure, on days 7 and 30, and subsequently at a 6-mo interval during follow-up. To determine a possible effect on liver function, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), albumin, and prothrombin time (PT) in serum were measured before and after the procedure at the same follow-up time point as evaluating the peripheral blood cells.

Abdominal CT scans were routinely performed before and 1 mo after the procedure, and subsequently every 6 mo during follow-up. CT images were obtained using a multislice GE Pro Speed machine (GE Medical Systems, Milwaukee, WI, United States) and included unenhanced and enhanced images (arterial phase, parenchymatous phase) with multiplanar 3D reconstruction. Based on enhanced CT images, we measured and compared the pretreatment splenic volume and the post-embolization residual splenic volume on a 3.1 workstation (GE Medical Systems) using volumetric analysis software. The infarcted splenic volume (mL) was calculated by subtracting the noninfarcted splenic volume from the pretreatment splenic volume. The splenic infarction rate was calculated by dividing the infarcted splenic volume by the pretreatment splenic volume (× 100%).

Data regarding (1) technical success; (2) liver function; (3) changes in splenic volume and (4) complications were evaluated at the time of the report or patient death. Technical success was defined as complete exclusion of the aneurysm on the post-embolization arteriogram, without major complications. Post-embolization syndrome was defined as fever, abdominal pain, or elevation of pancreatic enzymes over preprocedural values after splenic infarction.

Any potential complications following coil embolization, such as splenic abscess, splenic rupture, pneumonia, refractory ascites or pleural effusion, gastrointestinal bleeding, post-embolization syndrome, abdominal fullness, or appetite loss, were recorded.

Statistical analysis

All the data are expressed as mean ± SD. Dichotomous and categorical data are reported as numbers and percentages. The end points were determined technically and clinically. The technical end point was failed performance of the procedures. The clinical end points were loss to follow-up or death during or after the procedures. Comparisons of the variables between the two groups were performed using the Mann-Whitney test, χ^2 test or Fisher's exact test as appropriate. All statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL, United States).

RESULTS

Safety

The technical and initial clinical outcomes of the two groups are shown in Table 1. Coil embolization of the SAAs was technically successful in all 35 patients, with no procedure-related complications. The 30-d mortality rate was zero.

Complications after the procedures are listed in Table 1. The post-embolization syndrome, including abdominal pain, fever and vomiting, occurred in six patients (37.5%) in group A and three in group B (15.8%). One patient in group A had pleural effusion, which was resolved by thoracocentesis. One patient in group A suffered from persistent high fever, and was found to have splenic abscess on abdominal CT scan. The abscess was drained with a catheter and absorbed within 1 mo. There was no significant difference in hospital stays between the two groups (Table 1).

Follow-up clinical results

At the time of writing this manuscript, all patients were followed and assessed at 1 wk, 1 mo, 6 mo, and 1 year; 28 were assessed for 2 years and 13 were assessed for ⩾ 4 years, prospectively, with a mean follow-up of 37.34 ± 15.47 mo (range, 13-66 mo). There were no significant differences in the number of patients in each follow-up period between the two groups.

Changes in peripheral blood cell counts

Table 2 summarizes the follow-up clinical outcome between the two groups. In comparison with pre-procedural level of peripheral blood cell counts, including WBC and platelet counts in each of the two groups, there were no significant differences at each follow-up point after the procedures. There were also no significant differences in average WBC and platelet counts between the two groups at each follow-up point.

Changes in liver function parameters

In comparison with pre-procedural level of liver function parameters values, including AST, ALT, TB, albumin and PT, in each of the two groups, there were no significant differences at each follow-up point after the procedures.

Table 2 Comparison of variables between the two groups during follow-up (mean ± SD)

Evaluation	Initial		1 mo		6 mo		1 yr		2 yr		⩾ 4 yr	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
WBC count ($\times 10^9$)	6.1 ± 0.9	6.2 ± 1.0	6.2 ± 1.2	6.2 ± 1.1	6.2 ± 0.7	6.3 ± 1.0	6.0 ± 0.9	5.8 ± 0.8	5.9 ± 1.0	5.9 ± 1.0	5.9 ± 0.9	5.9 ± 1.1
PLT count ($\times 10^9$)	172.1 ± 52.9	178.7 ± 48.4	190.3 ± 28.3	198.9 ± 28.2	191.2 ± 23.7	194.3 ± 27.8	191.2 ± 31.6	196.5 ± 29.3	196.6 ± 28.6	190.1 ± 34.1	180.6 ± 3-6.6	192.8 ± 27.8
Splenic volume (cm^3)	289.8 ± 28.2	290.5 ± 26.5	222.3 ± 47.9[a]	285.2 ± 29.2[c]	202.3 ± 43.0[a]	283.6 ± 30.5[c]	199.1 ± 40.9[a]	275.4 ± 38.2[c]	197.7 ± 40.9[a]	276.4 ± 43.0[c]	201.3 ± 25.1[a]	281.7 ± 42.1[c]

[a]$P < 0.05$, Compared splenic volume in group A between preoperatively and at each follow-up point; [c]$P < 0.05$, Compared splenic volume between group A and group B at each follow-up point. WBC: White blood cell; PLT: Platelet.

There were also no significant differences in average AST, ALT, TB, albumin and PT between the two groups at each follow-up point.

Changes in splenic volume after embolization

All patients were followed and assessed at 6 mo and 1 year; 28 were assessed for 2 years and 13 were assessed for ⩾ 4 years, with a mean follow-up of 35.5 ± 15.4 mo (range, 12-66 mo). After the procedures, splenic volume decreased significantly in group A, peaked at 1 mo after the procedures, and was then maintained at about the same size during follow-up (Table 2). In group B, there was no obvious decrease in splenic volume after the procedures. There were significant differences in splenic volume at each follow-up point between the two groups. In addition, there were also significant differences in splenic volume in group A between preoperatively and at each follow-up point (Table 2, Figure 1).

DISCUSSION

There are three main findings from this study: (1) no significant differences were found in peripheral blood cell counts, including WBCs and platelets, between preoperatively and at each follow-up point after the procedures, and between the two groups at each follow-up point; (2) no significant differences were found in liver function between preoperatively and at each follow-up point after the procedures and between the two groups at each follow-up point; and (3) splenic volume in group A was significantly smaller after embolization. To the best of our knowledge, this is the first report to evaluate the clinical outcome of total embolization of the main splenic artery for patients with SAAs and normal spleen.

Therapeutic options available for patients with SAAs include conventional open surgery, endovascular treatment, and the more recently introduced technique of laparoscopic surgery. The traditional treatment was ligation of the artery or aneurysm or excision of the lesion, with or without partial or total splenectomy, and occasionally with distal pancreatectomy[12,13]. However, the mortality rate associated with this procedure was approximately 1.3% and the morbidity rate was 9% for aneurysms[12].

During the past decade, endovascular techniques including transcatheter embolization and covered stent placement were introduced as alternatives to conventional surgery[2,12,14,15]. These techniques can be used to treat most SAAs regardless of clinical presentation, etiology, and location. Endovascular treatment is also indicated for ruptured SAAs, especially when the site is difficult to access during conventional surgery or when the operative risk is high because the patient is in poor general health[3,16]. Advantages over surgery include accurate localization of the aneurysm, assessment of collateral flow, low risk for patients who are poor surgical candidates[17], and easier approach to aneurysms whose surgical exposure is difficult.

Nowadays, transcatheter coil embolization is the commonly used method and has been shown to be safe and effective for the treatment of the SAAs[1,4-8,18-23]. Although percutaneous treatment with covered stents has produced favorable results[24,25] with main splenic arterial patency, they are difficult to deploy in tortuous vessels, and thrombosis may occur after stenting.

There are two embolization techniques: coil embolization of the aneurysmal sac with patency of the splenic artery, and embolization of the main splenic artery with complete occlusion of the artery and the SAAs[1,5,9,10]. The former technique was often used for single and small SAAs, and the latter was often applied for large or multiple SAAs. In this study, although shrinkage of the splenic volume in group A was obtained after total embolization of the main splenic artery, no mortality occurred, and the complications were under control, which was confirmed by the patency of the collateral arteries.

In addition, an increase in WBC and platelet counts after total embolization of the main splenic artery for patients with hypersplenism has been reported by He *et al*[26,27] and Gu *et al*[28]. In the present study, there were no significant differences in WBC and platelet counts between preoperatively and at each follow-up point. In other words, there was no increase or decrease in WBC and platelet counts in group A after embolization. Furthermore, there were no differences in liver function parameters between preoperatively and at each follow-up point, which was in agreement with He *et al*[26,27] and Gu *et al*[28]. These results indicated that total embolization of the main splenic artery is a safe procedure for patients with SAAs and normal spleen.

In this study, we found that the splenic volume in

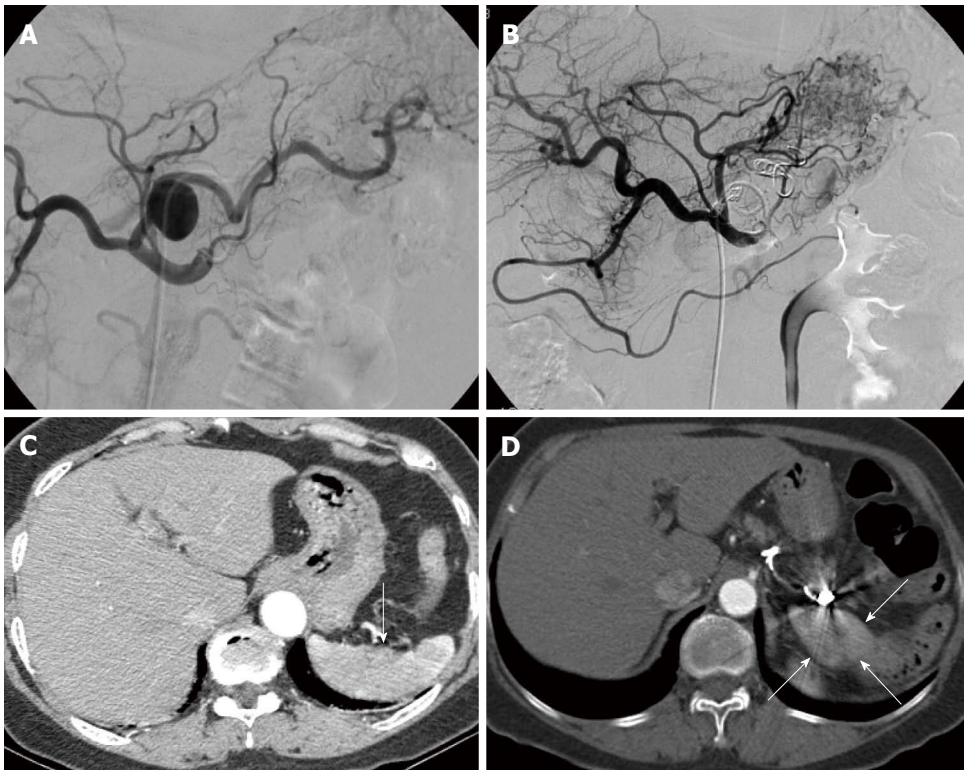

Figure 1 Group A. A splenic artery aneurysm (SAA) in a 56-year-old man with normal spleen. A: Celiac arteriogram prior to coil embolization demonstrated an SAA located at the proximal splenic artery; B: Celiac arteriogram after coil embolization showed total embolization of the main splenic artery with disappearance of the SAA; C: Before embolization, arterial phase of computed tomography angiography (CTA) showed that the spleen was normal (arrow); D: Arterial phase of CTA showed that the spleen had decreased size (arrows) 2 years after embolization.

three patients in group A did not decrease after total embolization of the main splenic artery. We thought that the main reason may be the recanalization of the splenic artery. Another possible reason was that there was sufficient blood supply for the spleen from these connections.

Our study had the following limitations. First, the study was retrospective, and the patient population was small, which may prevent generalization of our results. Second, severe complications may occur when the collateral arteries are lacking or not consummate. Confirmation of the patency of the collateral arteries, which connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery, was a necessary step prior to total embolization of the main splenic artery for SAAs. Third, CT angiography involves exposure of the patients to ionizing radiation and the administration of intravenous contrast material. Lastly, follow-up angiography was not performed in this study, thus, the possibility of recanalization of the main splenic artery or the SAAs could not be determined.

In conclusion, in this retrospective study, except for shrinkage of splenic volume in patients undergoing total embolization of the main splenic artery, no changes in WBC and platelet counts and liver function parameters were found. These results indicated that total embolization of the main splenic artery is a safe and feasible procedure for patients with SAAs and normal spleen. Further clinical trials and extended follow-up studies are needed.

COMMENTS

Background

Coil embolization is now a widely accepted treatment for splenic artery aneurysms (SAAs); however, for patents with large or multiple SAAs, total embolization of the splenic artery sometime must be performed to achieve complete occlusion of the SAAs.

Research frontiers

Total embolization of the splenic artery has been shown to be a safe and effective method for the treatment of SAAs, but the changes in splenic function and morphology after embolization have rarely been reported, especially in patients with SAAs and normal spleen. We retrospectively evaluated the clinical outcomes of total embolization of the main splenic artery for patients with SAAs and normal spleen.

Innovations and breakthroughs

The use of total embolization of the splenic artery was devised for the management of patients with SAAs and normal spleen. All procedures were performed under fluoroscopic control. This is believed to be the first report to evaluate the clinical outcomes of total embolization of the main splenic artery for patients with SAAs and normal spleen.

Applications

Total embolization of the main splenic artery is a safe and feasible procedure for the patients with SAAs and normal spleen.

Terminology

Total embolization of the main splenic artery is the technique of embolization of the main splenic artery with complete occlusion of the artery and SAAs.

Peer review

The authors reported total embolization of the main splenic artery for patients with SAAs and normal spleen. Thirty-five consecutive patients with SAAs were classified into two groups: coil embolization of the main splenic artery with complete occlusion of the artery and aneurysms (group A), and coil embolization of the aneurysmal sac with patency of the splenic artery (group B). The results

reveal that except for shrinkage of splenic volume in patients with total embolization of the main splenic artery, no changes in white blood cell and platelet counts or liver function parameters were found. These results suggest that total embolization of the main splenic artery is a safe and feasible procedure for the patients with SAAs and normal spleen.

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META-ANALYSIS

Xiangshaliujunzi Decoction for the treatment of diabetic gastroparesis: A systematic review

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Abstract

AIM: To assess the current clinical evidence of the effectiveness of Xiangshaliujunzi Decoction (XSLJZD) for the treatment of diabetic gastroparesis (DGP).

METHODS: Randomized controlled trials (RCTs) were retrieved from seven major electronic databases including Medline, the Cochrane Library, Embase, Chinese Biomedical Literature Database (CBM), Chinese National Knowledge Infrastructure, Chinese Scientific Journal Database (VIP), and Wanfang Databases, using search dates from the beginning of the databases to May 2013. No language limitations were applied. We included RCTs that used XSLJZD or a modified XSLJZD compared with a control group for the treatment of DGP. The control groups included conventional treatment (Western medicinal treatment), placebo, and no treatment (blank), but not acupuncture. The main outcome index was clinical effectiveness, which was based on the gastric emptying test and variations in the gastrointestinal (GI) symptoms between the treatment and control groups after intervention. Data extraction, analysis, and quality assessment were conducted according to the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0.

RESULTS: Ten RCTs involving 867 patients (441 in the experimental groups, and 426 in the control groups) were identified, and the overall methodological quality was evaluated as generally low. In the treatment groups, all 10 trials used herbs alone as the treatment, whereas all control groups used prokinetic medicine. The period of intervention ranged from 2 to 8 wk. Three classes were used to evaluate treatment efficacy: significant effective, effective, and ineffective, and all trials used the clinical effective rate (based on the gastric emptying test and changes in GI symptoms) to evaluate efficacy. The data showed that the effects of XSLJZD for the treatment of DGP were superior to the control group (n = 867, RR =1.33, 95%CI: 1.24-1.42, Z = 8.11, P < 0.00001). Two trials recorded adverse events, and one trial reported follow-up.

CONCLUSION: XSLJZD could restore the gastric emptying rate and improve symptoms. However, the evidence remains weak due to the poor methodological quality of the included studies.

Key words: Diabetic gastroparesis; Xiangshaliujunzi Decoction; Gastric emptying rate; Gastrointestinal symptoms; Systematic review

Core tip: This article is a systematic review of Xiangshaliujunzi Decoction (XSLJZD), which is used in traditional Chinese medicine to treat stomach discomfort

and diabetic gastroparesis. This article aims to evaluate the efficacy of XSLJZD for the treatment of diabetic gastroparesis. The incidence of diabetes has increased significantly, and so it is necessary to take active steps to prevent and treat diabetic complications. The clinical manifestations of diabetic gastroparesis are extremely apparent. Traditional Chinese medicine, an alternative approach for improving the symptoms of patients, has specific advantages and so it its efficacy should be carefully assessed. In addition to the improvement of patient symptoms and clinical indications, improved quality of life is of interest of this article.

Tian JX, Li M, Liao JQ, Liu WK, Tong XL. Xiangshaliujunzi Decoction for the treatment of diabetic gastroparesis: A systematic review. *World J Gastroenterol* 2014; 20(2): 561-568 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/561.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.561

INTRODUCTION

Delayed gastric emptying affects a substantial proportion of patients with long-standing diabetes. Kassander used the term "gastroparesis diabeticorum" to describe abnormally increased gastric retention in these patients in 1958[1]. The American Gastroenterological Association and the American Nuclear Medicine Society define gastroparesis as a syndrome characterized by delayed gastric emptying in the absence of mechanical obstruction[1]. Patients often present with upper gastrointestinal symptoms such as early satiety, weight loss, abdominal bloating, abdominal discomfort, nausea, frequent vomiting, and impaired glycemic control. As such, quality of life is severely impacted[2]. Symptoms attributed to gastroparesis are reported by 5%-12% of patients with diabetes[3]. Several diabetic abnormalities could result in gastric motor dysfunction including autonomic neuropathy, enteric neuropathy involving excitatory and inhibitory nerves, abnormalities of the interstitial cells of Cajal, acute fluctuations in blood glucose, incretin-based medications, and psychosomatic factors[4]. The disorder can range from being mildly symptomatic to having severe symptoms leading to malnutrition, electrolyte imbalance, and impaired glycemic control[5].

Gastroparesis is increasingly being recognized as a significant health problem. Based on blood glucose control, the available treatment options of modern medicine include nutritional support, improvement of gastric emptying using prokinetics, control of symptoms, and the use of a gastric electric stimulator[6,7]. Medications for gastroparesis include metoclopramide, domperidone, cisapride, and erythromycin. However, these agents are of only limited efficacy, and many patients cannot tolerate them because of their side effects[8,9]. Surgical procedures, such as gastrectomy and antrectomy, are the last treatment options, but are controversial and need additional studies[10,11]. Providing nutritional support such as Jejunostomy tube feeding cannot cure gastroparesis[12]. Considering the high recurrence rate and the increasing incidence of diabetic gastroparesis (DGP)[13,14], finding drugs that show efficacy for the treatment of DGP are necessary.

Xiangshaliujunzi Decoction (XSLJZD) is, a traditional Chinese medicinal herbal containing eight commonly used herbs (Panax ginseng, Rhizoma atractylodis macrocephalae, Poria, Radix glycyrrhizae, Pericarpium citri reticulatae, Pinellia tuber, Fructus amomi, and Radix aucklandiae). It has long been used to treat gastrointestinal discomfort in clinical practice in China[15-19]. Its mechanism of action could be related to invigorating the spleen to resolve dampness, regulate the stomach, facilitate elimination and supplement to restore Qi based on the theory of Traditional Chinese Medicine (TCM)[20,21]. Recent studies revealed that XSLJZD could regulate gastrointestinal motility[22,23]. Biochemically, XSLJZD also increases the secretion of plasma motilin, lowers serum gastrin levels, and enhances smooth muscle contraction by increasing calcium levels[24-27].

Currently, we use XSLJZD as an alternative method for the treatment of DGP[28,29]. Several studies have suggested that XSLJZD is an effective treatment for DGP, but data are not yet definitive. This systematic review aims to assess the current clinical evidence for the efficacy of XSLJZD for the treatment of DGP by conducting literature reviews in databases for randomized controlled trials (RCTs).

MATERIALS AND METHODS

Databases and searches

A computer-based online search was conducted in the Medline, Cochrane Library, Embase, Chinese Biomedical Literature Database (CBM), Chinese National Knowledge Infrastructure, Chinese Scientific Journal Database (VIP), and Wanfang databases. The search terms used were ("diabetic gastroparesis" OR "gastrointestinal changes" OR "gastrointestinal disease") AND ("herb" OR "Xiangshaliujunzi Decoction" OR "Xiangshaliujunzi" OR "Xiangshaliujunzi Tang") AND ("randomized controlled trial" OR "controlled clinical trial" OR "random" OR "randomly" OR "randomized" OR "control"). We searched all articles that were published prior to May 2013.

Study selection

The RCTs included were restricted to those that compared XSLJZD as the treatment with a control group. We evaluated all forms of XSLJZD, such as XSLJZD and modified XSLJZD, whereas the control groups included conventional treatment (western medicinal treatment), placebo, and no treatment (blank), but not acupuncture. Studies that evaluated DGP patients were included regardless of gender, age, or nationality, but patients with other gastrointestinal diseases were excluded. The main outcome was clinical effectiveness, which was based on the gastric emptying test and variations in gastrointesti-

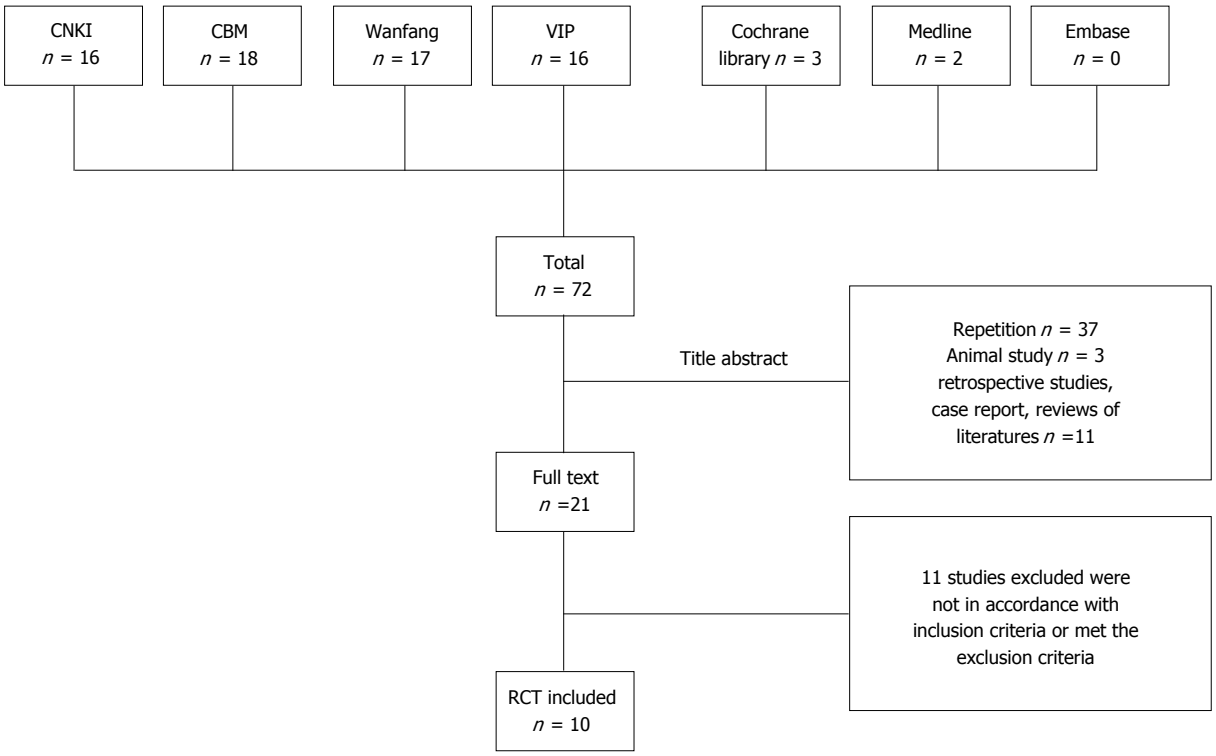

Figure 1 Flow chart of the trial selection process. CNKI: Chinese National Knowledge Infrastructure; CBM: China BioMedical Literature; VIP: VIP Database for Chinese Technical Periodicals; RCT: Randomized controlled trial.

nal (GI) symptoms. Studies used a 50% gastric emptying time. Any adverse events were also recorded. Duplicated publications reporting the same groups of patients were excluded.

Data extraction and quality assessment

Two authors carried out the literature searches (Tian JX, Li M), study selection (Tian JX, Liao JQ), and data extraction (Li M, Liao JQ) independently. The extracted data included the title of study, the authors, the year of publication, sample size, the gender and age of the participants, the names and components of the Chinese herbs, the details of the control interventions, the treatment process, outcomes, adverse effects, and details of the methods used. Discrepancies were resolved by consensus through discussion between the two authors and, if needed, by asking for additional information from a third party (Tong XL). The methodological quality of trials was assessed by two authors (Tian JX and Liu WK) independently based on the criteria from the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0[30]. Assessed parameters included random sequence generation (selection bias), allocation concealment (selection bias), the blinding of participants and personnel (performance bias), the blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. We judged each item on three levels ("Yes" for low bias, "No" for a high risk of bias, and "Unclear"). Then, we assessed the trials and categorized them into three levels: low risk of bias (all the items were categorized "Yes"), high risk of bias (at least one item ranked "No"), and unclear risk of bias (at least one item was "Unclear").

Statistical analysis

RevMan 5.1 software was used for data analyses, which was offered by the Cochrane collaboration. Dichotomous data were expressed as relative risk (RR), and continuous outcomes as weighted mean difference (WMD), both with 95%CI. Heterogeneity was assessed using the I^2 test with a significance level set at I^2 over 50% or $P < 0.1$. If there was no heterogeneity ($I^2 < 50\%$) we selected the fixed effect model. Possible causes of heterogeneity ($I^2 > 50\%$) were explained using the random effects model. Publication bias was explored by funnel plot analysis if sufficient studies were found[30].

RESULTS

Description of studies

A total of 72 studies were initially identified from the electronic databases. The search results are summarized in Figure 1. After screening the titles and abstracts, 51 potentially relevant studies were found. However, most of these were excluded due to repetition, retrospective studies, animal studies, case reports, and literature reviews. Thirty-seven studies were excluded because of duplicated publications, three were excluded because they were animal studies, and the remaining 11 were non-controlled clinical trials including retrospective studies, case reports, and literature reviews. After a detailed evaluation of the full text, a further 11 studies were excluded. Three trials

Table 1 Characteristics of included randomized controlled trials

Trials	Sample size	Gender (E/C)	Experimental group	Control group	Period	Outcome measure	Balance report of baseline
Cai[31]	54 (27/27)	(15M:12F) /(14M:13F)	Modified Xiangshaliujunzi Decoction, *bid*	Metoclopramide (10 mg, *tid*)	4 W	Clinical effective rate Gastric emptying test GI symptoms Adverse events	$P > 0.05$
Feng[32]	128 (64/64)	(30M:34F) /(34M:30F)	Modified Xiangshaliujunzi Decoction, *bid*	Cisapride (10 mg, *tid*), Metoclopramide (10 mg, *bid*)	4 W	Clinical effective rate GI symptoms	$P > 0.05$
Guo *et al*[33]	62 (32/30)	(35M:27F)	Modified Xiangshaliujunzi Decoction, *bid*	Domperidone (10 mg, *tid*)	8 W	Clinical effective rate GI symptoms	$P > 0.05$
Lu[34]	94 (48/46)	(28M:20F) /(24M:22F)	Modified Xiangshaliujunzi Decoction, *bid*	Domperidone (10 mg, *tid*)	4 W	Clinical effective rate GI symptoms	$P > 0.05$
Lu *et al*[35]	142 (72/70)	(30M:42F) /(31M:39F)	Modified Xiangshaliujunzi Decoction, *bid*	Mosapride (5 mg, *tid*)	4 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$
Meng[36]	62 (32/30)	(15M:17F) /(14M:16F)	Modified Xiangshaliujunzi Decoction, *bid*	Cisapride (10 mg, *tid*), Metoclopramide (10 mg, *bid*)	4 W	Clinical effective rate GI symptoms	$P > 0.05$
Wen[37]	87 (44/43)	(25M:19F) /(23M:20F)	Modified Xiangshaliujunzi Decoction, *bid*	Cisapride (5 mg, *tid*)	4 W	Clinical effective rate Gastric emptying test GI symptoms Adverse events	No significant differences
Dai[38]	70 (36/34)	(22M:14F) /(20M:14F)	Modified Xiangshaliujunzi Decoction, *bid*	Domperidone (10 mg, *tid*)	3 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$
Ji[39]	56 (30/26)	(14M:16F) /(12M:14F)	Modified Xiangshaliujunzi Decoction, *bid*	Domperidone (10 mg, *tid*)	2 W	Clinical effective rate GI symptoms	No significant differences
Hou *et al*[40]	112 (56/56)	(44M:12F) /(43M:13F)	Modified Xiangshaliujunzi Decoction, *bid*	Domperidone (10 mg, *tid*)	8 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$

GI: Gastrointestinal; M: Male; F: Female.

predominantly used Liujunzi decoction, which is similar to XSLJZD, two did not clarify the intervention used in the control group, three were excluded because they only reported the differences after treatment, and three trials were not in accordance with our inclusion criteria. Finally, 10 studies involving 867 patients were in accordance with our inclusion criteria without meeting the exclusion criteria. All studies were conducted in China and published in Chinese between 2003 and 2012. The bibliographic details of the included studies are shown in Table 1.

Among the 10 studies, all participants came from the inpatient and/or outpatient Department of Gastroenterology or Endocrinology, and the experimental interventions were oral administration. The trials included 473 male and 394 female subjects with ages ranging from 30 to 85 years. The diagnostic criteria of the studies were as follows: seven trials[31-37] described the World Health Organization diabetes mellitus (DM) diagnostic criteria involving a certain duration of gastrointestinal discomfort such as postprandial fullness, nausea, vomiting, bloating, and delayed gastrointestinal emptying in the absence of other gastrointestinal diseases. Two trials[38,39] described DM that was diagnosed with a certain duration of gastrointestinal discomfort such as postprandial fullness, nausea, vomiting, bloating, and delayed gastrointestinal emptying in the absence of other gastrointestinal diseases. Finally, one trial[40] described matching the "Interpretation of TCM prevention Guide for diabetes"[41].

All 10 trials used herbals alone (modified XSLJZD) as the treatment group. In the control group, all studies used prokinetic medicine alone: one used Cisapride, one used Mosapride, and the others used Domperidone. The period of intervention ranged from 2 to 8 wk. Three classes were used to describe the treatment efficacy: significantly effective, effective, and ineffective. In addition, all trials used the clinical effective rate (including significant effective and effective) based on the gastric emptying test (50% emptying time) and variations in GI symptoms to evaluate efficacy, which was the main outcome index. Adverse events were also recorded.

Methodological quality of included trials

The quality assessments are summarized in Table 2. The sample size of the included trials varied from 50 to 150 patients. None of the 10 studies reported details for sample size calculation, and none were double-blinded placebo controlled studies. One study described adequate methods of randomization using random number tables[40], whereas the remaining nine simply reported "randomly allocating" participants as the method of randomization. No trials had clear a description of the method used for allocation concealment or the blinding procedures. All trials described the patient characteristics, and described similarities between comparison groups at baseline, but none reported the loss of any participants, which made it difficult to determine whether these studies had an attrition bias. Two trials reported adverse events, and one trial[34] reported follow-up. The method-

Table 2 Quality assessment of included randomized controlled trials

Trials	Randomization	Allocation concealment	Blinding of participants and personnel	Blinding of assessors	Incomplete outcome data	Selective reporting	Other sources of bias	Risk of bias
Cai[31]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Feng[32]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Guo *et al*[33]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Lu[34]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Lu *et al*[35]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Meng[36]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Wen[37]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Dai[38]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Ji[39]	Unclear	Unclear	High	Unclear	Yes	No	Unclear	High
Hou *et al*[40]	Table of random number	Unclear	High	Unclear	Yes	No	Unclear	Unclear

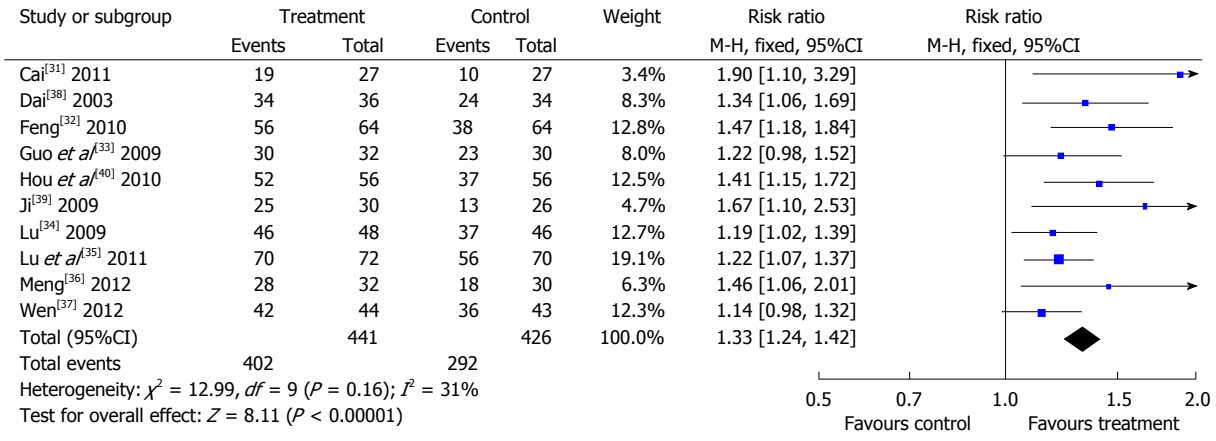

Figure 2 Effective rates of comparison between Xiangshaliujunzi Decoction and the control group.

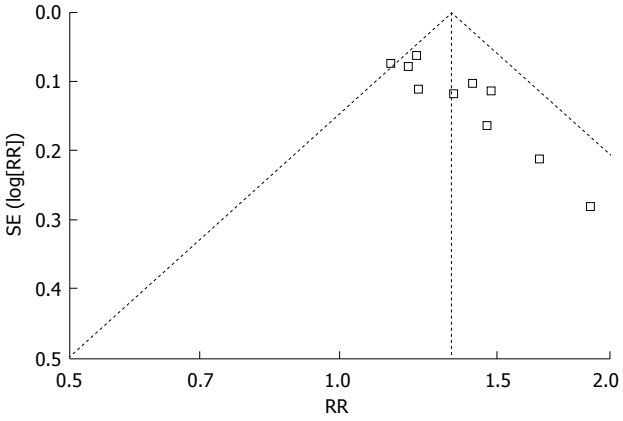

Figure 3 Funnel plot of publication bias.

ological quality of the included studies was determined to be generally low according to the predefined quality assessment criteria, which indicated that further investigations might influence the confidence intervals of the meta-analysis, and the results would likely be reversed.

Effect of the interventions

All the included studies compared the clinically effective rate between the treatment and control groups after intervention, which was based on the variations in the gastric emptying test and GI symptoms. Three classes were used to evaluate the effects of treatment: significant effective, effective, and ineffective. The total effective rate was the combination of the significant effective and effective rate, which was considered to be the main outcome index. The included trials exhibited homogeneity in the consistency of their results (χ^2 = 12.99, P = 0.16, I^2 = 31%). Thus, the fixed-effects model should be used for statistical analysis, and the treatment group scored significantly higher than the control group (n = 867, RR= 1.33, 95%CI: 1.24-1.42, Z = 8.11, P < 0.00001) (Figure 2).

Publication bias

Funnel plots based on the data of the effective rate are shown in Figure 3. The Figure was asymmetrical, which indicated that publication bias might influence the results of our analysis. Although we carried out comprehensive searches and tried to avoid bias, since all trials were published in Chinese we could not exclude potential publication bias.

Adverse events

Two trials[31,37] described safety reports: the control groups reported that one patient suffered from nausea, two with

stomachache, one with headache, one with diarrhea, and four reported skin allergies. In treatment group there was only one report of an adverse effect, which was a patient with stomachache. All the side effects were cured spontaneously.

Follow-up

Only one trial[34] reported follow-up. Lu *et al*[34] reported that two patients reported recurrence 3 months after treatment, with a recurrence rate of 4.2%. In contrast, the recurrence rate in the control group was 28.3%, involving 13 patients. The difference between treatment group and control group was statistically significant ($P < 0.01$).

DISCUSSION

Disturbances in gastrointestinal motility with associated symptoms have long been recognized as a complication of diabetes mellitus, and typically develop after diabetes mellitus has been established for several years[1]. Once established, DGP tends to persist despite improvement of glycemic control[42], which reduces the quality of life for affected patients[3]. However, current management strategies are far from clinically satisfactory[10]. It was demonstrated that Chinese herbal medicines could not only promote gastric emptying, but also improve symptoms. Because XSLJZD is widely used to treat gastrointestinal discomfort in clinical practice[29,30], this study aimed to assess the current clinical evidence of the effectiveness of XSLJZD for the treatment of DGP.

We did not find many systematic reviews reporting the use of Chinese herbal medicines to treat DGP. This systematic review included 10 randomized trials and a total of 867 participants. The main findings of this study were that XSLJZD demonstrated potential effects on the promotion of gastric emptying ($n = 867$, RR = 1.33, 95%CI: 1.24-1.42, $Z = 8.11$, $P < 0.00001$) compared with the control group. However, the methodological quality of the trials was assessed to be generally low. Before reaching definitive conclusions, the following weaknesses must be considered.

None of the studies reported sample size calculations, and the efficacy could not be clarified on some outcome measurements due to the small number of studies. Thus, the reliability of the outcome might be questionable. All trials lacked a description of the methods used for randomization. Only one study mentioned the random form (Hou), whereas the others simply mentioned "randomly allocating" subjects with no additional information. Therefore, it is difficult to ascertain whether these studies were adequately randomized. In addition, no studies described allocation concealment, which may introduce some false "RCTs" in the review, which could mislead the results. We tried to contact the authors for further information about the trials, but we were unable to obtain any additional information. No studies described the method used for blinding, which could lead to performance and detection bias for subjective outcome measures if researchers were aware which patients were in the therapeutic intervention group.

DGP is a disease with complicated symptoms. The gastric emptying test is the gold standard for DGP diagnosis. However, patients that suffer from severe nausea and vomiting are unable to endure the test, which would leave any studies of DGP with a limited number of participants. We suggest that the scope of included patients should be expanded, specifically to include severe gastroparesis patients who could not undergo the gastric emptying test. Vomiting time and duration can be auxiliary indicators that could provide stronger evidence for wider clinical applications.

Gastrointestinal discomfort is the most important clinical characteristic, and improving the symptoms based on the gastric emptying are important for the evaluation of DGP. This is distinct from blood pressure, lipids, and blood sugar, which are expressed by specific numerical indices. This makes the determination of efficacy of gastrointestinal lesions complicated. The improvement of symptoms also needs to be standardized and quantified. The studies included in this review lacked a unified syndrome questionnaire to allow us to evaluate symptomatic variations in the syndrome. The Gastroparesis Cardinal Symptom Index is widely used to evaluate gastrointestinal lesions[43], but none of the included studies used this questionnaire. Thus, it is critical to standardize the evaluation of gastrointestinal lesions, which could help improve the consistency of future studies.

Only two studies[31,37] mentioned adverse effects including nausea, stomachache, headache, diarrhea, and skin allergies. Safety is a serious concern that should be recorded in detail. Thus, definitive conclusions about the safety of XSLJZD still cannot be drawn. No trials reported the loss of participants or used the intention to treat method, which made it difficult to determine whether these studies had attrition bias. Only one trial[34] reported follow up. Diabetic gastrointestinal disease can easily recur, and so long-term follow-up is required for accurate analysis. We tried to avoid language and location bias, but all the included studies were published in China, and so we cannot exclude potential publication bias.

In conclusion, we found that XSLJZD could improve the gastric emptying rate and improve diabetic gastrointestinal symptoms, and could therefore be considered as an alternative method for the treatment of DGP. However, there is no established efficacy determination system for assessing the use of TCM to treat DGP. The long-term efficacy and safety of XSLJZD for the treatment of DGP remains uncertain, because the methodological quality was generally low, and some possible bias existed. The results of our analysis should therefore be read with caution. Therefore, an efficacy determination system for using TCM to treat DGP should be established soon. The accumulation of clinical evidence of severe gastroparesis is increasingly necessary, and future studies should include improved randomization, safety reports, detailed follow-up, and blinded methods to improve their quality.

Well-designed, large-scale, and high-quality randomized controlled clinical trials with scientific rigor are required to provide additional evidence.

COMMENTS

Background

Diabetic gastroparesis can cause stomach discomfort and significantly affect the patient's quality of life. Traditional Chinese Medicine (TCM) has advantages for improving gastric emptying, particularly for the relief of symptoms. However, there are few systematic reviews of the use of TCM to improve diabetic gastroparesis. This study aimed to evaluate the efficacy of TCM Xiangshaliujunzi Decoction (XSLJZD) for the treatment of diabetic gastroparesis, and provide new options for the clinical diagnosis and treatment of diabetic gastroparesis (DGP).

Research frontiers

Research showed that XSLJZD could regulate gastrointestinal motility. Biochemically, XSLJZD also increases the secretion of plasma motilin, lowers serum gastrin levels, and enhances smooth muscle contraction by increasing calcium levels. This study aimed to evaluate XSLJZD for the treatment of gastric emptying disorders and relieving the symptoms of diabetic gastroparesis.

Innovations and breakthroughs

Few previous studies focused on systematic reviews of TCM for the treatment of diabetic gastroparesis. This study evaluated the effects of XSLJZD in diabetic gastroparesis. As the incidence of diabetes increases, the early prevention of diabetes-related complications is particularly important. The current modern medical treatments for diabetic gastroparesis are being explored and improved. This study explored the efficacy of XSLJZD for improving gastric emptying and stomach symptoms in patients with diabetic gastroparesis from the perspective of TCM. It should provide novel ideas and information for the diagnosis and treatment of diabetic gastroparesis.

Applications

Patients with diabetic gastroparesis symptoms are often plagued by stomach discomfort, severe nausea and vomiting, water and electrolyte imbalance, and the loss of nutrients, which can even be life-threatening. In this study, the authors assessed existing clinical studies, which suggest that TCM is a viable alternative treatment method that could improve the symptoms of diabetic gastroparesis. It may also improve the treatment of disease and improve the quality of life for patients.

Terminology

DGP is abnormal gastric retention caused by long-term diabetes. The mechanism of action of TCM in this model may be related to invigorating the spleen to resolve dampness, regulating the stomach, and promote gastric emptying and supplementation to restore Qi.

Peer review

This meta-analysis focuses on the Chinese herbal medicine XSLJZD and its clinical efficacy for the treatment of diabetic gastroparesis. This study evaluates the clinical efficacy of a traditional medicine, and the information obtained from this meta-analysis could enhance our understanding of XSLJZD for the treatment of diabetic gastroparesis.

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META-ANALYSIS

Effects of pentoxifylline on nonalcoholic fatty liver disease: A meta-analysis

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Author contributions: Li YM proposed the study and revised the manuscript; Du J and Ma YY designed study, analyzed the data and wrote the manuscript; Yu CH contributed to the discussion; all the authors have read and approved the final version to be published.

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Abstract

AIM: To evaluate the effects of pentoxifylline therapy in patients with nonalcoholic fatty liver disease (NAFLD).

METHODS: We searched PubMed, Medline, Google Scholar, Embase, Web of Science, the Cochrane Library and the Chinese Biomedicine Database for all relevant controlled trials of pentoxifylline in patients with NAFLD from 1997 to July 2013. Five studies (3 randomized, double-blind, placebo-controlled trials and 2 prospective cohort studies with concurrent controls) were included in this meta-analysis. Statistical analysis was performed using RevMan 5.0 software.

RESULTS: Five randomized trials of 147 patients with NAFLD/nonalcoholic steatohepatitis (NASH) were included. The results showed that compared to placebo, pentoxifylline therapy resulted in a significant decrease in body weight (P = 0.04), alanine aminotransferase (P < 0.00001), aspartate transaminase (P = 0.0006), glucose (P = 0.0008) and tumor necrosis factor-α (P = 0.007), but did not significantly affect body mass index (P = 0.28), total cholesterol (P = 0.80), triglyceride (P = 0.98), alkaline phosphatase (P = 0.29), γ-glutamyl transferase (P = 0.39) and interleukin-6 (P = 0.38). With regard to histological changes, pentoxifylline only reduced the NAFLD activity score (P < 0.00001) and improved lobular inflammation (P < 0.0001). Improvements in steatosis grade (P = 0.11), ballooning (P = 0.10) and fibrosis (P = 0.50) were not obvious.

CONCLUSION: Pentoxifylline therapy results in weight loss, improved liver function and histological changes in patients with NAFLD/NASH. Therefore, pentoxifylline may be a new treatment option for NAFLD.

Key words: Pentoxifylline; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Meta-analysis

Core tip: Recently, more researchers have been attempting to identify new treatments for nonalcoholic fatty liver disease (NAFLD), however, no firm conclusions have been reached. Thus, it is necessary to conduct a meta-analysis to assess the efficacy of pentoxifylline. Our analysis showed that pentoxifylline therapy significantly decreased body weight, alanine aminotransferase, aspartate transaminase, glucose and tumor necrosis factor-α. Pentoxifylline also reduced the NAFLD activity score and improved lobular inflammation in NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a condition of fat accumulation in the liver in the absence of excessive alcohol consumption and any other specific causes of hepatic steatosis[1]. The histological pattern of NAFLD can progress into nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and more rarely, liver carcinoma[2]. NAFLD is now one of the most common liver diseases worldwide. Approximately 20%-30% of patients with NAFLD have histological signs of NASH. The metabolic steps and underlying mechanisms of disease progression remain complex and poorly understood. Diet and lifestyle changes are primary therapies in the management of these patients. For many decades, studies have been aimed at discovering new treatments for NAFLD and many specific treatment strategies have been proposed, such as insulin-sensitizers[3-5], lipid-lowering drugs[6,7], antioxidants[8,9], endocannabinoid receptor antagonists[10], L-carnitine[11] and probiotics[12-14]. However, the current best treatment for the disease is unknown and most of the above treatments are not guideline therapies for NAFLD, because few randomized controlled studies are available. Recently, more attention has been paid to therapeutic strategies that influence the necroinflammatory activity in NAFLD.

Pentoxifylline is a methylxanthine derivative with potent hemorrheologic properties[15] and is commonly used in the treatment of intermittent claudication in western countries[16], based on its effects in enhancing red blood cell flexibility, decreasing blood viscosity, and enhancing aerobic glycolysis and oxygen consumption in ischemic tissues[17]. Furthermore, human and animal studies have shown that pentoxifylline, as a nonspecific phosphodiesterase inhibitor, results in a variety of physiological changes at the cellular level, increases levels of cyclic AMP and decreases tumor necrosis factor (TNF)-α gene transcription[18-20], affecting multiple steps in the cytokine/chemokine pathway. Increased serum TNF-α has been reported in humans and animal models of NAFLD[21,22] and may be important in treating NAFLD.

Therefore, in the present study, we conducted a meta-analysis of pooled data from studies to assess the effects of pentoxifylline on liver function, cytokines and liver histopathology.

MATERIALS AND METHODS

Search strategy

We searched Pubmed, Medline, Google Scholar, Embase, Web of Science, Chinese Biomedicine Database, and the China Journal Full Text Database with no language restriction. The search terms included: (NASH or NAFLD or nonalcoholic steatohepatitis or nonalcoholic fatty liver disease or fatty liver or steatosis) and (pentoxifylline or oxpentifylline or PTX or POF) and (Fatty Liver [MeSH]) AND Pentoxifylline [MeSH]. We also searched the reference lists of each selected study by hand.

Inclusion and exclusion criteria

Inclusion criteria were as follows: (1) adult patients of any sex or ethnic origin with NAFLD/NASH; (2) randomized controlled trials or prospective cohort studies using pentoxifylline; and (3) diagnosis of NASH determined by histology or ultrasonography. Patients with other causes of hepatic steatosis or steatofibrosis, such as alcoholic fatty liver disease, viral hepatitis, autoimmune hepatitis, liver decompensation or malignancy were excluded. The trials should have at least one of the following characteristics: BMI, body weight, alanine aminotransferase (ALT), aspartate transaminase (AST), total cholesterol (TC), triglyceride (TG), alkaline phosphatase (AKP), glucose, TNF-α, interleukin (IL)-6 and histology changes. Studies must have objective outcome measures otherwise they were excluded from this review.

Data extraction and methodological quality

Data were extracted independently by four reviewers and included the following: author, publication year, study sign, population, intervention, duration, outcome, and others. Disagreement was resolved by discussion. Agreement between investigators for selection of studies for the meta-analysis was > 95%. All reviewers assessed the quality of the studies. The randomized controlled trials (RCTs) were all high-quality studies, and were scored using the Jadad scale. Prospective cohort studies were regarded as moderate-quality studies.

Statistical analysis

We analyzed the data using Review Manager 5.0. Dichotomous data were presented as OR with 95%CIs. Statistical heterogeneity was measured using the χ^2 test and the inconsistency index (I^2). $P < 0.05$ was considered to indicate statistically significant heterogeneity. If there was obvious heterogeneity, the random effects model was chosen; otherwise, the fixed effects model was adopted.

RESULTS

We initially identified 183 relevant items in PubMed, Medline, Google Scholar, Embase, Web of Science, Chinese Biomedicine Database and China Journal Full Text Database. Publication dates ranged from 1997 to June 2013. After reviewing each publication, we selected five original studies that met the selection criteria. A flow chart is shown in Figure 1.

Table 1 shows the specific information on study design, methodological quality, sample size, intervention, control method, and duration of treatment and follow-up. Two of the included studies were prospective cohort studies with a concurrent control and the other three were RCTs. All the RCTs were double-blinded and included a follow-up period. All the studies gave detailed baseline information. Three studies used placebo as a control and two studies used ursodeoxycholic acid (UDCA): placebo (68.7%) *vs* UDCA (31.3%). The main characteristics of the patients included in the two groups

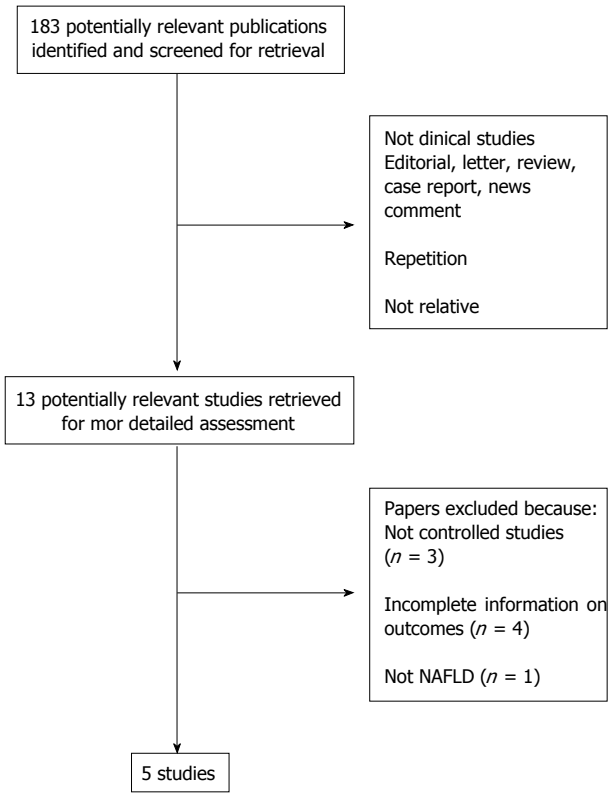


Figure 1 Selection of studies.

were well matched in all studies.

Two studies[23,25] evaluated changes in BMI after pentoxifylline treatment or placebo and showed no significant difference [weighted mean difference (WMD) 1.43, 95%CI: -1.19 to 4.05, $P = 0.28$]. The included studies were homogeneous ($I^2 = 0\%$, $P = 0.32$, Figure 2A).

Two studies[23,27] assessed the reduction of body weight in the experimental group and control group. The results showed a statistically significant difference between the experimental and control groups (WMD: -1.1, 95%CI: -2.16 to -0.05, $P = 0.04$). The included studies were homogeneous ($I^2 = 0\%$, $P = 0.44$) (Figure 2A).

Four studies[23-25,27] reported the effect of pentoxifylline on serum ALT reduction, however, compared with the control group, this reduction was not significantly different in the experimental group (WMD: -7.16, 95%CI: -19.67 to 5.34, $P = 0.26$). Significant heterogeneity among the studies was observed ($I^2 = 64\%$, $P = 0.04$, Figure 2B). Subgroup analyses were performed in order to evaluate the effect of the different controls. Two studies used placebo as the control and pentoxifylline was found to have a significantly better effect on reducing ALT (WMD: -13.64, 95%CI: -19.61 to -7.66, $P < 0.00001$). The studies were homogeneous ($I^2 = 0\%$, $P = 0.42$, Figure 2B). The other two studies used UDCA and the data were not significantly different (WMD: 7.51, 95%CI: -19.36 to 34.38, $P = 0.58$). The studies were homogeneous ($I^2 = 49\%$, $P = 0.58$, Figure 2B).

Three studies[23,24,27] assessed the effect of pentoxifylline on the level of serum AST and showed a significant difference in the treated group compared with the placebo group (WMD: -9.70, 95%CI: -15.24 to -4.16, $P = 0.0006$). The included studies were homogeneous ($I^2 = 0\%$, $P = 0.66$, Figure 2B).

Three studies[23-25] analyzed TC and TG in NAFLD/NASH patients treated with pentoxifylline compared with placebo, and two studies analyzed AKP and γ-glutamyl transferase (GGT). Pentoxifylline had no effect on normalizing TC (WMD: 0.26, 95%CI: -0.30 to 0.83, $P = 0.36$); TG (WMD: -0.07, 95%CI: -0.47 to 0.33, $P = 0.73$); AKP (WMD: -20.87, 95%CI: -59.33 to 17.59, $P = 0.29$); and GGT (WMD: -5.2, 95%CI: -17.05 to 6.64, $P = 0.39$). The included studies in all four analyses were homogeneous (TC: $I^2 = 0\%$, $P = 0.42$; TG: $I^2 = 0\%$, $P = 0.49$; AKP: $I^2 = 0\%$, $P = 0.96$; GGT: $I^2 = 0\%$, $P = 0.81$) (Figure 2B).

Three studies[24,25,27] reported the effect of pentoxifylline on serum glucose. Pentoxifylline had a significantly better effect on decreasing serum glucose (WMD: -8.27, 95%CI: -14.28 to -2.25, $P = 0.007$). The included studies were all homogeneous ($I^2 = 55\%$, $P = 0.11$) (Figure 2B).

Four[23,24,26,27] and three[23,24,27] studies, respectively, analyzed the cytokines: TNF-α and IL-6. Pentoxifylline significantly reduced TNF-α (WMD: -0.66, 95%CI: -1.14 to -0.18, $P = 0.007$) but not IL-6 (WMD: 1.35, 95%CI: -5.75 to 8.44, $P = 0.71$). Homogeneity among the studies was observed for TNF-α, but not for IL-6 (TNF-α: $I^2 = 11\%$, $P = 0.34$; IL-6: $I^2 = 69\%$, $P = 0.04$) (Figure 2C).

Three studies[25-27] provided sufficient data to compare the effects of pentoxifylline with placebo and showed a significant decrease in the NAFLD activity score (NAS) after treatment with pentoxifylline (WMD: -1.16, 95%CI: -1.51 to -0.81, $P < 0.00001$). Homogeneity among studies was observed ($I^2 = 10\%$, $P = 0.33$, Figure 2D).

Two studies[26,27] evaluated steatosis grade, lobular inflammation, ballooning and fibrosis before and after treatment. A significant improvement in lobular inflammation and ballooning was observed after treatment with pentoxifylline compared with placebo (steatosis grade: WMD: -0.49, 95%CI: -1.09 to 0.11, $P = 0.11$; lobular inflammation: WMD: -0.43, 95%CI: -0.64 to -0.23, $P < 0.0001$; ballooning: WMD: -0.32, 95%CI: -0.71 to 0.06, $P = 0.10$; fibrosis: WMD: -0.24, 95%CI: -0.92 to 0.45, $P = 0.50$). Significant heterogeneity was observed, with the exception of lobular inflammation (steatosis grade: $I^2 = 88\%$, $P = 0.004$; lobular inflammation: $I^2 = 0\%$, $P = 0.58$; ballooning: $I^2 = 78\%$, $P = 0.03$; Fibrosis: $I^2 = 85\%$, $P = 0.01$) (Figure 2D).

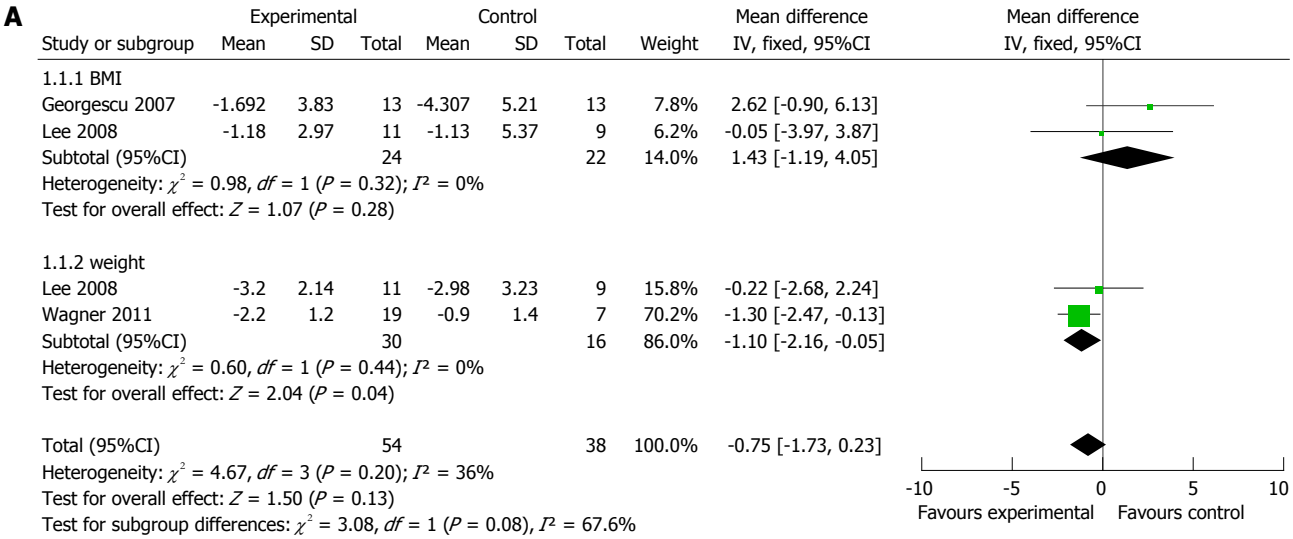
DISCUSSION

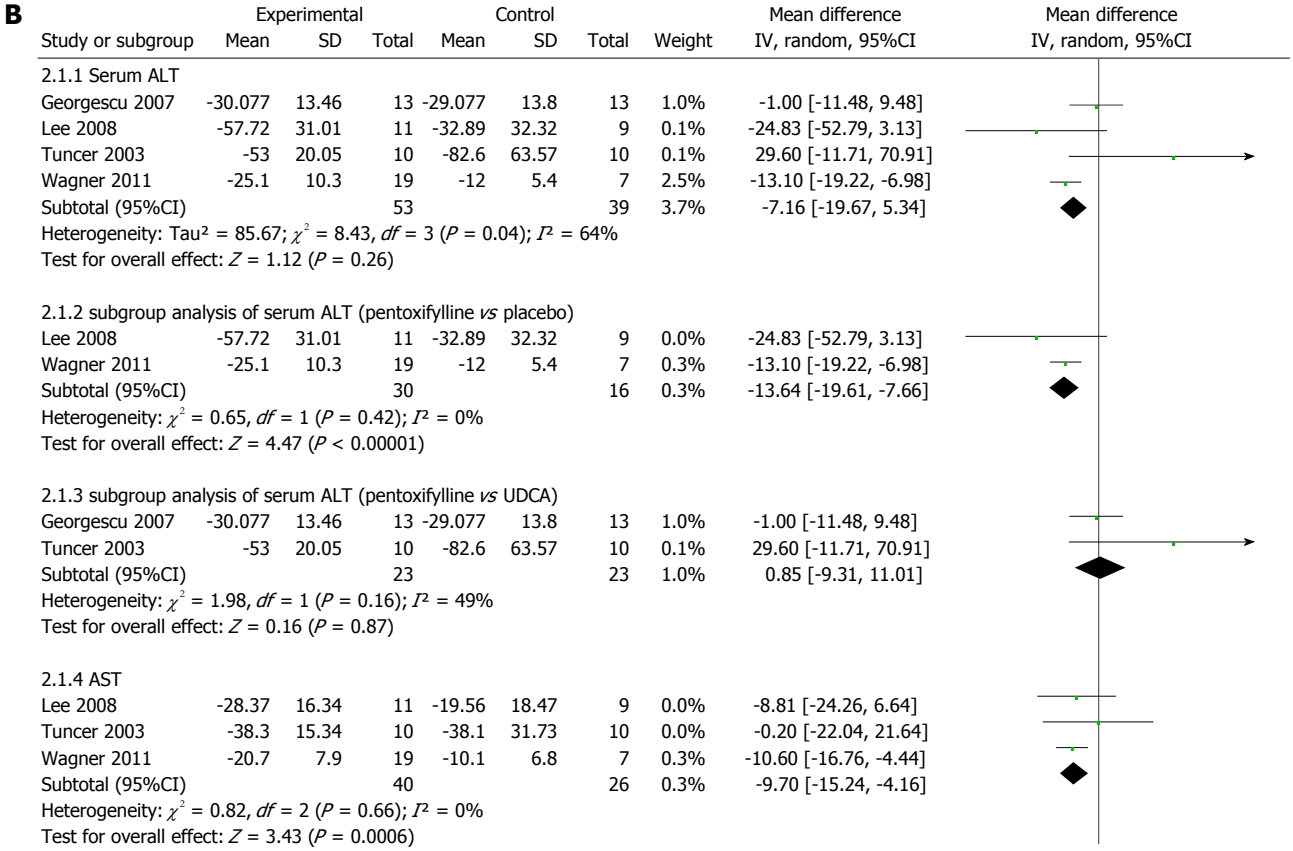
NAFLD is common and occurs in persons of all ages and ethnic groups, and is recognized as a major health issue. NAFLD is closely associated with obesity and insulin resistance, and is now recognized to represent the hepatic manifestation of the metabolic syndrome[28]. At present, there is no registered drug for the treatment of NAFLD. Although lifestyle intervention is often advocated[29,30], it is difficult to maintain. Socha *et al*[31] conducted a meta-

Table 1 Methodological characteristics of the included studies in this meta-analysis

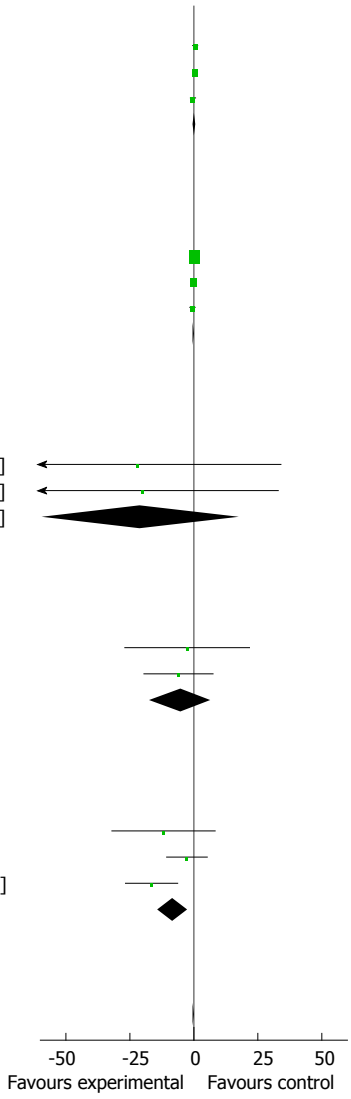
Ref.	Methodological quality	Sample size	Intervention	Control	Duration	Follow-up
Lee *et al*[23]	RCT	20 (11/9)	Pentoxifylline (1200 mg/d) plus low-calorie diet and exercise	Placebo plus low-calorie diet and daily exercise	12 wk	Yes
Tuncer *et al*[24]	Prospective cohort study with concurrent control	20 (10/10)	Pentoxifylline (20 mg/kg per day)	UDCA	24 wk	Yes
Georgescu *et al*[25]	Prospective cohort study with concurrent control	26 (13/13)	Pentoxifylline (800 mg/d)	UDCA	30 wk	Yes
Zein *et al*[26]	RCT	55 (26/29)	Pentoxifylline (1200 mg/d)	Placebo	12 mo	Yes
Van Wagner *et al*[27]	RCT	26 (19/7)	Pentoxifylline (1200 mg/d)	Placebo	12 mo	Yes

RCT: Randomized controlled trial; UDCA: Ursodeoxycholic acid.





2.1.5 TC								
Georgescu 2007	-0.631	1.378	13	-1.146	1.333	13	9.8%	0.51 [-0.53, 1.56]
Lee 2008	0.34	1.1	11	-0.15	0.86	9	14.4%	0.49 [-0.37, 1.35]
Tuncer 2003	0.039	1.25	10	0.385	1.181	10	9.3%	-0.35 [-1.41, 0.72]
Subtotal (95%CI)			34			32	33.5%	0.26 [-0.30, 0.83]
Heterogeneity: χ^2 = 1.75, *df* = 2 (*P* = 0.42); I^2 = 0%								
Test for overall effect: *Z* = 0.92 (*P* = 0.36)								
2.1.6 TG								
Georgescu 2007	-0.046	0.321	13	-0.169	0.92	13	37.8%	0.12 [-0.41, 0.65]
Lee 2008	-0.4	1.13	11	-0.16	0.57	9	18.2%	-0.24 [-1.00, 0.52]
Tuncer 2003	-0.869	0.79	10	-0.325	1.568	10	9.0%	-0.54 [-1.63, 0.54]
Subtotal (95%CI)			34			32	65.0%	-0.07 [-0.47, 0.33]
Heterogeneity: χ^2 = 1.43, *df* = 2 (*P* = 0.49); I^2 = 0%								
Test for overall effect: *Z* = 0.34 (*P* = 0.73)								
2.1.7 AKP								
Georgescu 2007	-79.538	67.64	13	-57.692	77.43	13	0.0%	-21.85 [-77.73, 34.04]
Tuncer 2003	-49	51.88	10	-29	67.99	10	0.0%	-20.00 [-73.01, 33.01]
Subtotal (95%CI)			23			23	0.0%	-20.87 [-59.33, 17.59]
Heterogeneity: χ^2 = 0.00, *df* = 1 (*P* = 0.96); I^2 = 0%								
Test for overall effect: *Z* = 1.06 (*P* = 0.29)								
2.1.8 GGT								
Georgescu 2007	-23.308	35.57	13	-20.693	27.4	13	0.0%	-2.61 [-27.02, 21.79]
Tuncer 2003	-54.8	7.48	10	-48.8	20.53	10	0.1%	-6.00 [-19.54, 7.54]
Subtotal (95%CI)			23			23	0.1%	-5.20 [-17.05, 6.64]
Heterogeneity: χ^2 = 0.06, *df* = 1 (*P* = 0.81); I^2 = 0%								
Test for overall effect: *Z* = 0.86 (*P* = 0.39)								
2.1.9 GLU								
Georgescu 2007	-15.231	23.55	13	-3.385	28.78	13	0.0%	-11.85 [-32.06, 8.37]
Tuncer 2003	-4.4	9.13	10	-1.7	9.1	10	0.2%	-2.70 [-10.69, 5.29]
Wagner 2011	-5.2	4.9	19	11.3	13.5	7	0.1%	-16.50 [-26.74, -6.26]
Subtotal (95%CI)			42			30	0.3%	-8.27 [-14.28, 2.25]
Heterogeneity: χ^2 = 4.47, *df* = 2 (*P* = 0.11); I^2 = 55%								
Test for overall effect: *Z* = 2.69 (*P* = 0.007)								
Total (95%CI)			302			244	100.%	-0.10 [-0.43, 0.23]
Heterogeneity: χ^2 = 75.33, *df* = 23 (*P* < 0.00001); I^2 = 69%								
Test for overall effect: *Z* = 0.60 (*P* = 0.55)								
Test for subgroup differences: χ^2 = 55.75, *df* = 8 (*P* < 0.00001), I^2 = 85.6%								



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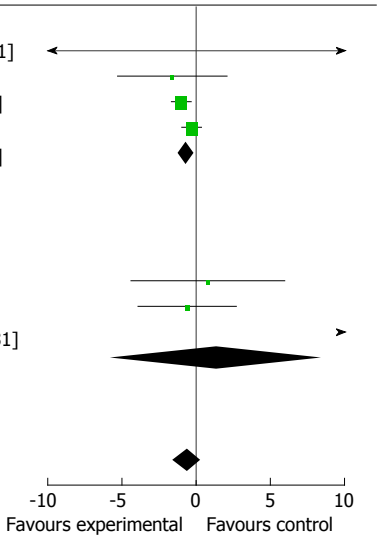
Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, fixed, 95%CI
3.1.1 TNF-α								
Lee 2008	-10.86	15.68	10	-31.35	54.54	8	0.0%	20.49 [-18.53, 59.51]
Tuncer 2003	-3.3	4.89	10	-1.7	3.43	10	1.6%	-1.60 [-5.30, 2.10]
Wagner 2011	-1.179	1.095	19	-0.183	0.644	7	47.6%	-1.00 [-1.68, -0.31]
Zein 2011	-0.1	0.9	23	0.2	1.5	26	47.9%	-0.30 [-0.98, 0.38]
Subtotal (95%CI)			62			51	97.1%	-0.66 [-1.14, -0.18]
Heterogeneity: χ^2 = 3.36, *df* = 3 (*P* = 0.34); I^2 = 11%								
Test for overall effect: *Z* = 2.69 (*P* = 0.007)								
3.1.2 IL-6								
Lee 2008	-7.47	6.81	10	-8.26	4.36	8	3.2%	0.79 [-4.40, 5.98]
Tuncer 2003	-0.5	4.77	10	0.1	2.46	10	7.1%	-0.60 [-3.93, 2.73]
Wagner 2011	-22.1	71	19	-82.9	47.5	7	0.0%	60.80 [13.29, 108.31]
Subtotal (95%CI)			39			25	10.3%	1.35 [-5.75, 8.44]
Heterogeneity: Tau² = 21.99; χ^2 = 6.50, *df* = 2 (*P* = 0.04); I^2 = 69%								
Test for overall effect: *Z* = 0.37 (*P* = 0.71)								
Total (95%CI)			101			76	100.%	-0.64 [-1.57, 0.33]
Heterogeneity: Tau² = 0.44; χ^2 = 10.09, *df* = 6 (*P* = 0.12); I^2 = 41%								
Test for overall effect: *Z* = 1.28 (*P* = 0.20)								



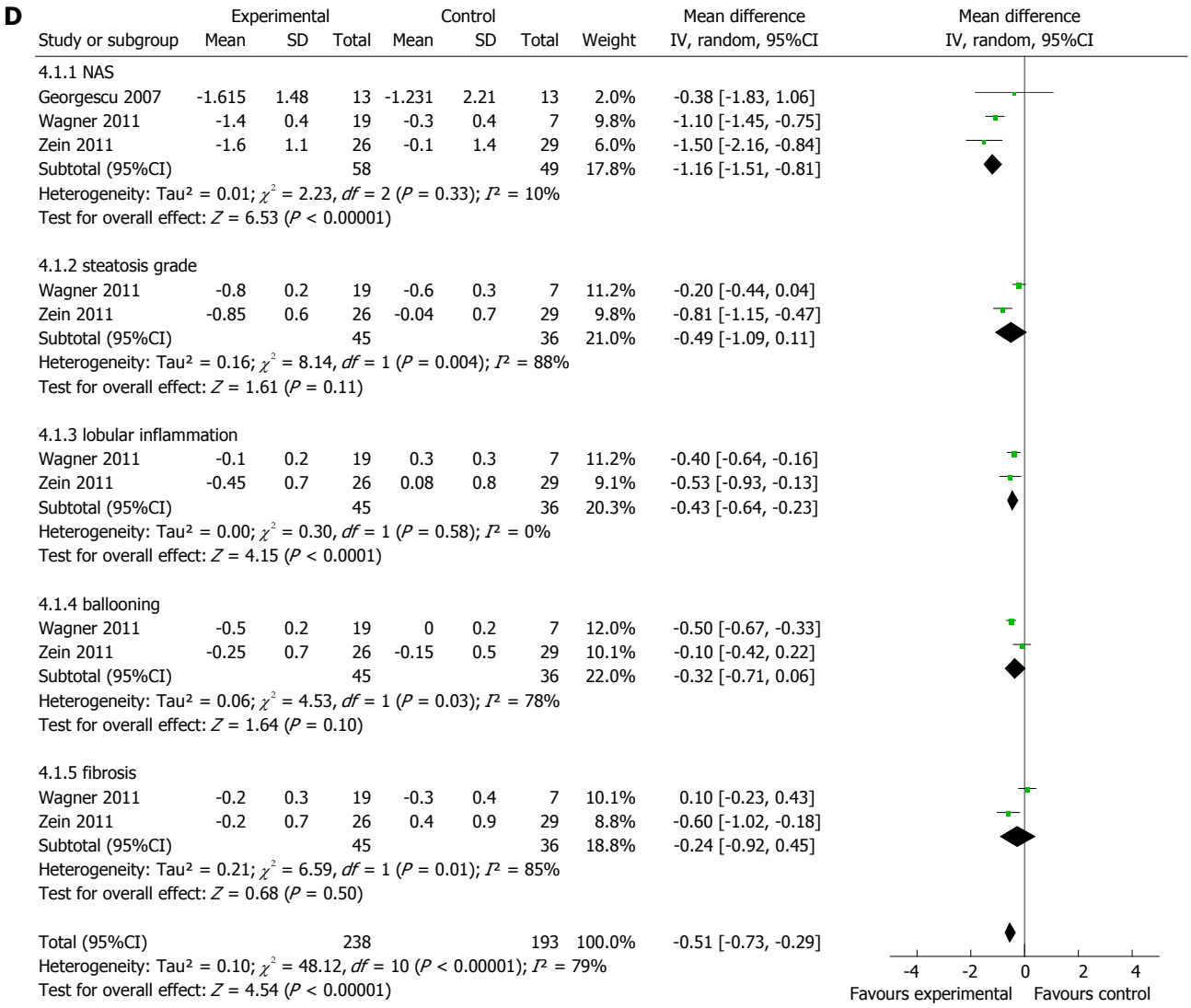


Figure 2 Forest plot of the effects of probiotics in patients with nonalcoholic fatty liver disease. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; UDCA: Ursodeoxycholic acid; TC: Total cholesterol; TG: Triglyceride; AKP: Alkaline phosphatase; GGT: γ-glutamyl transferase; GLU: Glucose; TNF-α: Tumor necrosis factor-α; IL: Interleukin; NAS: NAFLD activity score.

analysis on pharmacological interventions for NAFLD in adults and in children, including pioglitazone, vitamin E, UDCA, probucol, *N*-acetylcysteine, low doses of carnitine, as well as pentoxifylline. However, no firm conclusions on the efficacy of the various treatments for NAFLD have been drawn.

In 2011, a systematic review on pentoxifylline in NASH[32] was performed that found that pentoxifylline reduces AST and ALT levels and may improve liver histological scores in patients with NAFLD/NASH, but it does not appear to affect cytokines and histological improvement. The review only compared the post-treatment indexes, ignoring the differences from baseline. Moreover, the control group contained not only placebo, but also UDCA, which may have influenced the conclusion. Thus, it was necessary to conduct the present meta-analysis.

Oxidative stress and cytokine production play a vital role in the progression of NAFLD[33]. TNF-α is recognized to promote inflammatory, apoptotic and fibrogenic reactions in the development of NAFLD[21], and a large number of studies have shown that TNF-α is associated with the pathogenesis of NAFLD. Furthermore, TNF-α is an important cytokine that regulates insulin resistance in humans[34] by interfering with the insulin signaling transduction pathway[35,36]. Liver biopsy is currently considered the gold standard for the diagnosis of NAFLD/NASH. Lobular inflammation is one of the histological features of this disease, which is composed of lymphocytes, neutrophils and macrophages. The NAS is the sum of the scores of steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2) and ranges from 0 to 8, with the majority of patients with NASH having a NAS $\geqslant$ 5. The NAS is highly correlated with aminotransferase levels, commonly assumed to be markers of liver disease severity[37]. Our meta-analysis showed that pentoxifylline therapy significantly decreased body weight, lowered serum ALT, AST, glucose and TNF-α, and improved NAS and lobular inflammation on histological examination. Furthermore, pentoxifylline did not influence serum TC, TG, AKP and GGT. Thus, pentoxifylline may represent a novel therapeutic target in NASH by reducing acute inflammatory damage in the liver.

Selection and publication bias should be considered in this study, as two of the studies included were prospective cohort studies with a concurrent control and the other three were RCTs. In these studies, pentoxifylline was administered within the dose range of 800 to 1200 mg/d, and only Lee's study administered pentoxifylline with a low-calorie diet and exercise. The duration of treatment ranged from 12 wk to 12 mo. Given the use of different dosages and durations of treatment, the studies were difficult to reconcile. The diagnosis of NAFLD/NASH was confirmed by percutaneous liver biopsy in all studies, except for some patients who only underwent ultrasonography for diagnosis in Tuncer's research. Although ultrasonography is reasonably accurate, it cannot identify fatty infiltration of the liver below a threshold of 30%. Unfortunately, only three studies had post-treatment histology results.

In our meta-analysis, two studies used UDCA as the control treatment. UDCA is a naturally occurring bile acid with multiple hepatoprotective activities. It was first demonstrated to improve ALT, AST, AKP and GGT in an open-label pilot study compared with clofibrate[38]. However, the results were found to be controversial in later research[39-41]. UDCA is now recommended to improve liver biochemical tests in patients with a wide range of chronic liver and hepatobiliary diseases. In the present meta-analysis of TC, TG, AKP and GGT, there was no significant difference between the pentoxifylline and UDCA groups. Therefore, the effect of pentoxifylline on improving abnormal liver function may be similar to UDCA.

There were other limitations in this review. Pentoxifylline was administered with a low-calorie diet and exercise in one study[23], and the researchers ignored the effects of the dietary restriction and exercise/physical activities as they were not described. The sample sizes in some trials, as well as the number of trials for some comparisons, were small.

COMMENTS

Background

The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide. The metabolic steps and underlying mechanisms of disease progression remain complex and poorly understood. Diet and lifestyle changes are primary therapies in the management of NAFLD patients. Human and animal studies have shown that pentoxifylline, which is a nonspecific phosphodiesterase inhibitor, results in a variety of physiological changes and affects multiple steps in the cytokine/chemokine pathway, and may be important in treating NAFLD. Thus, it was necessary to conduct a meta-analysis to assess the effects of pentoxifylline on physiological indicators, liver function and histological changes in NAFLD patients.

Research frontiers

Pentoxifylline is a methylxanthine derivative with potent hemorrheologic properties and is commonly used in the treatment of intermittent claudication in western countries. However, recent human and animal studies have indicated that pentoxifylline may have an influence on a variety of physiological changes at the cellular level, cyclic AMP and tumor necrosis factor (TNF)-α gene transcription, affecting multiple steps in the cytokine/chemokine pathway, which are all related to the mechanism of NAFLD. Whether treatment with pentoxifylline is effective in patients with NAFLD has therefore become a research hotspot.

Innovations and breakthroughs

In 2009, a meta-analysis was conducted on pharmacological interventions for NAFLD in adults and children. However, no firm conclusions could be drawn. In 2011, a systematic review on pentoxifylline in nonalcoholic steatohepatitis (NASH) was performed, but it did not appear to affect cytokines or histology. The control group contained placebo and ursodeoxycholic acid and had no subgroup analysis, which may have influenced the conclusions. Thus, a meta-analysis was necessary.

Applications

Use of pentoxifylline can reduce body weight, serum alanine aminotransferase, aspartate transaminase, glucose and TNF-α. With regard to histological changes, pentoxifylline only reduced the NAFLD activity score and improved lobular inflammation. Thus, pentoxifylline may represent a new method for treating or preventing NAFLD.

Terminology

NAFLD is characterized by large vacuoles of triglyceride that accumulate in liver cells *via* the process of steatosis in non-alcohol users. NAFLD can progress into NASH, liver fibrosis, cirrhosis, and more rarely, liver carcinoma. Pentoxifylline is a nonspecific phosphodiesterase inhibitor and can cause a variety of physiological changes at the cellular level, affect the levels of cyclic AMP and decrease *TNF-α* gene transcription, which are all important mechanisms in the progression of NAFLD.

Peer review

This meta-analysis is interesting and it extends the present knowledge on pentoxifylline and NAFLD. However, the authors need to improve some aspects of the manuscript. The most important section of the manuscript, the results section, is too confusing and difficult to read. The figures are confusing. The authors could reduce the number of figures and include two or three figures with all the variables. Also, there needs to be significant editing on the grammar and spelling.

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META-ANALYSIS

Fast-track program *vs* traditional care in surgery for gastric cancer

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Abstract

AIM: To systematically review the evidence for the effectiveness of fast-track program *vs* traditional care in laparoscopic or open surgery for gastric cancer.

METHODS: PubMed, Embase and the Cochrane library databases were electronically searched for published studies between January 1995 and April 2013, and only randomized trials were included. The references of relevant studies were manually searched for further studies that may have been missed. Search terms included "gastric cancer", "fast track" and "enhanced recovery". Five outcome variables were considered most suitable for analysis: postoperative hospital stay, medical cost, duration to first flatus, C-reactive protein (CRP) level and complications. Postoperative hospital stay was calculated from the date of operation to the date of discharge. Fixed effects model was used for meta-analysis.

RESULTS: Compared with traditional care, fast-track program could significantly decrease the postoperative hospital stay [weighted mean difference (WMD) = -1.19, 95%CI: -1.79--0.60, P = 0.0001, fixed model], duration to first flatus (WMD = -6.82, 95%CI: -11.51--2.13, P = 0.004), medical costs (WMD = -2590, 95%CI: -4054--1126, P = 0.001), and the level of CRP (WMD = -17.78, 95%CI: -32.22--3.35, P = 0.0001) in laparoscopic surgery for gastric cancer. In open surgery for gastric cancer, fast-track program could also significantly decrease the postoperative hospital stay (WMD = -1.99, 95%CI: -2.09--1.89, P = 0.0001), duration to first flatus (WMD = -12.0, 95%CI: -18.89--5.11, P = 0.001), medical cost (WMD = -3674, 95%CI: -5025--2323, P = 0.0001), and the level of CRP (WMD = -27.34, 95%CI: -35.42--19.26, P = 0.0001). Furthermore, fast-track program did not significantly increase the incidence of complication (RR = 1.39, 95%CI: 0.77-2.51, P = 0.27, for laparoscopic surgery; and RR = 1.52, 95%CI: 0.90-2.56, P = 0.12, for open surgery).

CONCLUSION: Our overall results suggested that compared with traditional care, fast-track program could result in shorter postoperative hospital stay, less medical costs, and lower level of CRP, with no more complications occurring in both laparoscopic and open surgery for gastric cancer.

Key words: Fast-track program; Traditional care; Gastric cancer; Meta-analysis; Laparoscopic and open surgery

Core tip: Our overall results suggested that compared with traditional care, fast-track program could result in shorter postoperative hospital stay, less medical cost, and lower level of C-reactive protein, with no more complications occurring in both laparoscopic and open surgery for gastric cancer.

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INTRODUCTION

China, Japan, South America, Eastern Europe and parts of the Middle East are reported with the highest incidence of gastric cancer[1]. Over the past 20 years, there have been two important developments in elective major abdominal surgery; the introduction of laparoscopic surgery and the implementation of an enhanced recovery after surgery program, also referred to as "fast track" (FT) perioperative care, both focusing on accelerated recovery resulting in shorter hospital stay.

Laparoscopic surgery has definite advantages and has been used widely since its advent. And it is well known to be associated with less postoperative pain than open surgery and postoperative pain can be controlled without opioids[2]. In recent years, the advantages of laparoscopic surgery have been recognized in gastric cancer[3-6].

FT surgery is an integrated application of various medical interventions that can enhance recovery after surgery. The FT perioperative care, or the enhanced recovery program after surgery, initiated by Bardram *et al*[7] in 1995, consists of a multidisciplinary approach, including preoperative counseling, no bowel preparation, perioperative high oxygen concentrations, active prevention of hypothermia, and no routine use of nasogastric tubes or drains. In recent years, FT surgery has been successfully applied to general[8], urological[9], cardiovascular[10], gynecological[11], orthopedic[12] and thoracic surgery[13].

This study aims to systematically review the evidence for the effectiveness of FT program *vs* traditional care in surgery for gastric cancer.

MATERIALS AND METHODS

Search and selection strategies

PubMed, Embase and the Cochrane library databases were electronically searched for published studies between January 1995 and April 2013. The references of relevant studies were manually searched for further studies that may have been missed. Search terms included "gastric cancer," "FT" and "enhanced recovery". No language restriction was applied.

Inclusion and exclusion criteria

Randomized controlled trials (RCTs) comparing FT program with traditional care in adult patients (aged > 18 years) undergoing laparoscopic or open surgery for gastric cancer were eligible for inclusion. Excluded studies (1) were not RCTs (such as nonrandomized, quasi-randomized, pseudorandomized, or controlled clinical trials or cohort or retrospective studies); (2) had no documentation of individual items of the FT programs; or (3) had no data available for the present meta-analysis.

Methods of review

Each article was critically reviewed by two researchers independently using the double-extraction method for eligibility. Any conflict was resolved before final analysis. Five outcome variables were considered most suitable for analysis: postoperative hospital stay, medical costs, duration to first flatus, C-reactive protein (CRP) level, and complications. Postoperative hospital stay was calculated from the date of operation to the date of discharge. The quality of the RCTs was assessed with the Jadad scoring system by two authors[14].

Statistical analysis

Weighted mean differences (WMDs) and their 95%CIs were used for analyzing continuous variables presented in the same scale (postoperative hospital stay, medical costs, duration to first flatus, and CRP level). Data reported as medians and ranges or medians and interquartile ranges were converted to means and standard deviation (SD)[15]. We calculated the lower and upper ends of the range by multiplying the difference between the median and upper and lower ends of the interquartile range by 2 and adding or subtracting the product from the median[16]. For dichotomous data (complications), relative risk (RR) with 95%CI was calculated. The effect measures were pooled using the fixed-effects model. Level of statistical significance was set at $P < 0.05$. Heterogeneity was quantified by calculating I^2 where $P < 0.10$ was deemed significant. Publication bias was not evaluated by a funnel plot, because the number of included trials in the present review was limited. All statistical analyses were executed using STATA version 11. Some outcomes were not analyzed but presented in a descriptive way.

RESULTS

According to the searching strategy, three trials were included in our study. We divided the Chen's study into two comparisons, *i.e.*, FT program *vs* conventional care in laparoscopic surgery (Chen 2012), and FT program *vs* conventional care in open surgery [Chen 2012 (2)]. Two trials evaluated the effectiveness of FT program *vs* traditional care in laparoscopic surgery for gastric cancer[17,18], while two trials assessed the effectiveness of FT program *vs* traditional care in open surgery for gastric cancer[17,19]. The sample size was small in all trials, ranging from 41 to 92. All studies were conducted in Asia, including China and Korea. Detailed characteristics of each trial are given in Table 1.

Methodological assessment

No trials described the detailed methods of randomization, and allocation concealment was not performed in all trials. The incidence of withdrawal and dropouts was low, and the reasons were clearly reported. Blinding design was not applied in any trial. The methodological assessment by Jadad scale suggested that all trials were considered to be of moderate risk of bias.

FT program in laparoscopic surgery for gastric cancer

In 2012, Chen *et al*[17] reported one RCT that evaluated the safety and effectiveness of FT program combined with

Table 1 Main characteristics of included trials

Ref.	Location	Sample size	Age (yr)	BMI (kg/m^2)	Intervention group	Control group	Follow-up (wk)	Jadad score
Chen *et al*[17]	China	19/22	59/63	22.9/22.9	FT + LADG	LADG	4	2
Kim *et al*[18]	South Korea	22/22	53/57	23.4/23.8	FT + LADG	LADG	2	2
Chen *et al*[17]	China	21/20	64/64	23.5/23.5	FT + ODG	ODG	4	2
Wang *et al*[19]	China	45/47	59/57	23.8/23.2	FT + OG	OG	4	2

BMI: Body mass index; FT: Fast-track.

Table 2 Meta-analysis results

Gastric cancer	WMD/RR (95%CI)	*P* value	Heterogeneity	
			χ^2 (*P* value)	I^2 (%)
Outcomes for laparoscopy surgery				
Postoperative hospital stay	WMD -1.19 (-1.79--0.60)	0.000	10.60 (0.001)	90.6
Medical cost	WMD -2590 (-4054--1126)	0.001	0.13 (0.72)	0.0
CRP	WMD -17.78 (-32.22--3.35)	0.016	0.51 (0.48)	0.0
Duration to first flatus	WMD -6.82 (-11.51--2.13)	0.004	0.29 (0.59)	0.0
Complication	RR 1.39 (0.77-2.51)	0.270	1.22 (0.27)	18.2
Outcomes for open surgery				
Postoperative hospital stay	WMD -1.99 (-2.09--1.89)	0.000	2.44 (0.12)	59.1
Medical cost	WMD -3674 (-5025--2323)	0.000	2.21 (0.14)	54.7
CRP	WMD -27.34 (-35.42--19.26)	0.000	5.10 (0.02)	80.4
Duration to first flatus	WMD -12.0 (-18.89--5.11)	0.001	-	-
Complication	RR 1.52 (0.90-2.56)	0.120	0.16 (0.69)	0.0

WMD: Weighted mean difference; CRP: C-reactive protein.

laparoscopy-assisted radical distal gastrectomy for gastric cancer. They found that combination of FT with laparoscopy in gastric cancer is safe, feasible, and efficient and can improve nutritional status, lessen postoperative stress, and accelerate postoperative rehabilitation. Meanwhile, Kim and colleagues published another trial which also evaluated the safety and efficacy of FT in laparoscopic distal gastrectomy. They also found that FT surgery could enhance postoperative recovery and improve immediate postoperative quality of life, and was safe in laparoscopic distal gastrectomy. We made a meta-analysis for the following outcomes in these two trials.

Our overall results showed that compared with traditional care, FT program could significantly decrease the postoperative hospital stay (WMD = -1.19, 95%CI: -1.79--0.60, *P* = 0.0001, Figure 1A), duration to first flatus (WMD = -6.82, 95%CI: -11.51--2.13, *P* = 0.004, Figure 1B), medical cost (WMD = -2590, 95% CI: -4054--1126, *P* = 0.001, Figure 1C), and the level of CRP (WMD = -17.78, 95%CI: -32.22--3.35, *P* = 0.0001, Figure 1D) in laparoscopic surgery for gastric cancer. Furthermore, FT did not significantly increase the incidence of complications (RR = 1.39, 95%CI: 0.77-2.51, *P* = 0.27, Figure 1E). Additionally, there was no significant difference between the two groups for length of operative time and intraoperative blood loss. Most of the heterogeneity tests for those outcomes did not detect significant heterogeneity, which is detailed in Table 2.

FT program in open surgery for gastric cancer

In 2010, Wang *et al*[19] reported a trial which evaluated the feasibility and safety of FT program in patients with gastric cancer during the perioperative period. They suggested that FT open surgery could lessen postoperative stress reactions and accelerate rehabilitation in patients with gastric cancer. In 2012, Chen and colleagues also found that compared with conventional care in open distal gastrectomy, FT program could reduce postoperative stress and accelerate postoperative rehabilitation.

Our overall results found that compared with traditional care, FT program could significantly decrease the postoperative hospital stay (WMD = -1.99, 95%CI: -2.09--1.89, *P* = 0.0001, Figure 1A), duration to first flatus (WMD = -12.0, 95%CI: -18.89--5.11, *P* = 0.001, Figure 1B), medical cost (WMD = -3674, 95%CI: -5025--2323, *P* = 0.0001, Figure 1C), and the level of CRP (WMD = -27.34, 95%CI: -35.42--19.26, *P* = 0.0001, Figure 1D) in open surgery for gastric cancer. Furthermore, FT program did not increase the incidence of complications (RR = 1.52, 95%CI: 0.90-2.56, *P* = 0.12, Figure 1E). Additionally, there was no significant difference between the two groups for operative time and intraoperative blood loss. Most of the heterogeneity tests for those outcomes did not detect significant heterogeneity, which is detailed in Table 2.

DISCUSSION

There have been a lot of studies and systematic reviews which evaluated the safety, feasibility, efficacy of FT program in colorectal surgery, and they found that compared with traditional care, FT program is safe and effective, justifying perioperative care in colorectal surgery[8,20-26]. However, until recently, only several trials evaluated the feasibility of FT program in surgery for gastric cancer.

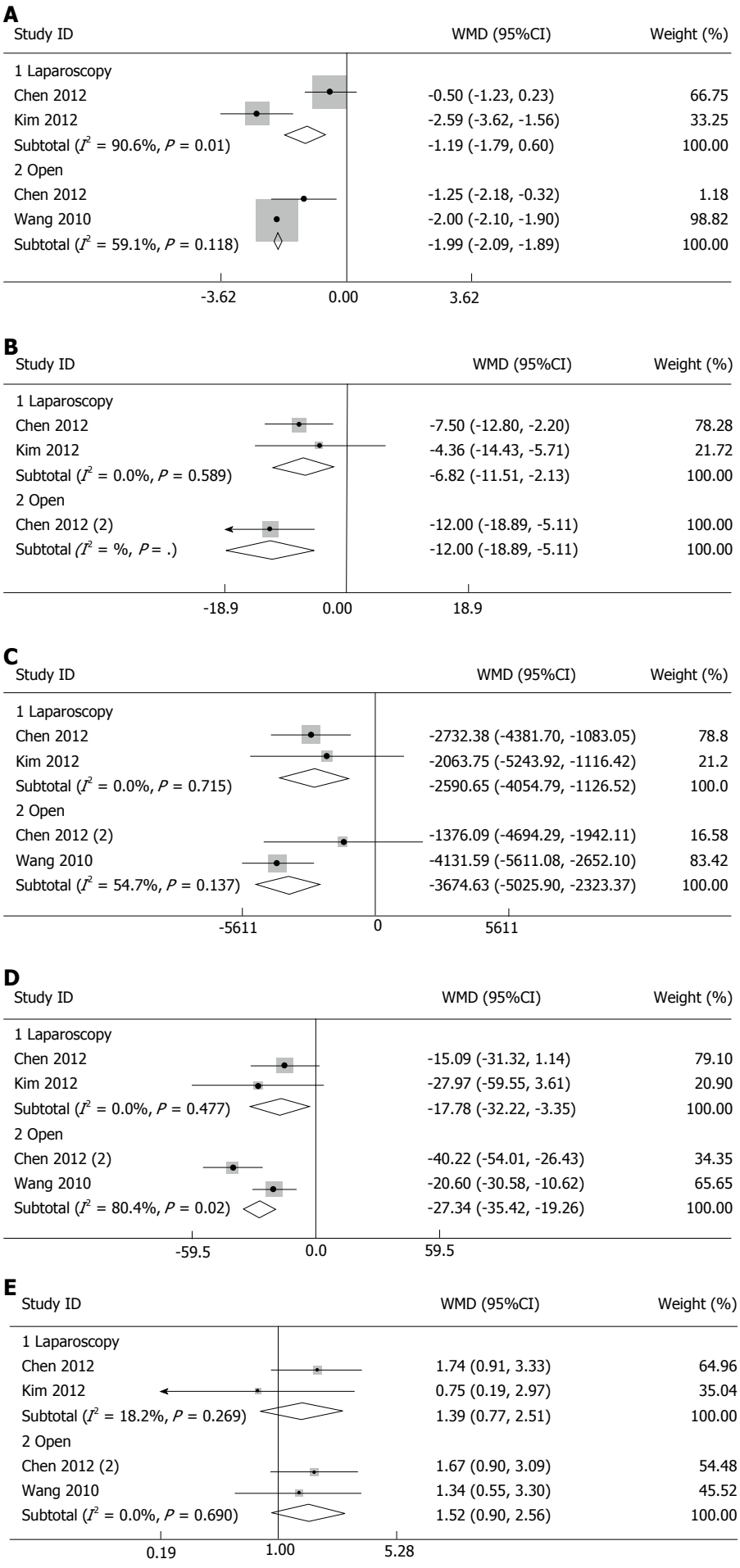

Figure 1 Meta-analysis. A: Postoperative hospital day; B: Duration to first flatus; C: Medical cost; D: Level of C-reactive protein; E: Complications.

Our present work is to systematically review the evidence for the effectiveness of FT program *vs* traditional care in laparoscopic or open surgery for gastric cancer. Our results showed that compared with traditional care, FT program resulted in more rapid postoperative recovery, less medical cost, and earlier discharge from hospital, with no more complications occurring.

There was significant heterogeneity for the outcome of postoperative hospital stay between the two laparoscopy trials. FT program did not decrease the postoperative hospital stay in Chen's study. In contrast, Kim's study suggested that FT could significantly decrease the postoperative hospital stay. Causes of the heterogeneity may result from the differences in population, surgeon, sampling, FT program or traditional care. However, we could not be sure which factor was the main source of heterogeneity. The FT program in these two laparoscopy trials was similar in most items, such as normal diet in the preoperative stage, and no opioid analgesics by intramuscular injection or patient-controlled analgesia in the postoperative period. There was some difference in the surgery day. In Chen's study, there was no routine use of abdominal cavity drainage in the FT program, while in the Kim's study, routine use of abdominal cavity drainage were applied. Additionally, the necessary data of mean ± SD in Chen's study were imputed according to the method described by Hozo *et al*[15], and it could introduce some heterogeneity. No significant heterogeneity was found for the outcome of postoperative hospital stay between the two open trials.

The duration to first flatus in the FT program group was also found to be shorter, which implied that the bowel function recovered faster. The medical cost was significantly less in the FT program group, which may be explained by shorter postoperative hospital stay in this group. The incidence rate of complications was more frequent in Chen's study than in Kim's study, which can be explained by the fact that the duration of follow-up in Chen's study was longer. However, there was no significant difference in the incidence rate of complications between the FT program and traditional care. Several cytokines such as interleukin-6, tumor necrosis factor-α, and CRP have been demonstrated to be involved in the response to surgical stress and therefore considered useful serum markers for evaluating the severity of surgery-induced stress. CRP was chosen as a serum marker in our study, and the overall results showed that the level of CRP was significantly lower in the FT program group than in the traditional group.

Our work is the first systematic review to discuss the FT surgery for gastric cancer. However, there were several main limitations in our study. First, the sample size was relatively small. Second, the number of included studies was limited. Third, the methodological assessment showed that all trials were of moderate risk of bias, because no trial described the details of randomization and allocation concealment. Finally, all trials were single-center studies.

In conclusion, our overall results suggested that compared with traditional care, FT program could result in shorter postoperative hospital stay, less medical cost, and lower level of CRP, with no more complications in both laparoscopic and open surgery for gastric cancer. Future trials need to include more participants in multiple centers to assess the effect of FT surgery for gastric cancer.

COMMENTS

Background

Fast-track (FT) surgery is an integrated application of various medical interventions that can enhance recovery after surgery. It has been applied in the surgery for gastric cancer.

Research frontiers

Several trials evaluated the effectiveness of FT program *vs* traditional care in surgery for gastric cancer.

Innovations and breakthroughs

Authors' overall results suggested that compared with traditional care, FT program could result in shorter postoperative hospital stay, less medical cost, and lower level of C-reactive protein, with no more complications occurring in both laparoscopic and open surgery for gastric cancer.

Applications

FT program can be applied in surgery for gastric cancer to enhance postoperative recovery.

Peer review

The manuscript was well written and concise. The title and abstract explain the manuscript very well. Their results suggested that FT program can be used in surgery for gastric cancer.

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META-ANALYSIS

Chemotherapy for patients with gastric cancer after complete resection: A network meta-analysis

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Abstract

AIM: To conduct a network meta-analysis to evaluate the effectiveness of different chemotherapy regimens for patients with gastric cancer.

METHODS: PubMed (1966-2011.12), the Cochrane Library (2011 Issue 2) and EMBASE (1974-2011.12) were searched with the terms "gastric cancer" and "chemotherapy", as well as the medical subject headings. References from relevant articles and conferences were also included. Patients who had previous gastric surgery, radiation before or after surgery or chemotherapy before surgery were excluded. In this study, only randomized controlled trials (RCTs) were considered, and the end-point was the overall mortality. Direct comparisons were performed using traditional meta-analysis whereas indirect comparisons were performed using network meta-analysis.

RESULTS: In total, 31 RCTs with 7120 patients were included. Five chemotherapy regimens, fluorouracil (FU) + BCNU, FU + methyl-CCNU (mCCNU), FU + cisplatin, FU + anthracyclines and FU + mitomycin c (MMC) + cytarabine (Ara-c), were found to be less beneficial in terms of overall mortality. In contrast, four chemotherapy regimens were effective for the patients after surgery, including FU + MMC + adriamycin (FMA), FU + MMC (FM), Tegafur and MMC, There was no significant difference in terms of overall mortality among these regimens. The evidence for the FM regimen and MMC regimen was poor. Additionally, the FMA regimen, which includes a variety of chemotherapy drugs and causes many side effects, was not better than the Tegafur regimen.

CONCLUSION: Although the four chemotherapy regimens were effective in patients with gastric cancer after surgery and the overall mortality revealed no significant difference among them in the network meta-analysis, thorough analysis of the results recommends Tegafur as the first-line adjuvant chemotherapy regimen for patients after complete resection.

Key words: Gastric cancer; Chemotherapy; Randomized controlled trials; Indirect treatment comparison; Network meta-analysis

Core tip: Although adjuvant chemotherapy after complete resection of gastric cancer is therapeutically useful, which of the many regimens is most effective? To date, no regimen has been clearly recommended as the standard procedure post-operation; therefore, we performed a network meta-analysis, which is a useful tool to summarize the different clinical trials and to evaluate the effectiveness of different chemotherapy regimens for patients after complete resection of gastric cancer.

Based on our findings, the Tegafur regimen, especially S-1, is the first therapy that should be recommend to the patients to reduce overall mortality.

Zhang YW, Zhang YL, Pan H, Wei FX, Zhang YC, Shao Y, Han W, Liu HP, Wang ZY, Yang SH. Chemotherapy for patients with gastric cancer after complete resection: A network meta-analysis. *World J Gastroenterol* 2014; 20(2): 584-592 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/584.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.584

INTRODUCTION

Gastric cancer (GC) remains the second leading cause of cancer-related deaths in the world and is the most common malignancy in Asia, South America and Eastern Europe. The overall outcome for patients with GC has not significantly improved over recent decades[1-4]. GC remains a considerable threat to public health around the world. Currently, complete resection still has the highest potential for curatively treating GC[5]. However, approximately 20%-60% of GC patients who have already had curative surgery develop recurrent diseases[6] and will need to undergo adjuvant chemotherapy.

No network meta-analysis has been conducted to compare the efficacy of different chemotherapy protocols for patients with GC. Network meta-analysis is a useful tool for summarizing different clinical trials[7], especially when many different regimens are effective for the same clinical condition. In this type of analysis, all binary comparisons are shown with labels indicating superiority, inferiority or no difference in a summary graph[8-12]. Some recent meta-analyses have indicated that adjuvant chemotherapy after complete resection produces a small survival benefit[13-18]. Several additional trials have also been conducted in this setting. However, they did not indicate which chemotherapy protocol had the best efficacy for treating patients who have undergone complete resection. There is no clearly recommended protocol for the standard treatment of patients with GC after complete resection, and a 5-fluorouracil (5-FU) and platinum-based regimen is usually administered. Surgeons need empirical evidence to determine the best treatment for GC patients. Therefore, it was deemed important to assess the benefits of various adjuvant chemotherapy regimens through a network meta-analysis based on data from all relevant randomized controlled trials (RCTs).

The purpose of this network meta-analysis was to evaluate the effectiveness of different chemotherapy regimens for patients with GC who had undergone surgery.

MATERIALS AND METHODS

Study selection

PubMed (1966.01-2011.12), the Cochrane Library (2011 Issue 12) and EMBASE (1974.01-2011.12) were searched with the terms "gastric cancer" and "chemotherapy", as well as the medical subject headings. The relevant articles referenced in these publications were downloaded from the databases. The related article function was also used to widen the search results. All abstracts, comparative studies, non-randomized trials, and citations scanned were searched comprehensively. Additional searches were conducted by reviewing abstract booklets and review articles. Trials were included irrespective of the language in which they were reported.

Data extraction

Each article was critically reviewed by two researchers for eligibility in our network meta-analysis (Table 1). Only RCTs on palliative or adjuvant chemotherapy for treating GC patients who had undergone surgery were analyzed in this network meta-analysis. The two researchers extracted the data separately, which were then confirmed by a third researcher.

Inclusion criterion: Patients with GC after complete resection and age < 71 years.

Exclusion criteria: Patients who had previous gastric surgery, radiation before or after surgery, chemotherapy before surgery, a history of deep venous thrombosis or pulmonary embolism and severe cardiovascular, respiratory, hepatic or renal disease.

End point: Overall mortality was defined as the time from randomization to death from any cause, or to the last follow-up, which was used as the date of censoring.

Quality evaluation

The quality of the studies included was assessed using the Jadad score[19].

Statistical analysis

The traditional meta-analysis method was used for extracting the crude rates of our pre-specified clinical endpoint for each treatment group when the trials reported suitable information. We summarized the available data on overall survival from the reported results in all trials, computing pooled odd ratios and their respective 95% confidence intervals (95%CI) by means of a fixed-effects model. All statistical analyses were performed using Review Manager (RevMan version 5.0), the Cochrane Collaboration's software for preparing and maintaining Cochrane systematic reviews. We used the chi-square statistic to assess the heterogeneity between trials and the I^2 statistic to assess the extent of inconsistency. Subgroup analysis was used to explore important clinical differences among trials that might be expected to affect the magnitude of the treatment effect.

Network meta-analysis was used after traditional meta-analysis. When efficient chemotherapy regimens were compared through network meta-analysis, the head-to-head comparisons (in this case, indirect comparisons) were handled and consequently assigned a statistical result in terms of superiority/inferiority or no difference

Table 1 Characteristics of randomized trials included in the network meta-analysis

Trial	Year	Postoperative chemotherapy regimens	Sample size		Overall mortality		Follow-up (mo)	Jadad score
			Chemotherapy group	Control group	Chemotherapy group	Control group		
Lawton *et al*[20]	1981	FU + BCNU	13	12	11/13	10/12	60	2
Stablein *et al*[21]	1982	FU + MCCNU	71	71	29/71	40/71	48	3
Higgins *et al*[22]	1983	FU + MCCNU	156	156	121/156	117/156	36	3
Nakajima *et al*[23]	1984	FM + Ara-c	128	124	11/128	17/124	60	3
Engstrom *et al*[24]	1985	FU + MCCNU	91	89	57/91	51/89	24	3
Schlag *et al*[25]	1987	FU + BCNU	42	53	21/42	28/53	72	2
Bonfanti *et al*[26]	1988	FU + MCCNU	75	69	63/75	56/69	84	4
Coombes *et al*[27]	1990	FMA	131	148	101/133	123/148	68	3
Estape *et al*[28]	1991	MMC	33	37	16/33	31/37	120	2
Krook *et al*[29]	1991	FA	61	64	41/61	43/64	60	3
Kim *et al*[30]	1992	MMC + FU	77	94	54/77	71/94	60	2
Grau *et al*[31]	1993	MMC	68	66	40/68	49/66	105	2
Hallissey *et al*[32]	1994	FMA	138	145	101/138	110/145	60	3
Macdonald *et al*[33]	1995	FMA	93	100	59/93	68/100	114	2
Lise *et al*[34]	1995	FMA	155	159	88/155	99/159	78	3
Tsavaris *et al*[35]	1996	FMA	42	42	27/42	34/42	60	3
Cirera *et al*[36]	1999	MMC + Tegafur	76	76	33/ 76	44/72	37	3
Nakajima *et al*[37]	1999	MMC + FU + UFT	288	285	41/288	49/285	72	3
Neri *et al*[38]	2001	Epirubicin + FU	69	68	48/69	59/68	60	2
Bajetta *et al*[39]	2002	FU + Adriamycin etoposide + cisplatin	137	137	66/137	71/137	66	2
Nashimoto *et al*[40]	2003	MMC + FU + Ara C	128	124	11/128	23/124	69	2
Popiela *et al*[41]	2004	FAM	53	52	42/53	47/52	120	2
Chipponi *et al*[42]	2004	Cisplatin + FU	101	104	62/101	63/104	60	2
Bouché *et al*[43]	2005	Cisplatin + FU	127	133	68/127	77/133	97.8	3
Nitti *et al*[44]	2006	FU + Adriamycin + methotrexate + LV	103	103	54/103	49/103	60	3
Nitti *et al*[44]	2006	FU + Epirubicin + methotrexate + LV	91	100	63/91	64/100	60	3
De Vita *et al*[45]	2007	FU + Epirubicin + LV + etoposide	112	113	58/112	64/113	60	2
Nakajima *et al*[46]	2007	Uracil-Tegafur	95	95	18/95	30/95	60	4
Di Costanzo *et al*[47]	2008	FU + Epirubicin + cisplatin + LV	130	128	69/130	70/128	60	3
Miyashiro *et al*[48]	2011	Cisplatin + FU	132	132	50/132	52/132	60	4
Sasako *et al*[49]	2011	S-1	529	530	149/529	206/530	60	4

FU: Fluorouracil; MCCNU: Methyl-CCNU; MMC: Mitomycin c; LV: Leucovorin; Ara-c: Cytarabine; CDHP: 5-Chloro-2,4-dihydropyrimidine; Oxo: Potassium oxonate; FM: FU + MMC; FMA: FU + MMC + adriamycin; S-1: Tegafur + CDHP + Oxo.

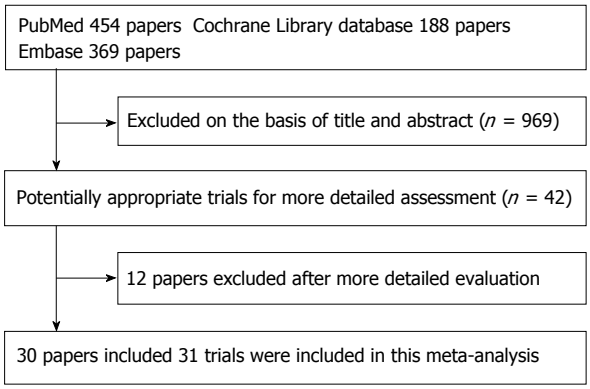

Figure 1 Flow diagram of trial selection.

along with the level of statistical significance. Statistical calculations and graph generation were carried out. The HR, with a 95%CI, for each indirect comparison was estimated according to the ITC software (Canadian Agency for Drugs and Technologies in Health, Indirect Treatment Comparison software, Ottawa, Ontario, Canada). This approach allows an indirect HR, with a 95%CI, to be estimated on the condition that both treatments included in the indirect comparison had been compared in actual trials against a common comparator.

Role of funding source

No sponsors were involved in the study design; during the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the report for publication. All authors had access to the raw data. The corresponding author had full access to all of the data and the final responsibility to submit the report for publication.

RESULTS

Flow diagram of trial selection

In total, 31 RCTs, with a total of 7120 patients, were included (Figure 1) from the electronic databases. Figure

1 shows a flow chart of studies from the initial results of the publication searches to the final inclusion or exclusion. The RCTs that met the criteria for our analysis are described in Table 1. There were 12 RCTs that had a Jadad score of 2, 15 RCTs that had a Jadad score of 3 and 4 RCTs that had a Jadad score of 4.

Analysis of regimen groups

In terms of direct comparisons, this analysis divided the chemotherapy regimens into 9 subgroups, and 8 subgroups were assessed by the fixed effects models, while only 1 was assessed by the random effects models. In terms of overall mortality, at least 5 chemotherapy regimens were found to be of equal efficacy when compared to a blank control. The values of HR were as follows: 0.92 (95%CI: 0.43-1.96) for FU + BCNU regimen, 1.00 (95%CI: 0.76-1.32) for FU + methyl-CCNU (mCCNU) regimen, 0.93 (95%CI: 0.69-1.24) for FU + cisplatin regimen, 0.92 (95%CI: 0.74-1.14) for FU + anthracyclines regimen, and 0.67 (95%CI: 0.41-1.10) for FU + mitomycin c (MMC) + AraC regimen. In contrast, in terms of overall mortality, 4 chemotherapy regimens were found to be more effective than the blank control. The values of HR were as follows: 0.74 (95%CI: 0.58-0.94) for FAM regimen, 0.68 (95%CI: 0.49-0.94) for FM regimen, 0.60 (95%CI: 0.47-0.76) for Tegafur regimen, and 0.33 (95%CI: 0.13-0.86) for MMC regimen. These outcomes are described in Figures 2 and 3.

In terms of indirect comparisons, 4 chemotherapy regimens were found to be equal in terms of overall mortality. The values of HR were as follows: 1.09 (95%CI: 0.73-1.63) for 5-FU + adriamycin + MCC (FAM) regimen *vs* FM regimen; 1.23 (95%CI: 0.88-1.73) for 5-FU + MMC + adriamycin (FMA) regimen *vs* Tegafur regimen; 2.24 (95%CI: 0.85-5.95) for FMA regimen *vs* MMC regimen; 1.13 (95%CI: 0.76-1.70) for FM regimen *vs* Tegafur regimen; 2.06 (95%CI: 0.76-5.60) for FM regimen *vs* MMC regimen; and 1.82 (95%CI: 0.67-4.80) for Tegafur regimen *vs* MMC regimen. These outcomes are described in Figure 4.

DISCUSSION

In total, 31 RCTs, with a total of 7120 patients, were included in this analysis, and 12 RCTs had a Jadad score of 2, 15 RCTs had a Jadad score of 3, and 4 RCTs had a Jadad score of 4. This study divided these chemotherapy regimens into 9 subgroups. The result of this analysis indicated that 5 chemotherapy regimens had little benefit to the patients, including the FU + BCNU, FU + mCCNU, FU + cisplatin, FU + anthracyclines, and FU + MMC + AraC regimens. In contrast, 4 chemotherapy regimens were effective for patients after surgery, including the FMA, FM, Tegafur, and MMC regimens. In this study, Tegafur and the S-1 regimen were assigned to one regimen because S-1 was composed of Tegafur, CDHP and Oxo, as CDHP and Oxo reduced the side effects of Tegafur. As Tegafur is a fluorouracil derivative, the FM regimen was included in 3 RCTs. Additionally, anthracyclines, including adriamycin, epirubicin and doxorubicin, were part of the FMA regimen, which was included in 6 RCTs. Indirect comparisons were estimated according to the ITC software, and the results indicated that there was no difference among these four chemotherapy regimens in the terms of overall mortality.

Although this analysis indicated that MMC was effective for patients after surgery, the evidence for this result was poor because of the low quality of the 2 RCTs included. Specifically, one trial had a small sample size, and only 204 patients were contained in the subgroup analysis. Additionally, because there was also significant heterogeneity among the trials ($P = 0.14$, $I^2 = 54\%$), the analysis was carried out using the random effects models. The curative effect of MMC needs to be further validated. The evidence for the Tegafur regimen included 1249 patients, the RCTs were of high quality, and there was no significant heterogeneity among the trials ($P = 0.59$, $I^2 = 0\%$). Accordingly, the analysis was carried out using the fixed effects model, and we found strong evidence to confirm the efficacy of the Tegafur regimen. The joint application with 5-chloro-2,4-dihydropyrimidine (CDHP) and potassium oxonate (Oxo) reduced the side effects of Tegafur; therefore, the S-1 regimen (Tegafur + CDHP + Oxo) is recommended.

The combination of Tegafur and MMC in the FM regimen was similar to treatment with each component individually, as determined by indirect comparison, and further studies are needed to confirm which treatment is the primary effector. Additionally, if the side effects of Tegafur and MMC will reduce the overall efficacy, further studies are needed to identify an adjuvant that can reduce these side effects, as in the case of S-1. If the treatments have a mutual antagonist effect on each other, they should be used separately. As the evidence for the FM regimen is not very strong, larger sample sizes and multicenter RCTs are still needed. While the FMA regimen is available, surprisingly, it is not better than Tegafur or MMC. Traditional analysis indicated that the FU + anthracyclines regimen is not available, and thus, MMC may contribute to the efficacy of the FMA regimen to a great extent. Accordingly, based on these results, FMA is not recommended.

In summary, chemotherapy regimens, especially Tegafur, are available for GC. However, the efficacy of the FM regimen and MMC regimen needs to be further validated. The evidence for the Tegafur regimen is more credible, and S-1 may be the best current choice. Future studies should focus on identifying better adjuvants that can reduce the side effects of MMC as much as possible. Their combination could be a better regimen than S-1, and perhaps, the combination of MMC, Tegafur and adjuvant can achieve better outcomes than mono-chemotherapy alone. However, based on recent evidence, the Tegafur regimen, especially S-1, is most commonly recommended to patients after complete resection.

In conclusion, this analysis indicated that four che-

Study or subgroup	Adjuvant chemotherapy Events	Total	Control Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
1.1.1 FU + BNCU						
Lawton *et al*[20] 1981	21	42	28	53	1.5%	0.89 [0.40, 2.01]
Schlag *et al*[25] 1987	11	13	10	12	0.2%	1.10 [0.13, 9.34]
Subtotal (95%CI)		55		65	1.7%	0.92 [0.43, 1.96]
Total events	32		38			
Heterogeneity: χ^2 = 0.03, *df* = 1 (*P* = 0.86); I^2 = 0%						
Test for overall effect: *Z* = 0.23 (*P* = 0.82)						
1.1.2 FU + mCCNU						
Bonfanti *et al*[26] 1988	47	75	40	69	1.9%	1.22 [0.62, 2.38]
Stablein *et al*[21] 1982	29	71	40	71	2.9%	0.54 [0.27, 1.04]
Engstrom *et al*[24] 1985	57	91	51	89	2.3%	1.25 [0.69, 2.27]
Higgins *et al*[22] 1983	121	156	117	156	3.2%	1.15 [0.68, 1.94]
Subtotal (95%CI)		393		385	10.3%	1.01 [0.75, 1.37]
Total events	254		248			
Heterogeneity: χ^2 = 4.52, *df* = 3 (*P* = 0.21); I^2 = 34%						
Test for overall effect: *Z* = 0.09 (*P* = 0.93)						
1.1.3 FU + Cisplatin						
Bouché *et al*[43] 2005	68	127	77	133	4.3%	0.84 [0.51, 1.37]
Chipponi *et al*[42] 2004	62	101	63	104	2.9%	1.03 [0.59, 1.81]
Miyashiro *et al*[48] 2011	50	132	52	132	3.9%	0.94 [0.57, 1.54]
Subtotal (95%CI)		360		369	11.1%	0.93 [0.69, 1.24]
Total events	180		192			
Heterogeneity: χ^2 = 0.31, *df* = 2 (*P* = 0.86); I^2 = 0%						
Test for overall effect: *Z* = 0.52 (*P* = 0.61)						
1.1.4 FU + Anthracyclines + others						
Bajetta *et al*[39] 2002	66	137	71	137	4.5%	0.86 [0.54, 1.39]
De Vita *et al*[45] 2007	58	112	64	113	3.7%	0.82 [0.49, 1.39]
Di Costanzo *et al*[47] 2008	69	130	70	128	4.0%	0.94 [0.57, 1.53]
Krook *et al*[47] 1991	41	61	43	64	1.7%	1.00 [0.47, 2.11]
Neri *et al*[38] 2001	48	69	59	68	2.2%	0.35 [0.15, 0.83]
Nitti *et al*[44] 2006a	54	103	49	103	2.8%	1.21 [0.70, 2.10]
Nitti *et al*[44] 2006b	63	91	64	100	2.3%	1.27 [0.69, 2.32]
Subtotal (95%CI)		703		713	21.3%	0.92 [0.74, 1.14]
Total events	399		420			
Heterogeneity: χ^2 = 7.15, *df* = 6 (*P* = 0.31); I^2 = 16%						
Test for overall effect: *Z* = 0.78 (*P* = 0.43)						
1.1.5 FU + MMC + AraC						
Nakajima *et al*[23] 1984	11	128	17	124	1.9%	0.59 [0.27, 1.32]
Nashimoto *et al*[40] 2003	102	156	52	72	3.0%	0.73 [0.39, 1.34]
Subtotal (95%CI)		284		196	4.9%	0.67 [0.41, 1.10]
Total events	113		69			
Heterogeneity: χ^2 = 0.16, *df* = 1 (*P* = 0.69); I^2 = 0%						
Test for overall effect: *Z* = 1.59 (*P* = 0.11)						
1.1.6 FU + MMC + Anthracyclines						
Coombers *et al*[27] 1990	73	133	91	148	4.7%	0.76 [0.47, 1.23]
Hallissey *et al*[32] 1994	101	138	110	145	3.5%	0.87 [0.51, 1.48]
Lise *et al*[34] 1995	88	152	99	154	5.0%	0.76 [0.48, 1.21]
Macdonald *et al*[33] 1995	59	93	68	100	2.9%	0.82 [0.45, 1.48]
Popiela *et al*[41] 2004	42	53	47	52	1.2%	0.41 [0.13, 1.27]
Tsavaris *et al*[35] 1996	27	42	34	42	1.5%	0.42 [0.16, 1.15]
Subtotal (95%CI)		611		641	18.9%	0.74 [0.58, 0.94]
Total events	390		449			
Heterogeneity: χ^2 = 2.76, *df* = 5 (*P* = 0.74); I^2 = 0%						
Test for overall effect: *Z* = 2.44 (*P* = 0.01)						
1.1.7 FD (fluorouracil derivative) + MMC						
Cirera *et al*[36] 1999	33	76	46	72	3.3%	0.43 [0.22, 0.84]
Kim *et al*[30] 1992	54	77	71	94	2.3%	0.76 [0.39, 1.50]
Nakajima *et al*[37] 1999	41	288	49	285	5.2%	0.80 [0.51, 1.26]
Subtotal (95%CI)		441		451	10.7%	0.68 [0.49, 0.94]
Total events	128		166			
Heterogeneity: χ^2 = 2.38, *df* = 2 (*P* = 0.30); I^2 = 16%						
Test for overall effect: *Z* = 2.32 (*P* = 0.02)						

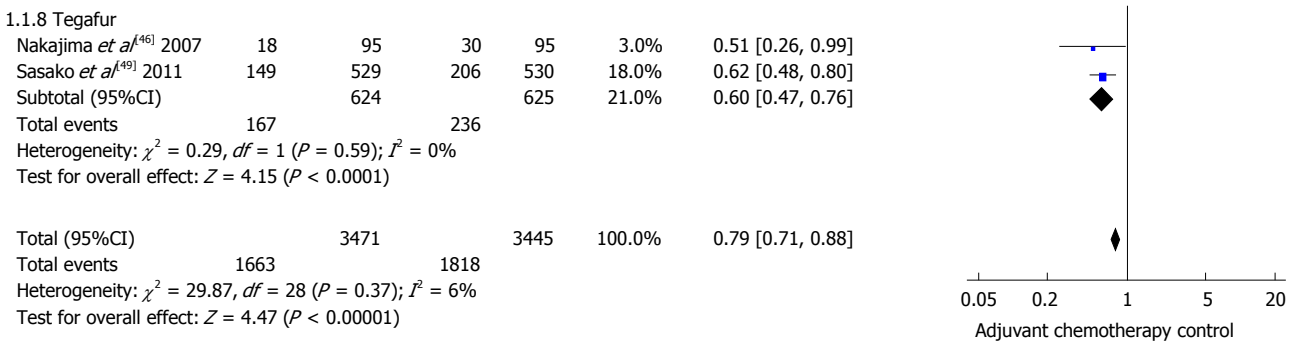

Figure 2 Eight subgroups in the fixed effects models.

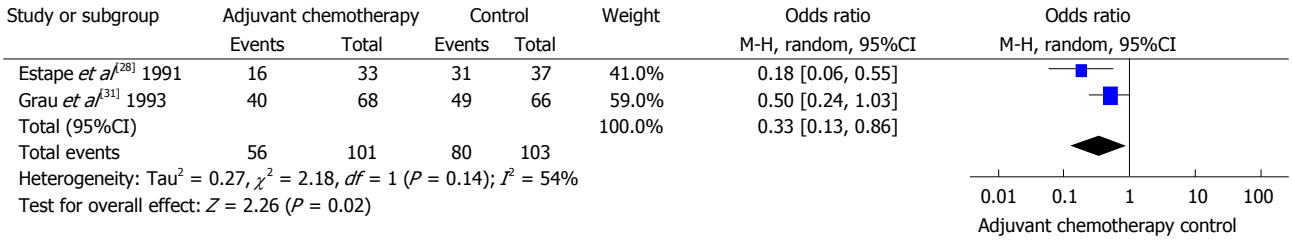

Figure 3 One subgroup in the random effects model. FU: Fluorouracil; mCCNU: Methyl-CCNU; MMC: Mitomycin c.

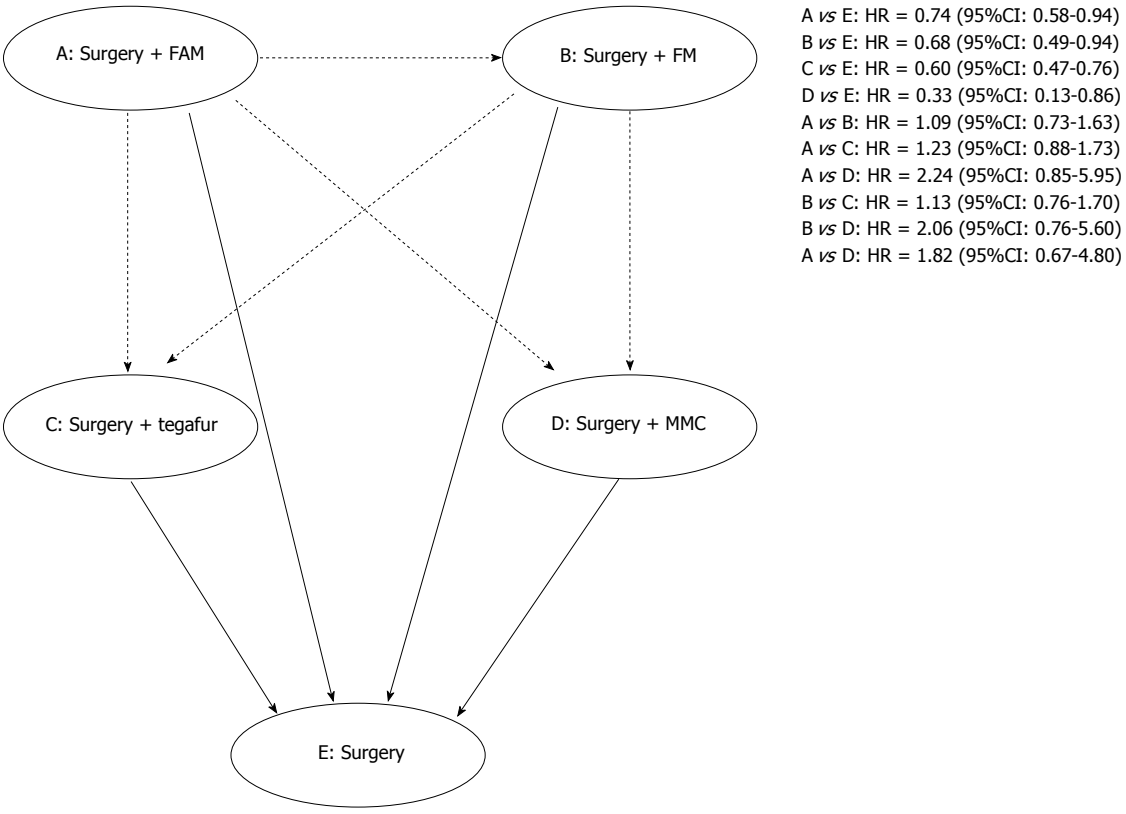

Figure 4 Network meta-analysis in terms of mortality. MMC: Mitomycin c; FAM: 5-fluorouracil, adriamycin, and mitomycin c.

motherapy regimens are effective for patients with GC after surgery, including the FMA regimen, FM regimen, Tegafur regimen and MMC regimen. However, the evidence for the FM regimen and MMC regimen was poor in terms of overall mortality. The FMA regimen, which includes many chemotherapy drugs and thus has many side effects, is not better than the Tegafur regimen. Based on this study, the Tegafur regimen is recommended as a better choice for doctors when dealing with GC patients after complete resection.

COMMENTS

Background

Gastric cancer is very common worldwide and, in most cases, will lead to serious health problems, even after complete resection. Currently, treatment with adjuvant and palliative chemotherapies are essential to prevent and treat recurrence disease. A standard chemotherapy regimen has not been established; therefore, the evaluation of which regimens may be better for gastric cancer patients is needed.

Research frontiers

This network meta-analysis was performed to evaluate the effectiveness of different chemotherapy regimens for patients with gastric cancer. The end point was overall mortality, which was defined as the time from randomization to death from any cause, or to the last follow-up.

Innovations and breakthroughs

The meta-analysis shows the following: four chemotherapy regimens [fluorouracil (FU) + mitomycin c + adriamycin, fluorouracil + mitomycin c (FM), tegafur and mitomycin c (MMC)] are effective for patients after surgery, whereas the other five regimens [fluorouracil + BCNU, FU + methyl-CCNU (mCCNU), FU + cisplatin, FU + anthracyclines and FU + mitomycin c + cytarabine] were found to be less beneficial.

Applications

From the analysis, Tegafur is recommended as the first-line adjuvant chemotherapy regimen for patients after complete resection. This recommendation is due to the high quality of the randomized controlled trials (RCTs), homogeneity among trials and fewer side effects.

Peer review

The current network meta-analysis evaluated the effectiveness of different chemotherapy regimens for gastric cancer patients after curative surgery, and we found that the outcomes and analysis were good. However, further RCTs are needed to study the FM regimen, MMC regimen and combination chemotherapy.

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CASE REPORT

An extremely rare case of pancreatic metastasis of esophageal squamous cell carcinoma

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Abstract

We report a rare case of a 68-year-old male with metachronous pancreatic metastasis that was resected 2 years after salvage esophagectomy for local recurrence of esophageal squamous cell carcinoma (ESCC). Two years and 8 mo ago, he had undergone definitive chemoradiotherapy for the lower thoracic ESCC and achieved a complete response. Chemoradiotherapy used the protocol of the Japan Clinical Oncology Group trial 9906. Approximately 8 mo later, he developed a local recurrence of the ESCC and underwent thoracoscopic salvage esophagectomy followed by reconstruction with a conduit colon graft via a subcutaneous route. Recently, a tumor of the pancreatic body was found on routine follow-up computed tomography (CT). The tumor diameter was 15 mm on CT, and the maximum standardized uptake value of the lesion was 5.49 at 18F-2-fluoro-2-deoxy-D-glucose positron-emission tomography, strongly suggesting pancreatic cancer. In addition, all tumor markers were within the reference intervals. Therefore, distal pancreatectomy was performed with the resultant histological diagnosis being confirmed as pancreatic metastasis of the ESCC. He was treated with adjuvant chemotherapy, and there has been no evidence of recurrence 9 mo after the surgery. Resection of pancreatic metastasis offers a good prognosis and should be considered for solitary ESCC metastasis.

Key words: Metachronous pancreatic metastasis; Esophagus; Squamous cell carcinoma; Pancreatectomy; Salvage esophagectomy; Definitive chemoradiotherapy

Core tip: This report a 68-year-old male presenting with a pancreatic tumor following esophageal squamous cell carcinoma (ESCC) treated with curative chemoradiotherapy and thoracoscopic salvage esophagectomy approximately 2 years previously. He was treated with distal pancreatectomy and histologically diagnosed as a rare case of metachronous pancreatic metastasis of ESCC. Resection of pancreatic metastasis offers a good prognosis and should be considered for solitary ESCC metastasis. Both this case and a review of the relevant literature support this view.

Okamoto H, Hara Y, Chin M, Hagiwara M, Onodera Y, Horii S, Shirahata Y, Kamei T, Hashizume E, Ohuchi N. An extremely rare case of pancreatic metastasis of esophageal squamous cell carcinoma. *World J Gastroenterol* 2014; 20(2): 593-597 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/593.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.593

INTRODUCTION

Pancreatic metastasis of esophageal carcinoma is rare. The disease occurs reportedly at a frequency of 0.12% in esophageal carcinomas and 0.68% in metastatic tumors of esophageal carcinoma[1]. Another study has reported the rate of pancreatic metastasis of esophageal carcinoma to be 3.9% of that of esophageal carcinomas according to pathological examination of autopsy samples[2]. Furthermore, it has been observed in 0%-4.9% of pancreatic metastatic malignancies[3-7]. To the best of our knowledge, there are few English literature reports of pancreatic metastasis of esophageal squamous cell carcinoma (ESCC). Here, we describe a case of metachronous pancreatic metastasis of ESCC with a brief review of the literature.

CASE REPORT

A 68-year-old male was found to have a tumor of the pancreatic body on routine enhanced computed tomography (CT), as follow-up for the treatment of esophageal carcinoma. Two years and eight months previously, he had been diagnosed and treated for lower thoracic ESCC (clinical T1bN0M0, stage I ; according to the seventh edition of the Union for International Cancer Control system). At that time, he underwent definitive chemoradiotherapy with a complete remission. The chemoradiotherapy protocol was consistent with that of the Japan Clinical Oncology Group trial 9906, and comprised two cycles of intravenous cisplatin infusions with continuous 5-fluorouracil infusion and concurrent irradiation with a total dose of 60 Gy[8]. Approximately 8 mo later, he developed local recurrence of the ESCC (pathological T1bN0M0, stage I), and underwent thoracoscopic salvage esophagectomy followed by reconstruction with a conduit colon graft via a subcutaneous route. Thoracoscopic salvage esophagectomy was performed with two-field lymph node dissection[9].

The pancreatic body tumor was evaluated prior to surgery by CT and 18F-2-fluoro-2-deoxy-D-glucose positron-emission tomography (FDG-PET). The tumor was low density and its diameter was 15 mm on CT examination, and the maximum standardized uptake value (SUVmax) of the lesion was 5.01 in the early phase and 5.49 in the late phase at FDG-PET (Figure 1). These results strongly suggested pancreatic cancer. In addition, all tumor markers were within the reference intervals, including SCC, CEA, CA19-9, SPan-1, DUPAN-2, and NCC-ST-439.

Distal pancreatectomy and splenectomy were performed to treat the pancreatic body tumor. Macroscopic findings of the specimen revealed a 15 mm × 11 mm tumor in the pancreatic body (Figure 2). Pathological examination revealed that the tumor was a squamous cell carcinoma with morphologic features similar to the previous ESCC; it was therefore diagnosed as a pancreatic metastasis of the ESCC (Figure 3). He was discharged uneventfully on the 11th postoperative day and later received one dose of adjuvant chemotherapy (intravenous cisplatin infusions with continuous 5-fluorouracil infusion). However, because of renal impairment the adjuvant chemotherapy was stopped and was followed up on an outpatient basis without adjuvant therapy. There have been no signs of recurrence 9 mo after surgical resection of the pancreatic metastasis.

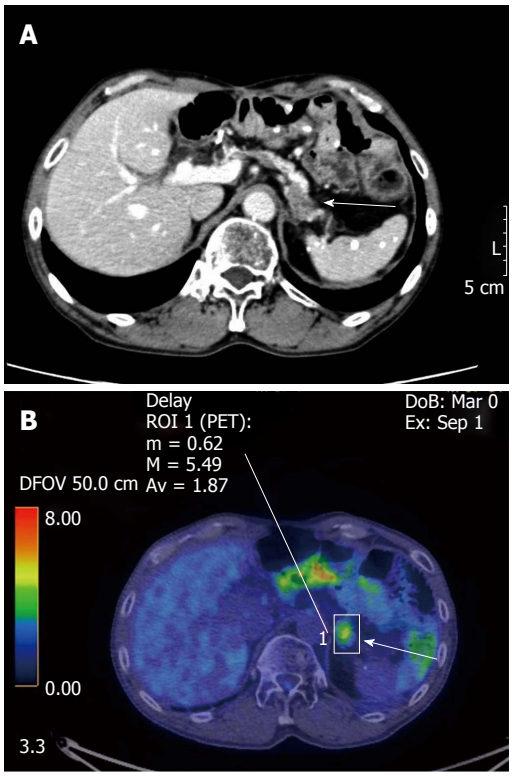

Figure 1 Enhanced computed tomography (A) and 18F-2-fluoro-2-deoxy-D-glucose positron-emission tomography (B) showed a tumor of the pancreatic body (arrows). The major axis of the tumor was 15 mm. Maximum standardized uptake value of the lesion was 5.49.

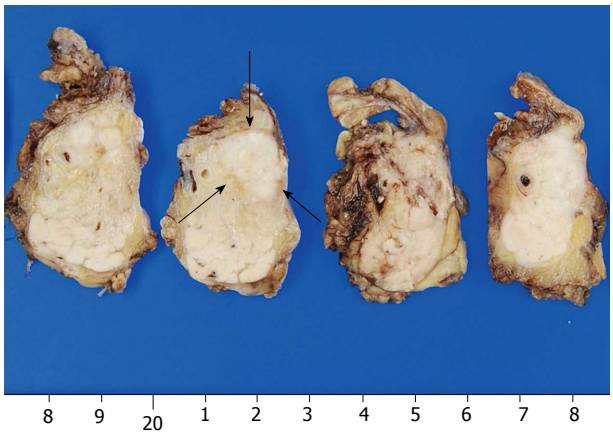

Figure 2 Macroscopic findings of the specimen revealed a 15 mm × 11 mm tumor in the pancreatic body (arrows).

DISCUSSION

To the best of our knowledge, only 3 cases of pancreatic

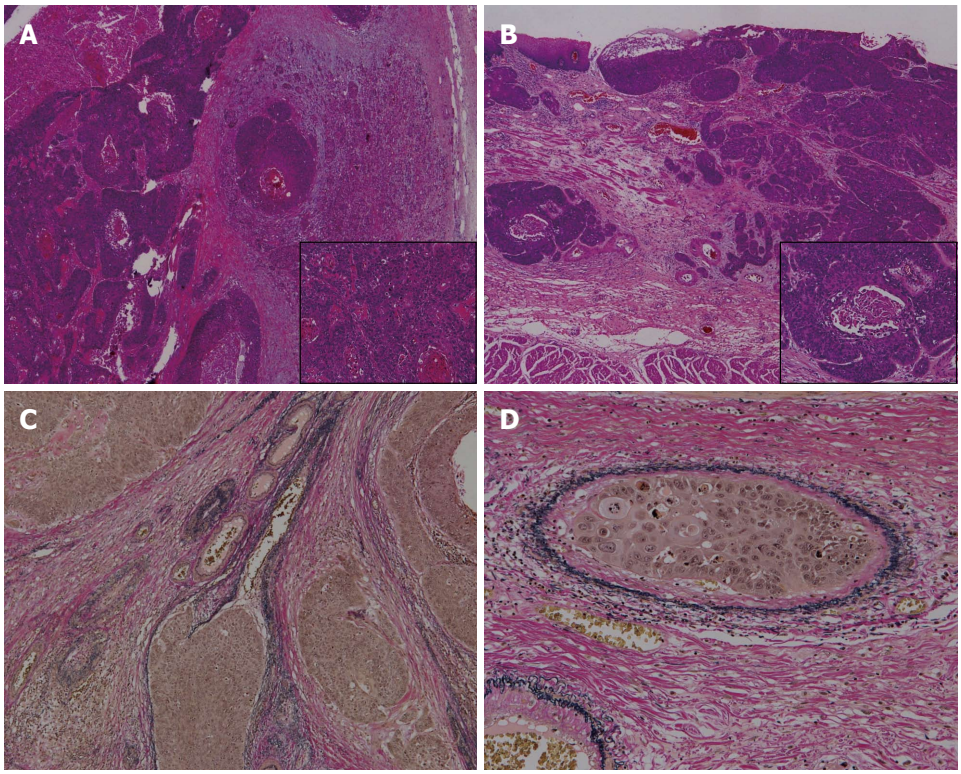

Figure 3 Pathological examination revealed squamous cell carcinoma. A: Squamous cell carcinoma in the pancreas; B: Squamous cell carcinoma in the esophagus resected previously (hematoxylin-eosin staining, A: × 40; B: × 400); C: Vascular invasion in the pancreatic metastasis; D: Esophageal carcinoma (elastica van gieson staining, C: × 100, D: × 400).

metastasis of ESCC have been reported in the English literature till date[10-12]. Park *et al*[11] reported the case of 58-year-old male who underwent an esophagectomy for early ESCC followed by distal pancreatectomy for pancreatic metastasis with radiofrequency ablation for hepatocellular carcinoma. Adjuvant chemotherapy was given for 4 mo after the surgery in this case, without any evidence of recurrence. Sawada *et al*[12] reported the case of 73-year-old male with advanced ESCC who developed pancreatic metastasis that caused hepatic portal venous gas; he received supportive care only. Esfehani *et al*[10] reported a 59-year-old female with a pancreatic metastasis of ESCC who had undergone surgical treatment and adjuvant chemoradiotherapy 4 years earlier. In this case, she was treated with distal pancreatectomy and adjuvant chemotherapy for the pancreatic metastasis and showed no recurrence within the 6-mo follow-up. This is the only known case of metachronous pancreatic metastasis of ESCC reported in the literature[10]. Our report is the first case of metachronous pancreatic metastasis of ESCC following local recurrence treated with salvage esophagectomy and definitive chemoradiotherapy.

The differential diagnosis of pure squamous cell carcinoma of the pancreas is also a possibility. However, this is very rare, and it has been argued that before diagnosis, metastasis from another site should be excluded[13]. Most of the squamous differentiation that occurs in pancreatic carcinoma, exists as a component of adenosquamous carcinoma. Therefore, the World Health Organization classification of tumors of the pancreas does not specify it as a distinct entity. Al-Shehri *et al*[14] reported in his review that the development of pancreatic squamous cell carcinoma was explained by 5 postulated theories as follows:. (1) malignant change in a primitive cell capable of differentiating into either squamous or glandular carcinoma; (2) squamous change in a pre-existing adenocarcinoma; (3) malignant transformation in a squamous metaplasia of the ductal epithelium; (4) malignant change in an aberrant squamous cell; and (5) the theory of tumor collision. In this case, the histological morphology similar to the primary ESCC together with the history of ESCC were the basis for the diagnosis of pancreatic metastasis of ESCC. Vascular invasion was observed in the specimens of both the previously resected esophagus and the pancreatic metastasis. This fact supports the hematogenous distant metastasis of the ESCC. Although we initially diagnosed the patient with pancreatic cancer at first, primarily on the basis of the results of imaging, the tumor was ultimately diagnosed as pancreatic metastasis from the ESCC.

Finally, we need to consider whether resection of the ESCC pancreatic metastasis was the appropriate treatment option. Several investigators have reported favorable prognosis with resection of pancreatic metastasis[4,6,7,15]. However, the primary cancers in these reports consisted of renal cell cancer, colon cancer, melanoma, sarcoma, lung cancer, and breast cancer with limited consideration of esophageal cancer. Because of the rarity of the presentation, the optimal treatment regimen remains unknown. Reddy *et al*[6] stated that patients might benefit from pancreatic metastasectomy given the following: a

primary cancer type that was associated with good outcome, control of the primary cancer site, demonstration of isolated metastasis, resectability of the metastasis, and patient fitness to tolerate pancreatectomy. We previously reported that following salvage esophagectomy, the outcome was better in patients with recurrent disease after complete response to definitive chemoradiotherapy than in those with persistent disease[16]. In addition, it was reported that resection of solitary ESCC metastasis results in a longer survival[17]. Therefore, it is therefore likely that this case will have a good prognosis.

In conclusion, we report a rare case of pancreatic metastasis of ESCC and provide a brief review of the literature. From these results, we argue that surgical excision should be considered a valid treatment strategy for solitary pancreatic metastasis in ESCC. However, further investigation and an accumulation of case reports are needed to confirm this finding.

ACKNOWLEDGMENTS

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COMMENTS

Clinical diagnosis

Metachronous pancreatic metastasis that was resected 2 years after salvage esophagectomy for local recurrence of esophageal squamous cell carcinoma (ESCC).

Differential diagnosis

This report performed pancreatectomy in order to clarify whether the tumor was primary pancreatic cancer or pancreatic metastasis.

Laboratory diagnosis

Laboratory testing such as tumor makers could not contribute to the diagnosis.

Imaging diagnosis

Computed tomography (CT) and PET-CT had suggested that the tumor was pancreatic cancer, but it was pathologically pancreatic metastasis of ESCC.

Pathological diagnosis

Pathological examination revealed that the tumor was a squamous cell carcinoma with morphologic features similar to the previous ESCC; it was therefore diagnosed as a pancreatic metastasis of the ESCC.

Treatment

Pancreatectomy and adjuvant chemotherapy (intravenous cisplatin infusions with continuous 5-fluorouracil infusion).

Experiences and lessons

Surgical excision should be considered a valid treatment strategy for solitary pancreatic metastasis in ESCC, but further investigation and an accumulation of case reports are needed to confirm this finding.

Peer review

This report is the first case of metachronous pancreatic metastasis of ESCC following local recurrence treated with salvage esophagectomy and definitive chemoradiotherapy and revealed favorable prognosis with resection of pancreatic metastasis. However, because few cases of pancreatic metastasis of ESCC have been reported it could not insist on many things. Further investigation and an accumulation of case reports are needed.

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CASE REPORT

Intestinal anisakiasis treated successfully with conservative therapy: Importance of clinical diagnosis

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Abstract

Intestinal anisakiasis is not only a rare parasitic disease, but is also difficult to diagnose. The symptoms are not specific and are often very severe and abrupt, and the findings of clinical imaging are very remarkable. Therefore, intestinal anisakiasis is often misdiagnosed as acute abdomen or intestinal obstruction and is treated surgically. However, if intestinal anisakiasis could be diagnosed correctly, it is well treated conservatively. We experienced three cases of intestinal anisakiasis, which were diagnosed correctly and treated successfully with conservative therapy. A correct clinical history and imaging interpretation helped us diagnose intestinal anisakiasis correctly and thus treat the patients successfully with conservative therapy.

Key words: Acute abdomen; Computed tomography findings; Clinical diagnosis; Intestinal anisakiasis; Conservative therapy

Core tip: We conclude that a correct clinical history and imaging interpretation will enable us to diagnose intestinal anisakiasis correctly and successfully manage patients with conservative measures, avoiding unnecessary surgery.

Shrestha S, Kisino A, Watanabe M, Itsukaichi H, Hamasuna K, Ohno G, Tsugu A. Intestinal anisakiasis treated successfully with conservative therapy: Importance of clinical diagnosis. *World J Gastroenterol* 2014; 20(2): 598-602 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/598.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.598

INTRODUCTION

Anisakiasis is a parasitic disease caused in humans by the accidental ingestion of Anisakis larvae, present in fresh fish and squid. The larvae stick to the gastro-intestinal membrane and cause a set of symptoms, which are called anisakiasis. Depending on the site of the digestive system where the Anisakis larvae are stuck, anisakiasis can be divided into the following three types; gastric, intestinal, and ectopic anisakiasis[1-3]. Most of the cases consist of gastric anisakiasis. According to a series of 15715 cases of anisakiasis reported by Ishikura, gastric anisakiasis accounts for 95.6% of the cases, whereas intestinal and ectopic anisakiasis account for 4.1% and 0.3% of the cases respectively[4].

Patients with perforations or strangulation of the intestine require surgical therapy, but conservative therapy is the basic treatment of anisakiasis. Cases of non-gastric anisakiasis are not only very rare, but are also difficult to diagnose because the small intestine is an inaccessible zone for endoscopy. Thus, the diagnostic procedure required for anisakiasis, which is to detect the whole worm, is not feasible. As a result, patients with intestinal anisakiasis, have been diagnosed with acute abdominal

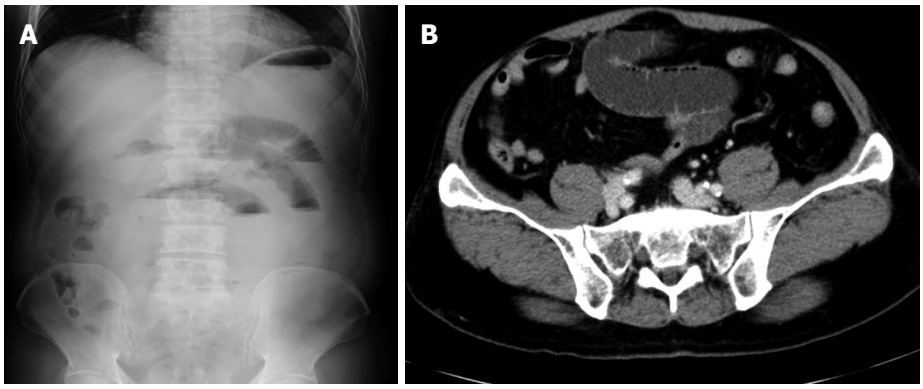

Figure 1 Abdominal X-ray (A) and computed tomography scan (B).

Table 1 Laboratory data of the three patients on the first hospital day

	Case 1	Case 2	Case 3
WBC (cells/μL)	7960	15460	11080
(Eosinophil; neutrophil)	(0.9%; 70%)	(1.0%; 88.2%)	(2.2%; 85.4%)
Hb (g/dL)	16.2	16.5	14.9
Plt (plt/μL)	287000	198000	223000
CRP (mg/dL)	0.78	0.25	2.52
GOT (AST) (U/I)	25	23	23
GPT (ALT) (U/I)	23	36	14
BUN (mg/dL)	14.8	16.8	13.4
Cre (mg/dL)	0.67	0.89	0.67
LDH (U/I)	214	182	196
CPK (IU/I)	88	150	66

WBC: White blood cells; Hb: Hemoglobin; Plt: Platelets; CRP: C-reactive protein; GOT (AST): Glutamin oxaloacetic transaminase (aspartate aminotransferase); GPT (ALT): Glutamate pyruvate transaminase (alanine aminotrasnferase); BUN: Blood urea nitrogen; Cre: Creatinine; LDH: Lactate dehydrogenase; CPK: Creatine phosphokinase.

or intestinal obstructions preoperatively, and have undergone unnecessary surgical operations[5-10]. We herein describe three cases of intestinal anisakiasis, which were correctly diagnosed clinically and treated successfully with conservative therapy.

CASE REPORT

Case 1

A 62-year-old man presented to the ER of our hospital because of abdominal pain that started abruptly. He was diagnosed with urinary tract stone, and a pain killer was prescribed. The abdominal pain was not relieved, and thus, he presented to our hospital the following day. He had a past history of hypertension, for which he was still being treated. He was a heavy smoker, and enjoyed alcohol with fresh sashimi everyday.

The patient was alert and his vital signs, except for his blood pressure (177/87 mmHg), were normal. On physical examination, tenderness with rebound tenderness and rigidity were revealed in the lower part of the abdomen. Laboratory examination was normal, except for a slight increase in C-reactive protein (CRP) levels (0.78 mg/dL) (Table 1). Abdominal X-ray showed gaseous dilatation of the small intestine (Figure 1A). An abdominal computed tomography (CT) scan demonstrated swelling of the partial segment of the small bowel and dilatation of the intestine with fluid collection on the oral side of the lesion (Figure 1B). No ascites was detected.

The patient was admitted to our hospital, and conservative therapy was started after a gastric tube was inserted into his stomach. On the second hospital day, the patient had to rely on pain-killers to control the abdominal pain. Therefore, he underwent another abdominal CT, that demonstrated ascites collection, in addition to the worsening of the observations from the first day (Figure 2A). By then, we had learned that the patient had raw fish (katsuo) 2 d before hospital admission. Therefore, we performed an anti-Anisakis IgG/A antibody test. Although imaging findings were worse, his vital signs were stable, which was another reason why we strongly suspected that the disease was intestinal anisakiasis and did not perform an emergency operation.

On the third hospital day, the abdominal symptoms were relieved, and an abdominal CT scan demonstrated a reduction in ascites (Figure 2B). On the fourth day, the gastric tube was removed and an oral diet was started. The serum titer of anti-Anisakis antibody was 1.68 (normal range < 1.50), and the patient was formally diagnosed with intestinal anisakiasis. No signs of the recurrence of the abdominal symptoms were observed, and

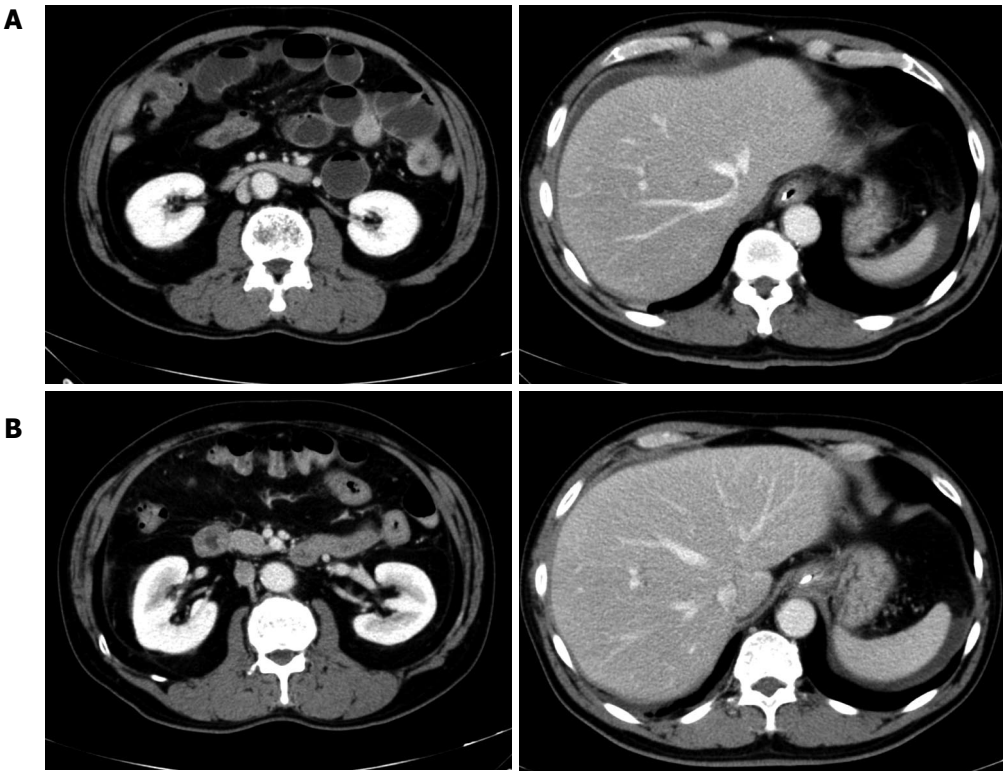

Figure 2 Abdominal computed tomography scan. A: Showing swelling of the partial segment of the small bowel and dilatation of the intestine (upper left) and ascites around liver and spleen (upper right); B: Demonstrating improvement in the partial swelling of the small bowel and the dilatation of the intestine and ascites collection.

the patient was discharged on the tenth hospital day.

Case 2

A 38-year-old male experienced abdominal pain that started abruptly. The abdominal pain was not relieved after the intake of an antacid. Therefore, he presented to the ER of our hospital. The patient had no particular past medical and family history. He had smoked 20 cigarettes/d for the past 18 years, and was a regular drinker.

The patient was alert and his vital signs were normal. On physical examination, tenderness was revealed in the upper abdomen. Laboratory examinations were normal except for a slight increase in white blood cells (WBC, 15460 cells/μL; Table 1). Abdominal X-ray showed gaseous dilatation of the small intestine (Figure 3A). An abdominal CT scan demonstrated swelling of the partial segment of the small bowel and dilatation of the intestine with fluid collection on the oral side of the lesion (Figure 3B). A slight amount of ascites was detected in the Douglas pouch.

The patient was admitted to our hospital, and conservative therapy was started. He had taken fresh ika the day before the symptoms developed, and the anti-Anisakis IgG/A antibody titer was 1.88 (normal range < 1.50). On the second hospital day, his abdominal pain had been relieved quite a lot. An oral diet was started from the third day, and he was discharged on the sixth hospital day, confirming no recurrence of the abdominal symptoms.

Case 3

A 47-year-old woman experienced an abdominal pain that started abruptly. The intermittent abdominal pain that was felt every 5-10 min was not relieved after the intake of a non-prescription drug. Thus, she presented to the ER of our hospital. The patient had acute hepatitis A 10 years ago. She neither drank nor smoked.

The patient was alert and her vital signs were normal. On physical examination, tenderness was revealed in the right side of the abdomen. Laboratory examinations were normal except for a slight increase in WBC (11080 cells/μL), and CRP levels (2.52 mg/dL) (Table 1). Abdominal X-ray (Figure 3C) and an abdominal CT scan (Figure 3D) demonstrated findings that were similar to those of the other two cases.

The patient was admitted to our hospital, and conservative therapy was started after a gastric tube was inserted into her stomach. She had taken fresh katsuo 2 d before she had the symptoms, and the anti-Anisakis IgG/A antibody titer was 1.91 (normal range < 1.50). On the second hospital day, the abdominal pain was relieved. On the third day, the gastric tube was removed, and an oral diet was started from the fifth day. She was discharged on the ninth hospital day, confirming no recurrence of the abdominal symptoms

DISCUSSION

The adult anisakis lives in the intestine of marine mam-

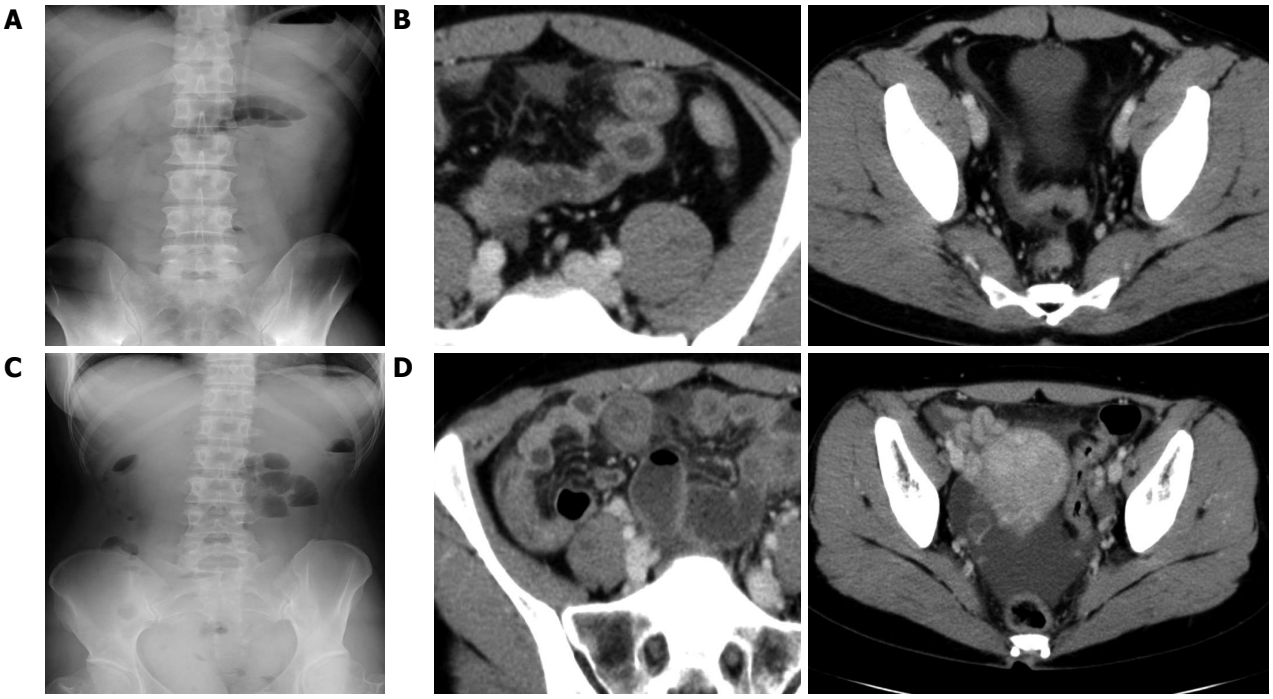

Figure 3 Abdominal X-ray (A, C) and computed tomography scan (B, D).

mals, such as whales and dolphins. The eggs that they lay, come out and spread in the ocean when these mammals defecate. L2 larvae hatch out-of the eggs and swim freely in the water. These larvae are ingested by crustaceans in which, they mature into L3 larvae. These crustaceans are eaten by fish and squid, which are finally eaten by humans and marine mammals. For those that are eaten by marine mammals, the larvae molt and mature into the adult worms, which lay eggs and enter into the life cycle again. Larvae eaten by humans cause anisakiasis.

Anisakiasis was first reported by Van Thiel *et al*[11] Since then, many cases have been reported in different parts of the world. Because of the tradition of eating raw fish (sushi and sashimi) in Japan, the incidence of anisakiasis is higher than that in other countries (2000-3000 cases per year according to the Ministry of Labor, Health, and Social Welfare).

The most common symptom of anisakiasis is severe abdominal pain, for which the patient often requires painkillers, as observed in case 1. Anisakiasis is diagnosed by the detection of the worm sticking to the gut wall. However, as far as intestinal anisakiasis is concerned, diagnosis is difficult because the small intestine is an inaccessible zone for endoscopy, although capsule endoscopy or double balloon endoscopy can be performed in a very few institutions. The only method for diagnosing anisakiasis, other than endoscopy, is immunological examination (*e.g.*, by examining Anisakis-specific IgA, IgG, and IgE). The sensitivity is as high as 70%-80%[12,13], but the results take time (a week) and, therefore are not at all helpful in the clinical field.

Because abdominal pain is severe and abrupt and the findings of clinical imaging are very remarkable, intestinal anisakiasis is often misdiagnosed as acute abdomen or intestinal obstruction and is treated surgically. According to the report by Ishikura *et al*[14] intestinal anisakiasis was correctly diagnosed postoperatively in only 23% cases, whereas 38% cases were acute appendicitis, 12% were intestinal obstructions and 10% were acute celiopathy. Anisakis larvae survive only for a few days in the intestinal tract of humans. Therefore, the symptoms due to acute inflammation subside within 2-3 wk and are treated well by conservative therapy[15]. However, there have been cases of strangulation or severe long segmental stenosis of the intestine caused by Anisakis, which of course required surgical therapy[10,16]. However, there are cases involving acute abdominal pain that are not intestinal anisakiasis. They should be correctly diagnosed and treated surgically.

Thus, we herein suggest methods to correctly diagnose intestinal anisakiasis and avoid unnecessary surgical intervention.

Collecting correct details of clinical history is the first step required for the correct diagnosis of intestinal anisakiasis. The onset of intestinal anisakiasis varies from 1-7 d after the ingestion of the raw fish and differs from that of gastric anisakiasis, which develops symptoms a few hours after the ingestion of raw fish[1,17]. However, there are cases in which the patients forget the food they ate, and thus, we should be very careful in collecting their clinical history details.

Accurate reading of the findings of the clinical images is the second step that is required for making a correct diagnosis of intestinal anisakiasis. CT shows swelling of a partial segment of the small bowel, dilatation of the intestine with fluid collection on the oral side of the

lesion, and collection of ascites, as observed in all three cases experienced[8,10,18]. Ultrasound shows marked local edema of Kerckring's fold, which is known as the corn sign, dilatation of the oral portion of the small intestine with fluid accumulation, and accumulation of ascites. We did not use ultrasound during the diagnostic procedure in our cases, but integrating the information from different modalities of images would certainly increase the rate of correct diagnosis.

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CASE REPORT

A completely isolated intestinal duplication cyst mimicking ovarian cyst torsion in an adult

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Abstract

Intestinal duplications are rare congenital anomalies that can occur anywhere in the gastrointestinal tract. They are most commonly located in the ileum and are usually detected in infancy or early childhood. Duplicated segments are usually firmly attached to and sometimes communicate with the normal gastrointestinal tract. Rarely, intestinal duplications are completely isolated, thus not associated at all with any part of the gastrointestinal tract. Such duplications do not share a common blood supply with the adjacent normal intestinal segment, unlike the usual form of duplication, but rather have a separate vascular pedicle. Reports of completely isolated duplication cysts in adults are extremely rare; we found only five such reports in the English-language medical literature. Here, we report a case of a completely isolated duplication cyst 12 cm long in an adult female. The cyst had no connection to any part of the intestinal tract and had a dedicated vascular pedicle.

Key words: Congenital abnormalities; Digestive system; Duplication; Cysts; Adult

Core tip: Intestinal duplications are rare congenital anomalies generally detected in infancy or early childhood. Duplicated segments are usually firmly attached to and sometimes communicate with the gastrointestinal tract. Rarely, intestinal duplications are completely isolated, thus not associated at all with the normal gastrointestinal tract. Such duplications do not share a common blood supply with the adjacent normal intestine, unlike the usual type of duplication. Reports on completely isolated duplication cysts in adults are extremely rare; we found only five in the English-language literature. Here, we report a case of a completely isolated duplication cyst mimicking an ovarian cyst in an adult female.

Park JY, Her KH, Kim BS, Maeng YH. A completely isolated intestinal duplication cyst mimicking ovarian cyst torsion in an adult. *World J Gastroenterol* 2014; 20(2): 603-606 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i2/603.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i2.603

INTRODUCTION

Enteric duplication cysts are uncommon congenital anomalies that occur anywhere from the tongue to the anus[1]. Such cysts occur most commonly in the small bowel and about half are in the mesenteric border of the ileum[1-3]. Such cystic duplications communicate only rarely with the intestinal lumen although the cysts are

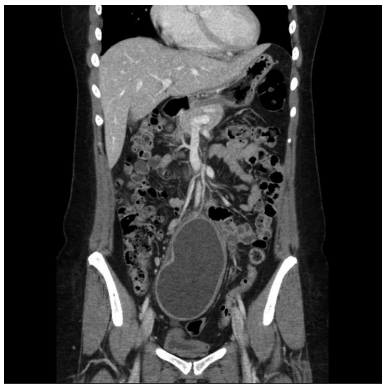

Figure 1 A contrast-enhanced coronal computer tomography image reveals a well-circumscribed and poorly attenuated dumbbell-shaped mass exhibiting peripheral enhancement and a trilaminar appearance in the pelvic cavity.

attached to the intestine and may even share a common wall with the adjacent alimentary tract[2,4,5]. The duplication cysts usually share a blood supply with the adjacent normal bowel[4]. However, completely isolated duplication cysts are not attached to any part of the intestine and have their own vascular supply[4]. They are usually detected in infancy and early childhood; adult patients are extremely rare. In the present report, we describe an exceptionally rare case of a completely isolated duplication cyst in an adult female.

CASE REPORT

A 36-year-old female patient presented with a 10-d history of abdominal pain. Clinical examination revealed pain and tenderness around the periumbilical area and a semi-mobile palpable mass in the lower abdomen. Her past medical history included two Cesarean sections and colchicine treatment for Behçet's disease, diagnosed before she attained the age of 20 years.

A contrast-enhanced abdominal computed tomography (CT) scan revealed a well-circumscribed and poorly attenuated lobulated mass approximately 12 cm long, located in the pelvic cavity (Figure 1). The mass exhibited peripheral enhancement and had a layered appearance. The center of the mass was of uniformly low attenuation (10 Hounsfield units) indicating the presence of a cystic cavity. The mass displaced the terminal ileum in an inferior direction. No right-side ovary was detected either ultrasonographically or on the CT scan. Ovarian cyst torsion was thus suggested both clinically and radiologically.

Upon laparotomy, a large cystic mass was located on the mesentery of the terminal ileum. The mass was not connected or attached to any part of the adjacent intestinal tract. The mass was dissected completely free of surrounding soft tissue without disturbing any normal intestinal or mesenteric structure. The mass had a dedicated vascular pedicle, which was simply ligated and removed. An enlarged lymph node was located in the vicinity of the cystic mass.

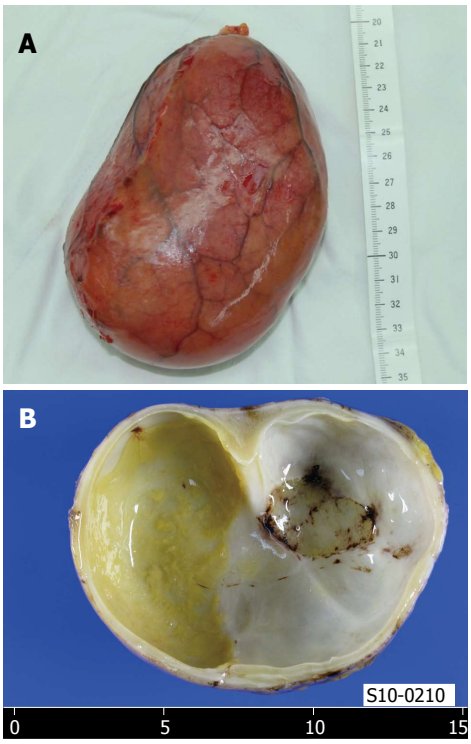

Figure 2 Gross findings of the resected mass. The 12-cm-long cystic mass has a smooth pinkish outer surface (A), and the cavity is uninoculated and incompletely septated (B).

The resected specimen was of dimensions 12 cm × 8.5 cm × 6 cm and weighed 413 g. The external surface was smooth and pinkish-gray in color (Figure 2A). When the specimen was opened, the cyst was found to be uninoculated and partly septated. The cavity contained a clear mucous fluid and a quantity of dirty-yellowish sludge-like material. The cystic wall was pale-grayish and smooth, and was relatively even in thickness (up to 0.5 cm; Figure 2B). Microscopically, the cystic wall consisted of mucosa, submucosa, two layers of smooth muscle, and the serosa. The mucosal layer was largely covered by simple cuboidal-to-columnar mucous epithelium and partly by a gastric-type mucosa, including oxyntic glands (Figure 3). Islands of myenteric plexus were also present between the two layers of muscle. No epithelial dysplasia or malignancy was evident.

The postoperative course was uneventful and the patient was discharged without any complications.

DISCUSSION

Intestinal duplication cysts are rare congenital anomalies that occur in one of every 4000-5000 live births[5]. Isolated duplications occur much less frequently, in approximately 1 in 10000 live births[6]. Duplications are most commonly detected in infancy and early childhood when symptoms such as abdominal pain, obstruction, or vomiting develop[7]. The duplications occur most frequently in the small bowel and over half are ileal duplications[3]. Enteric duplications are tubular or cystic in structure and

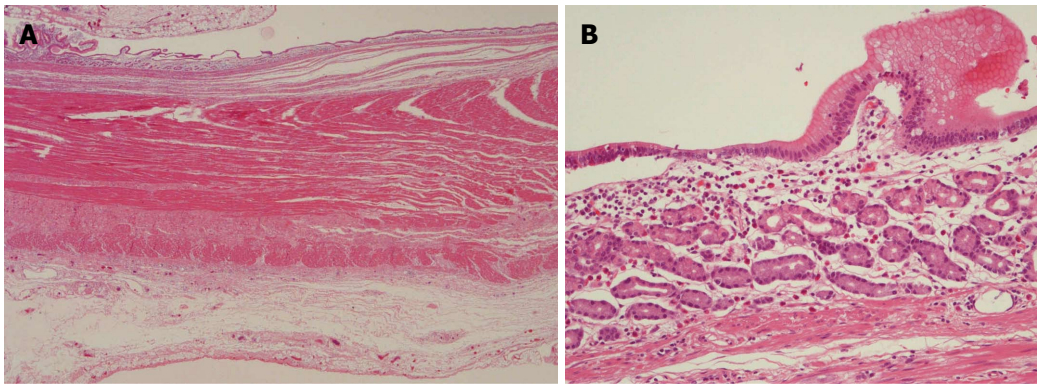

Figure 3 Microphotographs of the cystic wall. The wall consists of mucosa, submucosa, muscle layers, and serosa (A, HE stain, × 10), with regions of gastric-type mucosa containing oxyntic glands (B, HE stain, × 100).

Table 1 Summary of reports of completely isolated enteric duplication cysts in adults

Ref.	Age (yr)	Gender	Clinical feature	Dimensions (cm)	Site	Mucosal type
Blank *et al*[3]	51	M	Incidental	10 × 4	Mesentery of the ileum	Villi, crypts, numerous mucous cells
Kim *et al*[8]	28	M	Incidental	Not mentioned	Mesentery of the Treitz ligament	Gastric
Nichols *et al*[9]	27	F	Abdominal fullness	9 × 6 × 1	Mesentery of the descending colon	Simple columnar mucinous epithelium
Lee *et al*[10]	21	F	Palpable mass	3.5 × 2.5	Mesentery in the vicinity of the round ligament	No epithelial lining
Kyriakos *et al*[11]	20	M	Abdominal pain and fever	7 × 4	Lateral region of the ascending colon	Not mentioned
Present case	36	F	Abdominal pain	12 × 8.5 × 6	Mesentery of the terminal ileum	Mixed

M: Male; F: Female.

contain intestinal mucosa and muscle layers. The wall is usually attached to some part of the gastrointestinal tract and often shares a common muscle or serosal layer with the normal intestine of the region[4].

Completely isolated enteric duplication cysts are not connected or attached to any part of the intestinal tract. Such cysts have dedicated vascular pedicles, whereas the usual form of duplication cysts shares a blood supply with the adjacent bowel. Inevitably, then, the bowel must be resected upon cyst removal. Our 36-year-old female patient had a completely isolated enteric duplication cyst that was dissected without disturbing her intra-abdominal anatomy. Reports of completely isolated enteric duplication cysts in adults are extremely rare; only five cases have been described in the English-language medical literature[3,8-11] (Table 1). The cause of intestinal duplication is believed to be vascular compromise during early organogenesis. However, the precise mechanism remains to be elucidated[5].

Histopathologically, the wall of a duplication cyst is composed of a lining epithelium and thin layers of muscle[9]. The mucosa is often similar to that of the region of the bowel to which the cyst is attached[5], but some heterotopic mucosa is also commonly found[3,8,12]. In our case, the cystic mass was largely covered by a single layer of simple cuboidal-to-columnar mucous epithelium with some areas of gastric-type mucosa, including oxyntic glands. The wall contained two layers of smooth muscle and some islands of myenteric plexus were noted between the muscle layers. Thus, the wall was similar in structure to that of the normal intestine.

Enteric duplications are usually detected within the first year of life when infants present with abdominal pain, a palpable mass, or intestinal obstruction[1]. In some adult cases, however, the duplications are asymptomatic, remaining undiagnosed for years. Adenocarcinoma may arise in such adult asymptomatic cysts[3,13,14]. In the present case, our patient reported abdominal pain that we could not readily explain. Surface ulcers, distension of the cystic cavity, or impending vascular insufficiency, are among several possible causes. No dysplastic change or malignant transformation was evident.

Diagnosis of a duplication cyst is difficult even with the aid of modern imaging techniques such as CT or magnetic resonance imaging, especially when no structural connection exists between the cyst and the normal bowel. A recent report suggested that a barium meal could be used to differentiate noncommunicating from communicating cysts[7]. The cited authors reported that the alimentary tract was compressed by a noncommunicating cyst, whereas communicating cysts were themselves filled with contrast. However, it remains difficult to diagnose an enteric duplication cyst because the differential diagnosis includes all intra-abdominal cystic

masses, including mesenteric and omental cysts, pancreatic pseudocysts, and ovarian cysts[1,2,8]. In the present case, ultrasonography and CT suggested the possibility of an ovarian cyst rather than a rare intestinal duplication cyst. There has been one report of torsion of an ovarian cyst mimicking an enteric duplication cyst[15]. The author said that non-enteric cysts may have double-layered features on ultrasound, which are commonly found in duplication cysts.

No consensus has been reached on treatment of asymptomatic duplication cysts. However, surgical management of asymptomatic adult cases is recommended because various potential complications, including malignancy, may arise. Exploratory laparotomy may be indicated if the preoperative diagnosis is unclear. Completely isolated duplication cysts can be treated *via* simple ligation and division of the pedicle; no bowel resection is required[4]. A successful laparoscopic excision of a completely isolated enteric duplication cyst was reported recently[9]. We performed simple excision of the cystic mass without disturbing the normal intraperitoneal anatomy.

COMMENTS

Clinical diagnosis

A 36-year-old female patient presented with a 10-d history of abdominal pain and a palpable mass in the lower abdomen.

Differential diagnosis

Ultrasonography and computed tomography (CT) scan was performed for differential diagnosis between ovarian cyst and other intra-abdominal cystic lesions.

Laboratory diagnosis

Her complete blood count, blood chemistry, and urinalysis were within normal limits except mild anemia (Hb, 11.3g/dL; Hct, 33.9%).

Imaging diagnosis

A contrast-enhanced abdominal CT scan revealed a well-circumscribed and poorly attenuated lobulated mass having peripheral enhancement and a cystic cavity in the pelvis.

Pathological diagnosis

Microscopically, the cystic wall consisted of mucosa, submucosa, two layers of smooth muscle, and the serosa.

Treatment

A simple cystectomy was performed.

Experiences and lessons

A completely isolated intestinal duplication cyst should be included in the differential diagnosis of an intra-abdominal cystic lesion.

Peer review

The article presents an extremely rare but important case so that the readers could use the information in the practice. However, the pathogenesis of the disease was not fully investigated in this report.

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CASE REPORT

ERCP for patients who have undergone Billroth Ⅱ gastroenterostomy and Braun anastomosis

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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) is efficacious in patients who have undergone Billroth Ⅱ gastroenterostomies, but the success rate decreases in patients who also have experienced Braun anastomoses. There are currently no reports describing the preferred enterography route for cannulation in these patients. We first review the patient's previous surgery records, which most often indicate that the efferent loop is at the greater curvature of the stomach. We recommend extending the duodenoscope along the greater curvature of the stomach and then advancing it through the "lower entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater. Ten patients who had each undergone Billroth Ⅱ gastroenterostomy and Braun anastomosis between January 2009 and December 2011 were included in our study. The overall success rate of enterography was 90% for the patients who had undergone Billroth Ⅱ gastroenterostomy and Braun anastomosis, and the therapeutic success rate was 80%. We believe that this enterography route for ERCP is optimal for a patient who has had Billroth Ⅱ gastroenterostomy and Braun anastomosis and helps to increase the success rate of the procedure.

Key words: Optimal enterography route; Endoscopic retrograde cholangiopancreatography; Billroth Ⅱ; Gastroenterostomy; Braun anastomosis

Core tip: We recommend extending the duodenoscope along the greater curvature of the stomach and then advancing it through the "lower entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater. We believe that this enterography route for endoscopic retrograde cholangiopancreatography is optimal for a patient who has undergone Billroth Ⅱ gastroenterostomy and Braun anastomosis and helps to increase the success rate of the procedure.

Wu WG, Gu J, Zhang WJ, Zhao MN, Zhuang M, Tao YJ, Liu YB, Wang XF. ERCP for patients who have undergone Billroth Ⅱ gastroenterostomy and Braun anastomosis. *World J Gas-*

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is efficacious in patients having undergone Billroth Ⅱ gastroenterostomies, but the success rate decreases in patients who also have had Braun anastomoses. A previous study reported success rates of 83% in patients with simple Billroth Ⅱ gastroenterostomies but only 29% in patients with both a Billroth Ⅱ gastroenterostomy and an additional Braun anastomosis[1]. ERCP failures in such patients are caused by difficulties entering the afferent loop and accessing the papilla[2-4]. There are currently no reports describing the preferred enterography route for cannulation in these patients. Herein, we describe our method for identifying the optimal enterography route for ERCP in patients with both a Billroth Ⅱ gastroenterostomy and a Braun anastomosis.

CASE REPORT

In this study, we included patients who had had Billroth Ⅱ gastroenterostomies and Braun anastomoses and in whom therapeutic biliary intervention was planned between January 2009 and December 2011 in the ERCP unit in the Department of General Surgery, Xin Hua Hospital, which is affiliated with the Shanghai Jiaotong University School of Medicine. During this period, 1659 ERCP procedures were performed. Patients who had normal anatomies, Billroth Ⅰ / Ⅱ gastroenterostomies, Roux-en-Y gastroenterostomies, and choledochoduodenostomies were excluded from the study. A total of 10 patients who had undergone Billroth Ⅱ gastroenterostomies and Braun anastomoses were included in the study. These 10 patients [2 women and 8 men; mean age: 70.8 years (range: 55-83 years)] were admitted to our hospital as a result of upper right quadrant pain, fever and jaundice. Magnetic resonance cholangiopancreatography revealed intra- and extra-hepatic bile duct dilation (common bile duct stones were presented in all patients).

When conducting the procedure, we first review the patient's previous surgery records, which most often indicate that the efferent loop is at the greater curvature of the stomach. One major challenge is distinguishing between the afferent and efferent loops. Our solution is to extend the duodenoscope along the greater curvature of the stomach until the gastrojejunal anastomosis becomes visible, from which perspective the "lower entrance" is the entrance to the right efferent loop (Figure 1A). We are occasionally able to draw back the duodenoscope to "relax" the gastrojejunal anastomosis and thus differentiate the "upper entrance" from the "lower entrance." The efferent loop makes a better entrance for the duodenoscope because it is less angulated than the afferent loop.

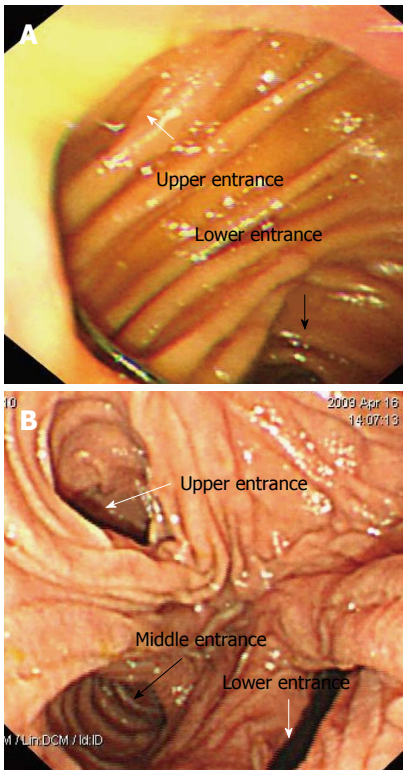

Figure 1 The gastrojejunal anastomosis is detected at the distal end of the stomach, and 2 stomal openings corresponding to an end-to-side anastomosis can be identified endoscopically. If the efferent loop was constructed at the greater curvature of the stomach in the previous surgery, the "lower entrance" is the entrance to the right efferent loop (A). Three stomal openings can be identified endoscopically at the site of the Braun anastomosis, and the "middle entrance" leads to the appropriate loop to reach the papilla of Vater. The "middle entrance" is unique irrespective of the endoscopic approach used (B).

Three stomal openings can be visualized endoscopically, but identifying the correct entrance is a major challenge (Figure 1B). The "middle entrance" is the entrance to the loop that can be used to reach the papilla of Vater when the endoscope is advanced from the efferent loop and is unique irrespective of the endoscopic approach used.

Ten patients who had each undergone a Billroth Ⅱ gastroenterostomy and a Braun anastomosis were included in our study. The overall success rate of enterography was 90% for the patients who had Billroth Ⅱ gastroenterostomies and Braun anastomoses, and the therapeutic success rate was 80%. One patient's procedure was unsuccessful because of a failure to access the papilla (due to a long afferent loop), and cannulation failure occurred another patient.

DISCUSSION

Many endoscopists enter the afferent loop *via* the site of the gastrojejunal anastomosis, but the sharp angulation caused by adhesions may make it impossible to advance the endoscope into the afferent loop. Premature entry into the afferent loop at the gastrojejunal anastomosis is

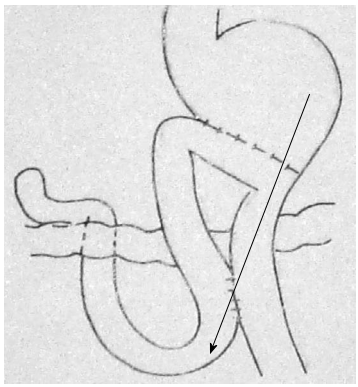

Figure 2 The duodenoscope should be extended along the greater curvature of the stomach and then advanced through the "lower entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater.

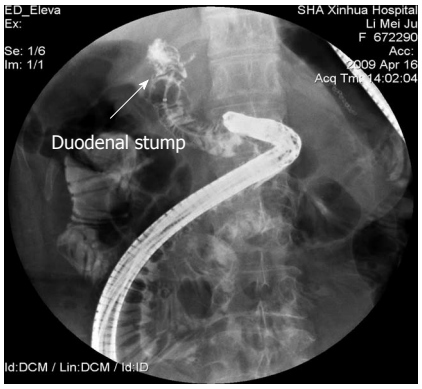

Figure 3 Retrieval-balloon-assisted enterography. A catheter is advanced into the middle limb and contrast injected into the loop to confirm that the limb is the duodenal stump.

the main cause of failure to access the papilla. We avoid this by extending the duodenoscope along the efferent loop until the Braun anastomosis is visible. We recommend extending the duodenoscope along the greater curvature of the stomach and then advancing it through the "lower entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater. We believe that this enterography route for ERCP is optimal for patients with Billroth Ⅱ gastroenterostomies and Braun anastomoses (Figure 2) and helps to increase the success rate of the procedure. Ten patients who received Billroth Ⅱ gastroenterostomies and Braun anastomoses were included in our study. The overall success rate of enterography was 90% for the patients who had undergone Billroth Ⅱ gastroenterostomies and Braun anastomoses, and the therapeutic success rate was 80%.

When the efferent loop is located at the lesser curvature of the stomach, we recommend extending the duodenoscope along the greater curvature of the stomach and then advancing it through the "upper entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater. If the previous surgery records do not specify the location of the efferent loop, we extend the duodenoscope along the greater curvature of the stomach until the gastrojejunal anastomosis is visible and then advance it through the "lower entrance" and along the jejunal loop until it reaches the 3 stomal openings at the site of the Braun anastomosis. We use a catheter (usually the wire-guided retrieval balloon that is used to remove the common bile duct stone) to explore the middle limb and inject contrast into the loop to confirm that the limb is the duodenal stump (Figure 3) and have therefore termed this procedure "retrieval-balloon-assisted enterography"[5,6]. It should be emphasized that when performing ERCP in patients with postsurgical anatomical changes, endoscopic guidance is insufficient and should be supplemented with radiographs. If the middle limb into which the catheter was advanced is the distal jejunum rather than the duodenal stump, the duodenoscope should be retracted to the gastrojejunal anastomosis and then advanced through the "upper entrance," along the limb, and then into the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater.

COMMENTS

Case characteristics

Ten patients were admitted to their hospital as a result of upper right quadrant pain, fever and jaundice.

Clinical diagnosis

Upper right quadrant pain, fever and jaundice.

Differential diagnosis

Magnetic resonance cholangiopancreatography.

Laboratory diagnosis

Liver function tests were outside normal limits.

Imaging diagnosis

Magnetic resonance cholangiopancreatography revealed intra- and extra-hepatic bile duct dilation (common bile duct stones were presented in all patients).

Treatment

Endoscopic retrograde cholangiopancreatography.

Related reports

Endoscopic retrograde cholangiopancreatography (ERCP) is efficacious in patients having undergone Billroth Ⅱ gastroenterostomies, but the success rate decreases in patients who also have had Braun anastomoses.

Term explanation

Braun anastomosis is an anastomosis between the afferent and efferent loops of the jejunum after a loop gastroenterostomy.

Experiences and lessons

The authors recommend extending the duodenoscope along the greater curvature of the stomach and then advancing it through the "lower entrance" at the site of the gastrojejunal anastomosis, along the efferent loop, and through the "middle entrance" at the site of the Braun anastomosis to reach the papilla of Vater. The authors believe that this is the optimal enterography route for ERCP in patients having undergone Billroth Ⅱ gastroenterostomies and a Braun anastomoses.

Peer review

In this paper, Wu *et al* describe a method for identifying the optimal route for ERCP in patients with both a Billroth Ⅱ gastroenterostomy and a Brown anastomosis. The methods used appear feasible and useful to increase success rate of ERCP in such setting. The figures are a good argument for their methods. The manuscript can be accepted for publication.

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Fecal calprotectin in coeliac disease

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Abstract

We would like to share with the readers the results of our experience in 50 celiac disease (CD) patients, enrolled between September 2012 and April 2013, who were referred to our third-level CD Unit. The fecal calprotectin (FC) concentration of 50 adults with newly diagnosed CD was compared to that of a control group of 50 healthy subjects. FC level was determined by enzyme linked immunosorbent assay with diagnostic cut-off of 75 μg/g. In addition, we tried to correlate the FC level with symptoms, histological severity of CD (Marsh grade) and level of tissue transglutaminase antibodies (aTg) in CD patients. Finally, FC level was increased in five CD patients and in four controls (10% *vs* 8%, *P* = NS); mean FC concentration of patients and controls were 57.7 (SD ± 29.1) and 45.1 (SD ± 38.4) respectively. Furthermore, no significant correlation was seen between FC levels and symptoms/Marsh grade/aTg. The five CD patients did not show inflammatory lesions (*e.g.,* ulcers, erosions) at upper endoscopy. The four healthy controls with positive FC were followed-up for further six months; in this observational period they did not show clinical signs of any underlying disease. On these bases, we think that FC is not able to investigate the subclinical inflammatory changes of active CD and FC should be considered a useless tool in the diagnostic work-up of uncomplicated CD but it should be accompanied by aTg when ruling out organic disease in patients with irritable bowel syndrome.

Key words: Coeliac disease; Calprotectin; Bowel inflammation; Small bowel

Core tip: High levels of fecal calprotectin (FC) have been found in several gastrointestinal diseases but only few and discordant reports investigated the role of FC in celiac disease (CD). So, we would like to share with the readers the results of our experience in 50 CD patients, who were referred to our third-level CD Unit. We think that FC is not able to investigate the subclinical inflammatory changes of active CD and FC should be considered a useless tool in the diagnostic work-up of uncomplicated CD but it should be accompanied by aTg when ruling out organic disease in patients with IBS.

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TO THE EDITOR

In recent years many studies have explored the role of fecal calprotectin (FC) in detecting the presence of bowel inflammation[1]. High levels of FC have been found in several gastrointestinal diseases but only few and discordant reports investigated the role of FC in celiac disease (CD). In effect, a first report by Montalto *et al*[2] focused on FC concentrations in 28 untreated CD patients compared with a control group of healthy subjects. The authors

reported that FC level of CD patients do not differ significantly from those in controls. On the contrary, the paediatric study by Balamtekın *et al*[3] found that FC level was higher in children with newly diagnosed CD compared to that of CD patients under gluten free diet and healthy controls. Similar results were also found by Ertekin *et al*[4].

So, we would like to share with the readers the results of our experience in 50 CD patients, enrolled between September 2012 and April 2013, who were referred to our third-level CD Unit. The FC concentration of 50 adults with newly diagnosed CD was compared to that of a control group of 50 healthy subjects. FC level was determined by enzyme linked immunosorbent assay with diagnostic cut-off of 75 μg/g. In addition, we tried to correlate the FC level with symptoms, histological severity of CD (Marsh grade) and level of tissue transglutaminase antibodies (aTg) in CD patients. Finally, FC level was increased in five CD patients and in four controls (10% *vs* 8%; P = NS); mean FC concentration of patients and controls were 57.7 (SD ± 29.1) and 45.1 (SD ± 38.4) respectively. Furthermore, no significant correlation was seen between FC levels and symptoms/Marsh grade/aTg. The five CD patients did not show inflammatory lesions (*e.g.*, ulcers, erosions) at upper endoscopy. The four healthy controls with positive FC were followed-up for further six months; in this observational period they did not show clinical signs of any underlying disease. On these bases, we think that FC is not able to investigate the subclinical inflammatory changes of active CD and FC should be considered a useless tool in the diagnostic work-up of uncomplicated CD but it should be accompanied by aTg when ruling out organic disease in patients with irritable bowel syndrome.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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

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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/index.htm

Patent (list all authors)

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

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