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EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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FRONTIER

Latest developments in precancerous lesions of hepatocellular carcinoma

Zhao-Shan Niu, Xiao-Jun Niu, Wen-Hong Wang, Jing Zhao

Zhao-Shan Niu, Laboratory of Micromorphology, Medical College of Qingdao University, Qingdao 266071, Shandong Province, China

Xiao-Jun Niu, Clinical Medicine Specialty, Medical College of Qingdao University, Qingdao 266071, Shandong Province, China

Wen-Hong Wang, Department of Pathology, Medical College of Qingdao University, Qingdao 266071, Shandong Province, China

Jing Zhao, Department of Pathology, the Affiliated Hospital of Qingdao University, Qingdao 266061, Shandong Province, China

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Correspondence to: Zhao-Shan Niu, MD, Laboratory of Micromorphology, Medical College of Qingdao University, Room 201, Building Boya, No. 308 Ningxia Road, Qingdao 266071, Shandong Province, China. niumiao1993@hotmail.com
Telephone: +86-532-83780012
Fax: +86-532-83780012

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Abstract

Hepatocarcinogenesis in human chronic liver diseases is a multi-step process in which hepatic precancerous lesions progress into early hepatocellular carcinoma (HCC) and progressed HCC, and the close surveillance and treatment of these lesions will help improve the survival rates of patients with HCC. The rapid development and extensive application of imaging technology have facilitated the discovery of nodular lesions of ambiguous significance, such as dysplastic nodules. Further investigations showed that these nodules may be hepatic precancerous lesions, and they often appear in patients with liver cirrhosis. Although the morphology of these nodules is not sufficient to support a diagnosis of malignant tumor, these nodules are closely correlated with the occurrence of HCC, as indicated by long-term follow-up studies. In recent years, the rapid development and wide application of pathology, molecular genetics and imaging technology have elucidated the characteristics of precancerous lesions. Based on our extensive review of the relevant literature, this article focuses on evidence indicating that high-grade dysplastic nodules are more likely to transform into HCC than low-grade dysplastic nodules based on clinical, pathological, molecular genetic and radiological assessments. In addition, evidence supporting the precancerous nature of large cell change in hepatitis B virus-related HCC is discussed.

Key words: Hepatocellular carcinoma; Precancerous lesions; High-grade dysplastic nodule; Large cell change; Small cell change

Core tip: The identification and characteristics of hepatic precancerous lesions may serve as early clues to malignant transformation. Over the last 10 years, studies of precancerous lesions have resulted in significant progress, especially in molecular biology and

imaging technology. Based on our extensive review of the relevant literature, this article focuses on evidence that supports the precancerous nature of dysplastic foci and dysplastic nodules from a clinical, pathological, molecular genetic and radiological point of view.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death around the world, and it is clinically characterized by a high incidence rate and poor prognosis. Despite the progress made in numerous treatments, the survival rate of HCC patients remains low because HCC is not easily detected prior to the advanced stage. Therefore, studies on the nature of precancerous lesions of HCC are important.

Hepatic precancerous lesions are currently recognized as nodular lesions that result from cirrhosis, and they are divided into two levels based on cytological and histological changes: microscopic dysplastic foci (DF) and macroscopic dysplastic nodules (DNs). DF are further classified as exhibiting large cell change (LCC) or small cell change (SCC)[1], the latter of which is widely observed in various precancerous lesions[2,3]. Specifically, molecular biology studies have shown SCC to be an intermediary stage between hepatocytes and malignant cells. DNs are further subdivided into low-grade dysplastic nodules (LGDNs) and high-grade dysplastic nodules (HGDNs) based on the degree of atypia[4], and the latter has a higher risk of malignant transformation.

Recent advances in pathology, molecular biology, genetics, and radiology have tremendously improved our understanding of hepatic precancerous lesions. Based on our extensive review of the relevant literature, this article focuses on the evidence showing that HGDNs are more likely to transform into HCC than LGDNs based on clinical, pathological, molecular genetic and radiological assessments.

DF

DF are microscopic lesions with an arbitrary diameter less than 1 mm, and they usually occur in a background of cirrhosis or chronic hepatitis[4]. DF often exhibit LCC (Figure 1A) or SCC (Figure 1B). According to a recent study[5], LCC is defined as hepatocytes displaying enlarged nuclei or cytoplasms that preserve their nuclear-cytoplasmic ratio, whereas SCC is defined as hepatocytes that exhibit decreased cytoplasmic volume, cytoplasmic basophilia, mild nuclear pleomorphism, hyperchromasia, and an increased nuclear-cytoplasmic ratio. Consequently, lesions with SCC are characterized by crowded nuclei and an increased cellular density.

LCC

LCC is currently thought to be a degenerative senescent change that results from chronic liver injury, but it could also be regarded as a predictive marker associated with HCC rather than a genuine premalignant lesion[6,7]. Conversely, LCC may actually be an important risk factor for HCC based on the following findings: (1) The patients with chronic hepatitis B exhibiting LCC were overall much more likely to develop HCC than patients without LCC[8]; (2) In most cases of human B viral chronic hepatitis/cirrhosis, hepatocytes exhibiting LCC had higher levels of proliferating cell nuclear antigen (PCNA)-labelling index (LI) and lower levels of transferase-mediated dUTP-biotin nick end labeling (TUNEL)-LI than cells without LCC[9], which suggests that HBV-related LCC is actively involved in hepatocarcinogenesis; (3) Abnormal DNA content (aneuploidy) is common in LCC, but this abnormality does not significantly differ from that observed in SCC[10]; (4) LCC and related molecular changes have been identified in a subset of patients infected with HBV, which suggests a direct premalignancy[11]; and (5) Compared with cholestatic LCC, HBV-related LCC exhibits significantly higher Tp53-LI, gamma-H2AX-LI and micronuclei index as well as shorter telomere length, decreased SA-beta-Gal activity and increased net cellular gain[12].

LCC is frequently detected in chronic hepatitis B cases with advanced histologic stage, particularly those with HCC[6]. Taken together, these findings indicate that LCC may not only be related to hepatocarcinogenesis but may be a precancerous lesion of HBV-related HCC.

SCC

SCC has long been thought to be a characteristic of early precancerous lesions of HCC based on the following findings: (1) SCC has frequently been identified in HGDNs and well-differentiated HCC (W-HCC). Specifically, the largest nuclei have been observed in W-HCC, and the nuclear/cytoplasmic ratio is higher in HGDNs and W-HCC than in other nodular lesions. Moreover, hyperchromasia is markedly more pronounced in W-HCC than in other nodules[2]; (2) Mosaicism for loss of X chromosomal inactivation has been demonstrated in nodules of altered hepatocytes (NAH) exhibiting SCC, and an array-based comparative genomic hybridization (array-CGH) analysis revealed that some of the chromosomal abnormalities in NAH with SCC coincided with the abnormalities in HCC[3], suggesting that NAH exhibiting SCC could be precursors of HCC; and (3) SCC is characterized

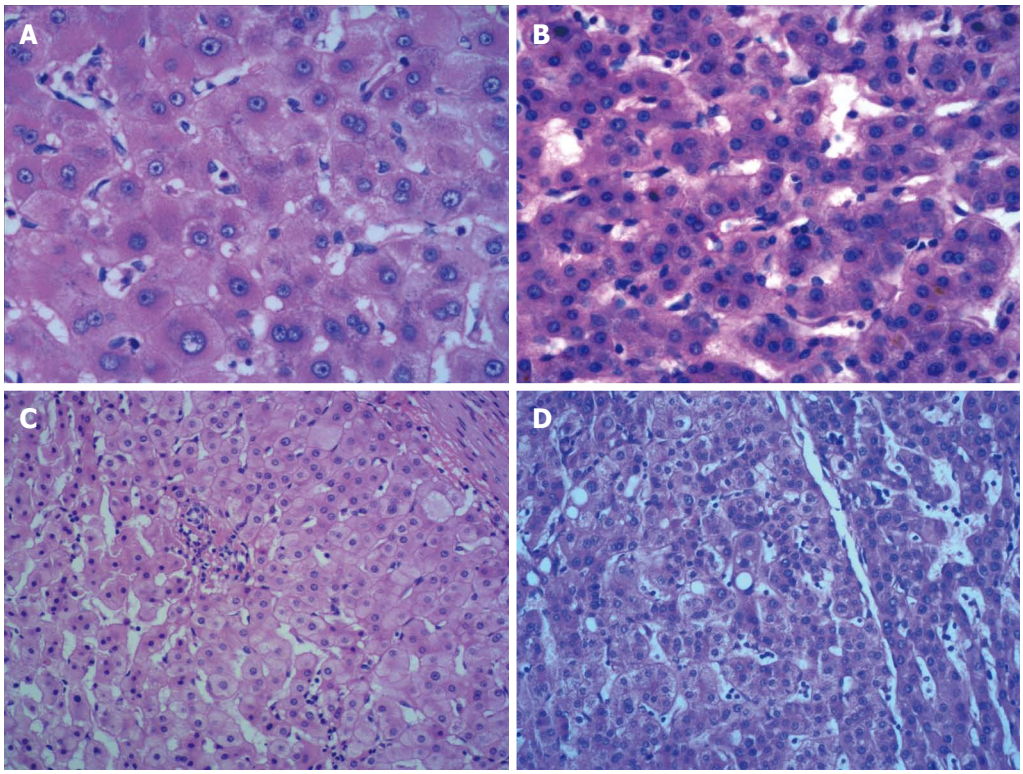

Figure 1 Histological characterization of large cell change (A), small cell change (B), low-grade dysplastic nodule (C) and high-grade dysplastic nodule (D). Large cell change is characterized by cellular and nuclear enlargement with preserved nuclear and cytoplasmic ratio, nuclear pleomorphism and hyperchromasia, and prominent nucleoli (A), and small cell change characterized by decreased cell volume, mild nuclear pleomorphism, an increased nuclear-cytoplasmic ratio, and increased nuclear density (B); Low-grade dysplastic nodule is characterized by minimal cytological atypia, slightly increased nuclear-cytoplasmic ratio and cell density (C), and high-grade dysplastic nodule characterized by high cell density, an increased nuclear-cytoplasmic ratio, hepatocytes organized in trabeculae that are two to three cells thick, and mild nuclear atypia (D). A-D: Hematoxylin-eosin staining, magnification × 200.

by high proliferative activity[13]. Conversely, the cumulative probability of developing HCC did not differ between patients with SCC and those without SCC in one study[8], suggesting that SCC in chronic hepatitis B should not be considered a risk factor for HCC development.

Surprisingly, a crosscheck of the literature revealed inconsistent or contradictory conclusions, even from the same group of authors, between different studies of the same marker. In one study[14], the expression of p21 and p16 in HBV/HCV-related cirrhosis was preserved in LCC, decreased in SCC, and absent in HCC, whereas gammaH2AX-DNA-damage foci were absent in LCC but present in SCC and in HCC. These data suggest that SCC occurs in more advanced precancerous lesions than LCC. In another study[12], the same group of authors found that p21 and p16 were activated in normal-looking cirrhotic hepatocytes (NLCH) of HBV-related cirrhosis and that this expression gradually diminished from LCC to SCC and HCC. They also reported that the expression of gamma-H2AX foci significantly increased during carcinogenesis, from normal hepatocytes to NLCH, LCC, SCC, and HCC. Thus, these data contradict the previous findings and suggest that HBV-related LCC may represent dysplastic hepatocytes rather than reactive hepatocytes. These conflicting findings may be attributed to the use of different racial and regional groups, *i.e.*, HBV (17 cases)/HCV (6 cases)-related cirrhosis samples in the former study and HBV-related cirrhosis samples in the latter study.

The nature of LCC and SCC as characteristics of true precancerous lesions and their relationship to HCC are fiercely debated[1]. Future studies should consider the following factors, which at least partly explain the inconsistency, if not conflict, in previous findings: SCC is a relatively rare finding that could easily be confused with liver regenerative cells. In some studies, the diagnosis of SCC was confirmed by fewer than two pathologists or the authors failed to give a definition and/or example of DF[15,16]. Therefore, SCC is not easily distinguished from liver regenerative cells based on current findings, which may limit the predictive value of SCC for HCC development[17]. Discrepancies in studies may also be due to differences in research methodology and regional groups under study.

Based on current studies, SCC may be a more advanced precancerous lesion than LCC, and LCC may be a very early precancerous lesion of HBV-related HCC. Accordingly, the identification of SCC and LCC in liver biopsies might be related to an increased risk of HCC over time, and such lesions warrant inclusion in pathology reports[18].

Table 1 Summary of the main pathological, molecular genetic and radiological features of distinction between low- and high-grade dysplastic nodules

	LGDNs	HGDNs	e/WD-HCC	Quoted literature examples
Cytologic features				
Small cell change	-	+	+	Roncalli *et al*[30]
Large cell change	±	±	-	Roncalli *et al*[30]
Architectural features				
Nuclear/cyto-plasmic ratio	±	+	+	Chang *et al*[2]
Increased cell density compared with surroundings	-	1.3 to 2 times	> 2 times or more	Park *et al*[4]
Pseudoglands	-	±	+	Nascimento *et al*[31]
Unpaired arteries	±	±	+	Park *et al*[4]
Portal tract	-	+	+	Kojiro *et al*[32]
Hyperchromasia/nuclear atypia	-	+	+	Nascimento *et al*[31]
Radiological features				
Hepatobiliary phase images	No hypointensity	Hypointensity (70%)	Hypointensity (97.5%)	Gatto *et al*[40]
Arterial phase	Iso/hyperintensity (100%)	Iso/hypointense (96.7%)	Iso/hypointense (27.5%)	Gatto *et al*[40]
Washout and arterial enhancement	No washout or arterial enhancement on CT or MRI	6 (6/6)showed arterial enhancement on CT, 4 (4/6) were hypervascular on MRI; 5 (5/6) on CT and 4 (4/6) on MRI	51 (51/74) were hypervascular on CT, 57 (57/74) were hypervascular on MRI; washout was not showed	Serste *et al*[25]
Molecular genetic biomarkers				
Overall FAL index	0.16 ± 0.07	0.33 ± 0.21	0.40 ± 0.23	Lee *et al*[27]
Chromosomal changes	Deletions and gains were not found	Deletions of 8p and gains of 1q	Deletions of 8p and gains of 1q	Tornillo *et al*[45]
TERT promoter mutations	6%	19%	61%	Nault *et al*[50]
TRF length	7.2 ± 1.97	4.0 ± 0.89	4.5 ± 0.85	Oh *et al*[53]
Telomerase activity	0.9 ± 0.56	1.7 ± 0.75	2.3 ± 1.39	Oh *et al*[53]
miRNAs (miR-224)	-	+ (50%)	+ (100%)	Gao *et al*[57]
DNA methylation Nuclear expression of Dnmt3a	-	+ (29.2%)	+ (53.8%)	Choi *et al*[62]
Immunohistological biomarkers				
VEGF	± (100%)	+ (100%)	+ (100%)	Park *et al*[67]
GPC3	± (5.48%)	+ (50%)	+ (57.1%)	Gong *et al*[1]
MN index (number of micronuclei per 3000 hepatocytes)	0.9 ± 0.16	3.1 ± 0.38	4.0 ± 0.58	Lee *et al*[77]

-: absent; ±: may be present; +: usually present. CT: Computed tomography; MRI: Magnetic resonance imaging; FAL: Fractional allelic loss; TERT: Telomerase reverse-transcriptase; TRF: Terminal restriction fragment; Dnmt3a: DNA methyltransferases 3a; VEGF: Vascular endothelial growth factor; GPC3: Glypican-3; MN: Micronucleus; LGDNs: Low-grade dysplastic nodules; HGDNs: High-grade dysplastic nodules; e/WD-HCC: Early/well-differentiated hepatocellular carcinoma.

DYSPLASTIC NODULES

Dysplastic nodules are usually found in chronic liver diseases. These lesions are primarily 1-1.5 cm in diameter and are classified as LGDNs (Figure 1C) or HGDNs (Figure 1D) based on the presence of cytologic and architectural atypia[19]. Pathologically, human HCC develops in the following multistep manner: from LGDNs to HGDNs, early HCC, W-HCC, nodule-in-nodule HCC, and finally, moderately differentiated HCC[20]. In recent years, the concept of multi-step human hepatocarcinogenesis has been well documented[21-23]. Advances in pathology and molecular genetics, as well as clinical follow-ups, have confirmed that DNs are precancerous lesions of HCC[4,24]. In particular, advances in medical imaging technology have provided a more intuitive understanding of the characteristics of DNs by facilitating the detection of such small nodular lesions.

For many years, HGDNs have been thought to be more closely related to HCC than LGDNs based on histopathological features and clinical follow-up studies[2,25,26]. In recent years, advances in pathology, molecular genetics, and imaging have further confirmed that HGDNs are more likely the advanced precursors of HCC than LGDNs[2,27]. The main pathological, molecular genetic and radiological features of distinction between LGDNs and HGDNs are summarized in Table 1.

CLINICAL FOLLOW-UP STUDIES

Clinically, LGDNs are considered precancerous lesions associated with a slightly elevated risk of malignant transformation, whereas HGDNs are considered advanced precursors of HCC associated with a high risk of transformation[4,28]. Moreover, HCCs have been demonstrated to occur more frequently in HGDNs than LGDNs[29].

Clinical follow-up studies have revealed that HGDNs are the most advanced precancerous lesions of the liver, with a risk of malignant transformation of approximately 30%-40% at 24 mo[24]. In another study, a total of 147 patients with non-malignant liver nodules were

followed over a median duration of 29 mo, and the HCC development rate was higher in patients with HGDNs than in patients with LGDNs[26]. Based on a multivariate analysis of histologic diagnosis and decreases in portal flow on computed tomographic arterial portography (CT-AP), the authors classified hepatic nodules into three groups: HGDNs, LGDNs, and regenerative nodules (RNs). The progression rate of HCC from HGDNs was high, and the annual HCC development rate exceeded 30% in the first 2 years. HCC developed more often from HGDNs than from LGDNs and RNs[28]. Therefore, HGDNs are true precancerous lesions of HCC.

HISTOPATHOLOGIC FEATURES

Compared with HGDNs, LGDNs lack appreciable cytologic and architectural atypia based on the following findings: (1) LCC is frequently observed inside and outside of LGDNs as microscopic (< 1 mm) DF. SCC is most frequently seen inside HGDNs[30]; (2) The nuclear/cytoplasmic ratio is higher in HGDNs and W-HCC than in LGDNs[2]; (3) HGDNs and HCC exhibit more pronounced basophilia in the cytoplasm than large regenerative nodules (LRNs) and LGDNs[31]; (4) Hyperchromasia and nuclear atypia are observed in HGDNs and HCC[31]; (5) A pseudoacinar pattern (or acinar formation) and thickened trabeculae are never seen in LGDNs but frequently appear in HGDNs and HCC[31]; and (6) The number of portal tracts is normal in LGDNs but reduced in HGDNs and early HCCs[32]. In addition, some HGDNs often contain one or more microscopic foci of W-HCC, whereas nodule-in-nodule lesions are absent in LGDNs, which suggests that HGDNs might be precancerous lesions of HCC[20,33,34]. In conclusion, these histopathologic features indicate that HGDNs represent borderline lesions and closely resemble W-HCCs[35].

IMAGING DIFFERENCES BETWEEN HGDNs AND LGDNs

Angiogenesis

Angiogenesis, such as sinusoidal capillarization and unpaired arteries, gradually increases during hepatocarcinogenesis from HGDNs to classic hypervascular HCC[36]. Compared with LGDNs, HGDNs and W-HCC contain more aberrant unpaired arterioles and capillarized sinusoids[25].

Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging findings

Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) has been shown to improve the detection and characterization of focal liver lesions. Specifically, this approach can differentiate LGDNs from pre-malignant HGDNs and early HCC[37].

The hypointense appearance in hepatobiliary phase is often considered a radiological marker of nodule differentiation[38]. The Gd-EOB-DTPA-enhanced MRI findings of nodules (LGDNs, HGDNs, and HCCs), which were histologically identified on cirrhotic and explanted livers, indicated nodular hypointensity at the hepatobiliary (HB) phase in 39/40 HCCs and 21/30 HGDNs but in none of the LGDNs, despite a lack of significant differences among the enhancement ratios (ERs) of HGDNs and HCCs at the HB phase[39]. Another study examined the ability of delayed phase imaging (DPI) gadobenate dimeglumine-enhanced MRI in addition to dynamic postcontrast imaging to improve the characterization of small HCCs, HGDNs and LGDNs. This study identified significant qualitative and quantitative differences in the hypointensity on DPI between LGDNs and the group consisting of HGDNs and HCCs[40].

In one study, 62 of 215 nodules exhibited atypical radiological behavior (atypical nodules refer to a lack of hypervascularization in the arterial phase and/or absent hypovasculature in portal phase[38]), and 19 out of 20 HGDNs/early HCC nodules were found to be hypointense in the HB phase[41]. Most HGDNs (20/30) were reported to be iso/hypointense in the arterial phase and hypointense in the late phase, where all LGDNs were characterized by iso/hyperintensity in both the arterial and late phases. The ERs of lesions did not significantly quantitatively differ between hypovascular HCCs and HGDNs in the arterial phase or between hypointense HCCs and HGDNs in the HB phase[39].

In addition, patients with chronic liver disease showing a hypointense hypovascular nodule in the liver on hepatocyte-phase Gd-EOB-DTPA-enhanced MRI are at a high risk of developing HCC[42]. Therefore, HGDNs, which represent hypointense hypovascular nodules, could be precancerous lesions of HCC.

"Washout" and arterial enhancement of HGDNs and LGDNs on CT or MRI

In one study, washout was observed in 5 HGDNs (5/6) on CT and in 4 HGDNs (4/6) on MRI, whereas washout or arterial enhancement was not observed in patients with LGDNs[25]. In another study, the frequency of washout increased to 93.8% (45/48) in HCCs and 69.3% (9/13) in HGDNs in cirrhotic livers, and these incidences were higher than that of arterial enhancement[43]. These findings suggest that lesions exhibiting washout or arterial enhancement in the cirrhotic liver may be malignant nodules.

Furthermore, arterial hypervascularization with washout in subcentimeter hypointense nodules in the HB phase on Gd-EOB-DTPA-enhanced MRI in patients with chronic liver disease has been shown to strongly correlate with progression to hypervascular HCC[44].

Therefore, the imaging differences between HGDNs and LGDNs indicate that HGDNs are closely associated with progression to HCC and likely are more advanced precursors of HCC than LGDNs.

BIOMARKERS FOR THE DISCRIMINATION BETWEEN LGDNs AND HGDNs - MOLECULAR GENETIC BIOMARKERS

Allelic losses

Allelic losses in some regions were found to be correlated to clinicopathologic features and HCC progression[27]. The overall fractional allelic loss (FAL) index represents the frequency of allelic losses.

Genome-wide allelotyping has been adopted to systematically evaluate allelic changes during hepatitis B virus-associated hepatocarcinogenesis. Specifically, these studies found that the overall FAL index of HGDNs was significantly higher than that of LGDNs and was very close to that of HCCs[27]. This result indicates that HGDNs are genetically closer to HCC in terms of allelic losses, suggesting that their biological behavior likely resembles that of early HCC. Another group of researchers investigated the genetic differences between macroregenerative nodules (MRNs), LGDNs, and HGDNs *via* comparative genomic hybridization and found that although allelic losses of 8p and gains of 1q were observed in three out of six HGDNs, chromosomal imbalances between LGDNs and MRNs were absent[45]. In addition, the array CGH analysis revealed that HGDNs and HCCs exhibited a loss of heterozygosity at 5q13.2 and 8p23.1[46], which suggests that the genetic changes in HGDNs and HCCs are similar. Overall, these studies provide genetic evidence to support the idea that HGDNs are precancerous lesions.

Telomerase reverse-transcriptase

Telomerase reverse-transcriptase (TERT) promoter mutations are the most frequent somatic genetic alterations in human HCC arising from both cirrhotic and normal livers. They are major early events in tumorigenesis that occur at the precancerous stages in cirrhosis[47-49]. One study found that TERT promoter mutations were highly correlated with hepatocarcinogenesis: mutations were identified in 6% of LGDNs, 19% of HGDNs, 61% of early HCCs and 42% of small and progressive HCCs[50].

In addition, the human TERT (hTERT) mRNA levels, as measured by real-time quantitative RT-PCR, positively correlated with hepatocarcinogenesis, and a significant induction in the transition between LGDNs and HGDNs was observed. Most HGDNs strongly expressed hTERT mRNA at levels similar to those of HCCs[51].

Telomerase expression and the maintenance of a critical telomere length (TL) in cancer initiation indicate that telomere shortening and telomerase expression initiate cancer by inducing chromosomal instability[52]. Several studies have demonstrated that many HGDNs have shorter telomeres and a higher telomerase activity (TA) than LGDNs, and the TL and TA in HGDNs are similar to those of DNs with HCC foci and HCCs[53,54]. These results suggest that HGDNs are more similar to HCCs than LGDNs.

MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNA molecules that are thought to play an important role in the regulation of gene expression[55]. A growing body of evidence indicates that the deregulation of miRNAs plays a crucial role in hepatocarcinogenesis[56]. Specifically, some miRNAs, such as miR-145 and miR-199b, are downregulated in the progression from LGDNs to small HCC; miR-224 showed no expression in LGDNs, moderate expression in HGDNs, and strong expression in small HCCs[57,58]. These results suggest that LGDNs are early lesions in the spectrum of hepatocarcinogenesis and that HGDNs could be considered advanced precursors of HCC within this spectrum.

Changes in DNA methylation

Epigenetic changes are reversible and heritable and include changes in DNA methylation. Aberrant epigenetic modifications occur at the earliest stages of neoplastic transformation and are now believed to be essential players in cancer initiation and progression[59]. The aberrant DNA methylation of CpG islands is catalyzed by DNA methyltransferases (DNMTs). In human hepatocarcinogenesis, the expression levels of DNMT1, DNMT3a and DNMT3b mRNAs progressively increase from LGDNs to HGDNs, HCCs in DNs, and advanced HCCs[60], suggesting that DNMTs are involved in hepatocarcinogenesis and that HGDNs may be more advanced precancerous lesions than LGDNs.

DNMT3A: DNMT3A may play a role in hepatocellular carcinogenesis by regulating the expression of some tumor-suppressor genes, such as PTEN[61]. Specifically, nuclear immunoreactivity to Dnmt3a has not been detected in non-neoplastic livers or LGDNs but was observed in some HGDNs and HCCs[62].

Ras association domain family 1 isoform A: Ras association domain family 1 isoform A (RASSF1A) is a tumor suppressor that is methylated in many human cancers, including HCC[63]. As human hepatocarcinogenesis progresses from chronic hepatitis/cirrhosis to LGDNs, HGDNs, and finally, HCC, the expression of RASSF1A tends to gradually decrease[63]. Moreover, aberrant DNA methylation and increased DNMT expression have been demonstrated to be features of tumor cells[64]. Overall, the above-mentioned findings indicate that HGDNs share some features with HCC and are precancerous lesions of HCC.

BIOMARKERS FOR THE DISCRIMINATION BETWEEN LGDNs AND HGDNs - IMMUNOHISTOCHEMICAL BIOMARKERS

Vascular endothelial growth factor

Angiogenesis refers to the process of blood vessel formation, and all tumors require new blood vessels

to grow. Evidence from human studies suggests that angiogenesis is not necessarily a characteristic of an invasive tumor but may be an early event during the pre-malignant stages of cancer[65]. Angiogenesis may be stimulated by several regulators, among which vascular endothelial growth factor (VEGF) seems to be the most important one[66]. The expression of VEGF is higher in HGDNs and early HCCs than in LGDNs[67], which suggests that VEGF expression may be a good indicator of precancerous changes and may be useful to better understand and prevent the transformation of HGDNs to HCC.

Glypican-3

Glypican-3 (GPC3) is an oncofetal protein that plays a key role in growth factor signaling to regulate the proliferative activity of cancer cells[68]. Several recent studies have demonstrated high GPC3 protein expression in HCC but not in hepatic para-carcinomatous and cirrhotic tissues[69-72], indicating that GPC3 may be used as an immunohistochemistry marker to differentiate HCC from benign hepatocyte nodules[73]. To date, several studies have reported higher GPC3 protein expression in HGDNs than in LGDNs[1,74,75]. These results suggest that GPC3-positive DNs, especially GPC3-positive HGDNs, are indeed precancerous lesions of HCC[74].

Micronucleus

Chromosomal damage and the formation of a micronucleus (MN) are believed to play a significant role in the pathogenesis of many malignancies[76]. An MN is a small nucleus, and increases in the formation of MNs are usually regarded as an indicator of chromosomal damage. In one study, the micronuclei index was significantly increased in HGDNs compared with LGDNs. HCC exhibited the highest micronuclei index compared with those of HGDNs and DNs with HCC foci[77]. Therefore, the micronuclei index may transition from LGDNs to HGDNs, DNs with HCC foci and HCC, suggesting that HGDNs harbor more chromosome damage than LGDNs during hepatocarcinogenesis.

PROBLEMS AND PERSPECTIVES

For many years, the differentiation of dysplastic lesions, particularly HGDNs, from early HCC has been a challenge for clinicians, radiologists and pathologists. A variety of imaging modalities are currently used to evaluate patients with chronic liver disease and suspected HCC. Of these modalities, CT, MRI and contrast-enhanced ultrasound (CEUS) have largely replaced biopsy for the diagnosis of HCC[78]. However, all imaging techniques may fail to detect and diagnose small HCCs, particularly in the presence of cirrhosis[79]. The detection and diagnosis of small nodules, such as DNs, by imaging techniques are also very difficult.

DNs are generally hypovascular in the arterial phase, but they can sometimes be hypervascular without "washout" during the portal/late phases of CEUS[80]. Therefore, imaging findings on CEUS overlap between DNs and W-HCCs[81]. HCC or HGDNs can be noninvasively diagnosed if arterial enhancement and washout are found in a single dynamic imaging examination; however, these findings are frequently discordant on both CT and MRI[25]. CEUS can be effectively used as a useful problem-solving method by utilizing its unique advantages when CT and MRI are contraindicated or their results are indeterminate[82]. Similarly, nodules considered indeterminate after CEUS have been evaluated by contrast-enhanced CT or MRI for diagnosis[83,84]. Despite the above-mentioned merits of these imaging modalities, the differentiation between "early" HCC and an HGDN using only imaging techniques is sometimes difficult[80]. Moreover, a recent study recommends performing a biopsy rather than additional imaging when the first imaging is inconclusive[25].

DNs, especially HGDNs, are borderline lesions, and the biopsy of DNs is associated with a high rate of false-negative findings due to histological heterogeneity within the nodules[85]. Therefore, differentiating from HGDNs based on a small biopsy specimen may be difficult or nearly impossible[86]. Consequently, the identification of potential biomarkers that reliably detect or diagnose HGDNs or early HCC is urgently needed.

An increasing body of evidence suggests that epigenetic changes contribute to hepatocarcinogenesis. Therefore, DNA methylation and miRNAs have been proposed as promising biomarkers[87]. Specifically, changes in DNA methylation are ubiquitous in human cancer and have been shown to occur early during carcinogenesis. As such, these changes may serve as biomarkers for the detection of HGDNs or early HCC[88]. The frequency of aberrant promoter methylation has been confirmed to increase during the progression from precancerous lesion to HCC[89]. Therefore, DNA methylation in precancerous or early neoplastic stages may serve as a biomarker for screening patients with an increased risk for HCC and detecting early cancer. Moreover, the deregulation of miRNAs plays an important role in human carcinogenesis. For example, the down-regulation of miR-145 and miR-199b and up-regulation of miR-224 are frequently observed in pre-malignant DNs, and these changes persist throughout HCC development[57], suggesting that miRNA expression may serve as a biomarker for the detection of HGDNs or early HCC.

However, the above-mentioned biomarkers are tissue markers for detecting HGDNs or early HCC in liver biopsy specimens. In other words, a biomarker will be useful for screening or the early detection of cancer only if it can be detected in a noninvasive or minimally invasive fashion without tissue biopsy[88].

Increasing evidence has verified that DNA methylation and miRNAs in the blood or other bodily fluids may serve as valuable biomarkers for the detection of early

HCC[90]. Hopefully, DNA methylation or miRNAs in the blood will be translated into clinical use in the near future, which would facilitate the early diagnosis of at-risk patients and the accurate assessment of disease progression[87]. In addition, when combined with imaging contrast, blood biomarkers will significantly enhance our ability to diagnose HGDNs or early HCC and screen patients who are at carcinogenetic risk. These advances would allow patients to receive early treatment and ultimately improve survival.

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MINIREVIEWS

Lipids in liver transplant recipients

Anna Hüsing, Iyad Kabar, Hartmut H Schmidt

Anna Hüsing, Iyad Kabar, Hartmut H Schmidt, Department of Transplant Medicine, University Hospital Münster, 48149 Münster, Germany

Author contributions: Hüsing A, Kabar I and Schmidt HH analyzed the literature and wrote the paper.

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Correspondence to: Hartmut H Schmidt, MD, Professor of Medicine, Department of Transplant Medicine, University Hospital Münster, Albert-Schweitzer-Campus 1, Gebäude A14, 48149 Münster, Germany. hepar@ukmuenster.de
Telephone: +49-251-8357770
Fax: +49-251-8357771

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Abstract

Hyperlipidemia is very common after liver transplantation and can be observed in up to 71% of patients. The etiology of lipid disorders in these patients is multifactorial, with different lipid profiles observed depending on the immunosuppressive agents administered and the presence of additional risk factors, such as obesity, diabetes mellitus and nutrition. Due to recent improvements in survival of liver transplant recipients, the prevention of cardiovascular events has become more important, especially as approximately 64% of liver transplant recipients present with an increased risk of cardiovascular events. Management of dyslipidemia and of other modifiable cardiovascular risk factors, such as hypertension, diabetes and smoking, has therefore become essential in these patients. Treatment of hyperlipidemia after liver transplantation consists of life style modification, modifying the dose or type of immunosuppressive agents and use of lipid lowering agents. At the start of administration of lipid lowering medications, it is important to monitor drug-drug interactions, especially between lipid lowering agents and immunosuppressive drugs. Furthermore, as combinations of various lipid lowering drugs can lead to severe side effects, such as myopathies and rhabdomyolysis, these combinations should therefore be avoided. To our knowledge, there are no current guidelines targeting the management of lipid metabolism disorders in liver transplant recipients. This paper therefore recommends an approach of managing lipid abnormalities occurring after liver transplantation.

Key words: Liver transplantation; Dyslipidemia; Lipid management; Immunosuppression; mTOR-inhibition; Treatment

Core tip: Lipid disorders after liver transplantation are common and can significantly increase the risk of cardiovascular events in liver transplant recipients. Furthermore, dyslipidemia may also impair graft function and survival. Therefore management of dyslipidemia is of great importance in preventing cardiovascular diseases and graft dysfunction in these patients. Knowledge of the different manifestations of lipid disorders after liver transplantation, the role of immunosuppressive agents and of drug-drug interactions is therefore essential for management and

follow-up of these patients.

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INTRODUCTION

Hyperlipidemia (HLP) is considered one of the most important risk factors for the development of atherosclerosis. The prevalence of HLP in liver transplant (LT) recipients is high and has been estimated to vary from 27% to 71%[1,2].

Development of HLP is multifactorial, including in LT recipients. Immunosuppressive agents administered after liver transplantation may lead to lipid metabolism disorders and HLP. Although some studies have reported that immunosuppressive therapy is not a risk factor for cardiovascular events[1], others have found that the adverse event profiles of immunosuppressive agents can contribute to cardiovascular risk in transplant recipients[3,4].

A meta-analysis reporting pooled estimates from population-based and nested case-control studies found that post-LT recipients have an approximately 64% greater risk of cardiovascular events than the general population[5]. Although the increased risk for atherosclerosis-associated events in transplant recipients suggests that all of these patients be treated prophylactically with lipid lowering drugs, drug interactions and other side effects limit this approach. In practice many transplant physicians remain cautious about treating LT recipients with lipid lowering drugs.

Cardiovascular diseases are emerging as the main cause of non-graft-related mortality in LT recipients, especially in older subjects, thus affecting their long-term outcomes and causes of death[6,7]. Optimal follow up of these patients should include continuous observation and management of cardiovascular risk factors such as dyslipidemia.

Post-LT HLP, however, occurs frequently after LT, independent of administration of immunosuppressive agents[8]. Additional causes of HLP include increased body weight, malnutrition, abnormal renal function, impaired glucose metabolism and genetic predisposition. Since genetically associated HLP also occurs in the general population, HLP may also result from a liver donor carrying, *e.g.*, a low-density lipoprotein-cholesterol (LDL-C) receptor deficient variant or an apolipoprotein E defective variant expressed by the liver[9,10]. To date, guidelines have not been formulated for the prevention and treatment of lipid metabolism disorders after LT. Based on the recommendations of the 2011 guidelines formulated by the Task Force for the management of dyslipidemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)[11], in addition to the guidelines of the American College of Cardiology (ACC) and the American Heart Association (AHA)[12], and our practical experience treating LT recipients, we here propose strategies to manage dyslipidemia in this patient population.

HLP classification

Various types of HLP have been identified, including HLP with increased triglycerides (hypertriglyceridemia), HLP with high LDL-C (hypercholesterolemia) and their combination, HLP with both hypertriglyceridemia and hypercholesterolemia. Statin treatment should be tailored to the type of HLP, as these agents, when administered independent of the type of HLP, may result in reduced efficacy and metabolic control, a secondary increase in triglycerides, and increased side effects.

LDL-C and atherogenesis

Epidemiological studies have shown that HLP with high serum LDL-C is a particular risk factor for the development of atherosclerosis[13,14]. Table 1 shows the relationship between LDL-C concentrations and potential atherogenicity in the general population. Indications for treatment of LT recipients with lipid lowering drugs depend on each patient's individual risk profile.

Cardiovascular risk shows a greater association with serum LDL-C than total cholesterol concentrations. Hypercholesterolemia may also be due to increases in serum high density lipoprotein-cholesterol (HDL-C) concentrations, which have no clinical relevance.

Alterations in diet may show have moderate effects on serum LDL-C concentrations, but they are often not sufficient. Familial hypercholesterolemia, for example, does not significantly respond to dietary approaches. Cardiovascular events such as myocardial infarction can occur as early as the fourth to sixth decade of life in the absence of lipid lowering treatment. Most trials of lipid-lowering agents have assessed response to therapy by measuring serum LDL-C concentrations, indicating the importance of reducing serum LDL-C concentrations in managing dyslipidemia. A meta-analysis by the Cholesterol Treatment Trialists' Collaboration of several trials involving > 170000 patients also showed that lowering LDL-C concentration reduced the risk of cardiovascular disease (CVD)[15]. Moreover, reducing LDL-C concentration to below 1.8 mmol/L (below approximately 70 mg/dL), or to < 50% of baseline, was optimal in reducing the risk of CVD[15].

HYPERTRIGLYCERIDEMIA AND ITS METABOLIC IMPACT

Metabolic complications occur frequently in patients with hypertriglyceridemia, with long-term hypertri-

Table 1 Hypercholesterolemia and its estimated atherogenic potential[25]

LDL-cholesterol (mg/dL)	
< 100	Optimal
100-129	Near/above optimal
130-159	Borderline high
160-189	High
⩾ 190	Very high

LDL: Low density lipoprotein.

Table 2 Hypertriglyceridemia and its estimated atherogenic potential[25]

Triglycerides (mg/dL)	
< 150	Optimal
150-190	Near/above optimal
200-499	High
⩾ 500	very high

Table 3 Proposed targeted triglyceride serum concentrations in liver transplant recipients[42]

Triglyceride-targets and -limits
Triglyceride-targeted serum concentrations < 200 mg/dL
Triglycerides < 400 mg/dL with life-style intervention[1]: tolerable
Triglycerides < 500 mg/dL despite lipid-lowering medication before start of immunosuppression: no initiation of therapy with mTOR-inhibitors
Triglycerides > 500 mg/dL under mTOR-inhibitors: if despite therapy triglycerides < 500 mg/dL, change to different group of immunosuppressant (or reduce doses)

[1]Life-style modification/ therapy (lipid-lowering medication): (1) weight loss; (2) increase physical activity; (3) optimize glucose-metabolism; and (4) lipid lowering agents. mTOR: Mammalian target of rapamycin.

glyceridemia often causing lipomatosis of the liver and pancreas[16]. These pathological alterations may impair glucose metabolism, especially peripheral insulin resistance, resulting in diabetes mellitus[16]. Table 2 shows a classification of serum triglyceride concentrations in the general population, and Table 3 shows serum concentration limits and treatment recommendations with mTOR inhibitors in LT recipients.

Specific treatment targets for hypertriglyceridemia have not yet been determined. Moreover, serum triglyceride concentrations are highly variable, increasing during the day in most individuals. Treatment to reduce the risk of pancreatitis, however, is recommended when serum triglyceride concentrations exceed 1000 mg/dL[17]. Causes of elevated serum triglyceride concentrations include obesity, alcohol consumption, high carbohydrate diet, diabetes mellitus, reduced kidney function, nephrotic syndrome; treatment with steroids[18], protease inhibitors, hormone substitutes, calcineurin inhibiting drugs[19], mTOR inhibiting drugs[20]; and predisposing genetic factors. In contrast to elevated LDL-C levels, hypertriglyceridemia can be influenced by diet. A weight loss of 2-3 kg can result in a significant reduction in serum triglyceride concentrations, of up to 200 mg/dL[21]. In contrast, a weight loss of more than 10 kg may reduce serum LDL-C concentrations by only 30 mg/dL. Fruit juices and other high-carbohydrate products, as well as small amounts of alcohol, may exacerbate hypertriglyceridemia temporarily.

COMBINED HLP

Treatment of patients with combined HLP depends on serum LDL-C and triglyceride concentrations and their risks of atherosclerosis and metabolic symptoms. As combinations of lipid-lowering agents may cause side effects, including rhabdomyolysis, both atherosclerosis and metabolic risks must be evaluated. Based on a patient's individual risk profile, we recommend lipid lowering treatment targeting either LDL-C or triglyceride concentrations. Combinations of drugs that lower serum cholesterol and triglyceride concentrations should be avoided in LT recipients due to their interactions with immunosuppressants. Liver enzymes and creatinine kinase should be measured weekly in patients started on both cholesterol and triglyceride lowering drugs.

Lipoprotein (a) and atherogenesis

Lipoprotein (a) [Lp(a)] is a risk factor for atherogenesis and may play roles in plasma viscosity and the coagulation cascade[22-24]. Lp(a) should be monitored regularly in patients with myocardial infarction and other cardiovascular end points such as cerebral insult. Serum Lp(a) concentrations < 30 mg/dL are regarded as normal. Elevated serum Lp(a) concentrations in patients with hypertriglyceridemia may respond to dietary modifications or treatment with nicotinic acid. However, statin treatment of patients with elevated Lp(a) and LDL-C does not significantly reduce Lp(a) concentrations. LDL-apheresis may be successful in treatment of patients with persistently high LDL-C and/or Lp(a). In Germany, LDL-apheresis is currently used to treat selected patients with manifest atherosclerosis and Lp(a) > 60 mg/dL.

HDL-C

Serum HDL-C concentration < 40 mg/dL is a known risk factor for coronary heart disease[11,25]. High serum HDL-C does not protect against atherosclerosis in patients with high serum LDL-C (Table 4)[26]. High serum triglyceride concentrations, overweight, obesity and a high carbohydrate diet may reduce serum HDL-C concentrations. Life style modifications, triglyceride lowering and antidiabetic interventions can improve and normalize serum HDL-C.

DIAGNOSTICS OF LIPID STATUS

Serum lipid concentrations should be measured in a

Table 4 Serum high density lipoprotein-cholesterol concentrations and predicted atherogenic impact[25]

HDL-cholesterol (mg/dL)	
> 60	Optimal
40-60	Near optimal
< 40	Atherogenic

HDL: High density lipoprotein.

Table 5 Criteria of the metabolic syndrome (3 of 5 criteria have to be fulfilled)[25]

Risk factor	Limit value
Abdominal obesity	Abdominal measurement
Men	> 120 cm
Women	> 88 cm
Triglycerides	⩾ 150 mg/dL
HDL-cholesterol	
Men	< 40 mg/dL
Women	< 50 mg/dL
Blood pressure	⩾ 130/⩾ 85 mmHg
Fasting blood sugar or insulin-glucose tolerance test after 60, 120 and 180 min	⩾ 110 mg/dL

fasting state. Postprandial synthesis of chylomicrons may increase serum cholesterol and especially triglyceride concentrations. Total serum cholesterol concentration is the sum of serum HDL-C, LDL-C, and very low density lipoprotein (VLDL)-C concentrations.

Targets for lipid lowering agents include serum LDL-C, total cholesterol (TC) and non-HDL-C concentrations. The 2011 ESC/EAS guidelines have reported that most studies of lipid lowering therapy strategies determine risk by measuring serum TC and LDL-C concentrations. Many clinical trials have shown that reducing TC or LDL-C, even in high-risk-patients, is associated with statistically and clinically significant reductions in cardiovascular mortality[11]. In addition, the 2013 ACC/AHA guidelines have targeted serum LDL-C, TC and non-HDL-C in reducing the risk of atherogenesis.

Non-modifiable risk factors for coronary heart disease include age, sex (male) and genetic factors (family history). Modifiable risk factors for cardiovascular events include arterial hypertension, nicotine consumption (smoking), diabetes mellitus, dyslipoproteinemia (high LDL-C, low HDL-C), obesity and physical inactivity. Measurement of serum lipid concentrations, evaluation of risk factors and diagnosis of glucose metabolism disorders can identify patients with metabolic syndrome (Table 5). Table 6 shows proposed target serum LDL-C concentrations as a function of individual risk factors. These patients have an increased risk of developing insulin resistance and subsequent cardiovascular events, suggesting they be regularly monitored for cardiovascular risk factors.

CHARACTERISTICS OF LIVER TRANSPLANT RECIPIENTS

Risk factors for atherosclerosis and cardiovascular events in LT recipients are qualitatively the same as those in the non-transplant population. Quantitatively, LT recipients are at abnormally high risk for the development of atherosclerosis and metabolic dysfunction than the non-transplant population. For example, the reported rate of coronary heart disease in LT recipients is 30%[27], compared with 9% in the general population[28].

Due to the increased life expectancy of LT recipients, it has become a challenge to manage metabolic disorders, which are also associated with immunosuppressive agents in these patients. Therefore, recommendations for lipid management in LT recipients are urgently required, including the consideration of additional cardiovascular risk factors and the effects of immunosuppressive agents.

Table 6 shows targeted serum LDL-C concentrations in LT recipients. These concentrations are dependent on patient risk category. Long-term serum LDL-C concentrations > 160 mg/dL should be avoided. Patients should begin by modifying diet and life style for at least three months; if serum LDL-C concentrations remain higher than recommended, patients would be started on lipid reducing agents.

IMMUNOSUPPRESSIVE THERAPY AND THE DEVELOPMENT OF HLP

HLP has been observed in up to 45% of LT recipients, regardless of immunosuppressive medication[8], and recent studies have reported HLP in 32%-49% of LT recipients after 10-23 mo[29-33]. Hypercholesterolemia has been reported in 13%-46% of LT recipients after 11-20 mo[34-36], and hypertriglyceridemia has been observed in 15%-50% of patients after 11-12 mo[35,36]. Many factors can influence the development of HLP in these patients, including nutrition, body weight, renal function, glucose metabolism, and genetic factors.

Immunosuppressants, including steroids and calcineurin inhibitors, have been shown to interfere with lipid metabolism and increase the risk of hyperlipidemia. Steroid treatment has been associated with both diabetes mellitus and dyslipidemia[37]. Steroids upregulate the synthesis of fatty acids, resulting in peripheral insulin resistance and increased VLDL-C synthesis within the liver. This can result in both isolated hypertriglyceridemia and combined HLP. Serum cholesterol concentrations are increased by activation of multiple indirect pathways. Hyperinsulinemia may stimulate VLDL-C synthesis in the liver, as well as downregulating LDL receptors, possibly by suppression of adrenocorticotropic hormone[38].

Table 6 Proposed low density lipoprotein-cholesterol-serum concentrations and limits for therapeutic interventions[25]

Risk category	LDL-targeted serum concentration (mg/dL)	Life style modification[1,2] starting at LDL-serum concentrations (mg/dL)	Medical treatment
CHD, PAD, CVD[3]	< 100	⩾ 100	⩾ 130
⩾ 2 risk factors	< 130	⩾ 130	⩾ 160
0-1 risk factor	< 160	> 160	> 190

[1]Aspects of life style modification: (1) nutrition: (a) saturated fats < 7% of calories, dietary cholesterol < 200 mg/d; (b) increase of soluble fibers (10-25 g/d) and sterols of vegetable origin (2 g/d) as an option of lowering LDL-levels; (2) weight management; and (3) physical activity; [2]If LDL-serum concentrations after 3 mo still elevated, consider medical treatment; [3]Documented vascular atherosclerosis. CHD: Coronary heart disease; CVD: Cardiovascular disease; LDL: Low density lipoprotein; PAD: Peripheral artery disease.

A calcineurin inhibitor based regimen may induce HLP independently of concurrent use of steroids[39]. Cyclosporine was shown to dose-dependently increase serum lipid concentrations. Moreover, increased total and LDL-C, along with reduced HDL-C, have been observed in patients with elevated cyclosporine blood levels[19]. Tacrolimus may have advantages over cyclosporine-based regimens in that the former does not increased serum lipid concentrations in LT recipients. Indeed, a study in which patients were switched from a cyclosporine- to a tacrolimus-based regimen found that serum LDL-C and triglyceride concentrations were reduced compared with a control group maintained on cyclosporine[40]. However, tacrolimus has been associated with hyperinsulinemia, which may result in HLP, especially hypertriglyceridemia. Both immunosuppressors have therefore been found to result in HLP in LT recipients.

The introduction of mTOR inhibitors as immunosuppressive agents has enhanced options in the treatment of transplant recipients. Both sirolimus and everolimus are very promising mTOR inhibitors in transplant patients, but also increase the risk for development of HLP[41]. Overall all these immunosuppressants may result in HLP, mostly hypertriglyceridemia and combined hyperlipidemia. HLP associated with mTOR inhibitors may, however, be transient in some patients. Although mTOR inhibitors affect lipid metabolism and are therefore more likely to result in HLP, patients with pre-existing HLP still can be treated using mTOR inhibitors. These agents do not exacerbate HLP in all of these patients. Furthermore, mTOR inhibitors may enhance renal recovery, improving lipid status by reducing calcineurin inhibitor concentrations.

HLP IN LIVER TRANSPLANT RECIPIENTS TREATED WITH MTOR INHIBITORS

Table 3 shows recommended target serum concentrations and upper limits of triglycerides in LT patients who receive mTOR inhibitors as immunosuppressive agents. Long-term triglyceride serum concentrations > 400 mg/dL should be avoided to prevent long term metabolic disorders such as non-alcoholic fatty liver diseases and insulin resistance. Furthermore, triglyceride serum concentrations > 1000 mg/dL may lead to acute or chronic pancreatitis. Immunosuppressive regimens using mTOR inhibitors should be avoided if triglyceride serum concentrations exceed 500 mg/dL despite changes in diet and lifestyle and despite administering lipid-lowering agents. In addition, mTOR inhibitors should not be administered to LT recipients, especially those with atherosclerosis, if their serum LDL-C concentrations are > 250 mg/dL despite treatment with lipid lowering agents and lifestyle modifications. mTOR inhibitors may also result in proteinuria, aggravating any preexisting HLP. Careful patient monitoring is therefore required to determine the clinical significance of proteinuria and associated HLP.

COMBINED HLP AFTER LIVER TRANSPLANT

Liver transplant recipients with severe combined HLP, including hypercholesterolemia and hypertriglyceridemia, should be preferentially treate for high serum LDL-C concentrations. However, if fasting serum triglyceride concentrations exceed 500 mg/dL, this condition should be treated first due to the increased risk of pancreatitis. If patients exhibit proteinuria > 1 g/d during treatment with mTOR inhibitors, these agents should be reduced or switched. HLP associated with immunosuppressants, including mTOR inhibitors, responds well to all lipid lowering interventions, including changes in diet, weight loss in obese patients, optimization of serum glucose concentrations and modulation of diabetes mellitus, dose reduction of co-medications, modification of immunosuppressants used, and lipid lowering agents[42]. It is not yet clear whether reducing the doses of mTOR inhibitors will lower serum lipid concentrations[43].

Patients with high serum triglyceride concentrations despite lifestyle interventions should be treated, if possible, with fenofibrate or nicotinic acid. Patients with high serum LDL-C concentrations can be treated with statins[44]. Statins have been shown safe in LT recipients[45], with pravastatin or fluvastatin showing fewer interactions than other statins with cytochrome P 450 3A4 and thus a reduced risk for interactions with immunosuppressants (Table 7).

Table 7 Lipid lowering agents, their impact on lipoprotein metabolism, and potential interaction profiles[49]

Agent (daily dose)	Interactions	Comment
HMG-CoA reductase inhibitors (statins)		
Lovastatin (20-80 mg) Pravastatin (20-40 mg) Simvastatin (20-80 mg) Fluvastatin (20-80 mg) Atorvastatin (10-80 mg) Rosuvastatin (5-40 mg)	Lovastatin, Simvastatin, Atorvastatin are mainly catabolized *via* hepatic CYP3A4: Caution in case of use of CYP3A4-Inhibitors (*e.g.*, Itraconazole, Ketoconazole, HIV-protease-inhibitors Erythromycin, Clarithromycin, Telithromycin, Nefazodon). Caution if fibrates or nicotinic acid are simultaneously used: high risk of myopathies. Simultaneous use of calcineurin-inhibiting agents might reduce the elimination of statins: high risk of myopathies and rhabdomyolysis. Monitoring is necessary and low statin doses at the beginning are recommended. Caution with dose escalation. No interactions have been observed between Sirolimus and Atorvastatin and between Everolimus and Atorvastatin respectively Pravastatin.	Class of drugs with the highest lipid lowering effect Contraindications: (1) advanced liver diseases; (2) Rosuvastatin: simultaneous use of Cyclosporine; and (3) Statin intolerance Caution: serious interactions due to competitive inhibition of CYP450 3A4-metabolism cannot be ruled out: Prefer Fluvastatin or Pravastatin for treatment due to absence of CYP450 3A4 metabolisis.
Bile acid binding anion exchange resins		
Colestyramine (4-16 g) Colesevelam (2.5-3.75 g)	Caution: may reduce or retard gastrointestinal absorption of simultaneous orally administered agents. If interactions are possible, agents should be taken > 1 before or > 4 h after Colestyramine intake. Colesevelam should be taken 4 h before or after taking other drugs. Blood level monitoring is required for agents with a narrow therapeutic window. Caution with simultaneous use of immunsuppressants or lipid lowering agents. *e.g.*, bioavailability of mycophenolic acid can be reduced due to the simultaneous use of bile acid binding anion exchange resins (40% in case of MMF + Colestyramine). Intervals of medication intake mentioned above are obligatory.	Lowering of LDL-cholesterol, also used in combination with Statins or Ezetemibe Contraindications: Ileus or occlusion of bile ducts
Nicotinic acid		
Sustained-release tablets (Niaspan®) (1-2 g) Nicotininc acid/ Laropiprant (Tredaptive®) (1-2 g)	In some cases simultaneous use of nicotinic acid and HMG-CoA reductase inhibitors was associated with myopathies/rhabdomyolysis: careful assessment of risks and benefits is required. Tredaptive® (according to medicinal product's professional information use was only evaluated in combination with Simvastatin): small increase of AUC and Cmax of Simvastatin (probably without any clinical relevance). Hot drinks, alcohol and spicy foods may favor flush. Simultaneous use with nicotinic acid should be avoided.	Lowering of LDL and triglyceride serum concentrations Contraindications (1) advanced liver diseases; (2) acute peptic ulcer; and (3) arterial bleeding
Fibrates		
Gemfibrozil (2 × 600 mg) Fenofibrate (200 mg) Bezafibrate (200-600 mg)	Caution: Simultaneous treatment with HMG-CoA reductase inhibitors leads to an increased risk for myopathies and rhabdomyolysis. Statin serum concentrations can rise: no combination with statins or monitor patients closely. Combination with Calcineurin-Inhibitors and mTOR-Inhibitors leads to an increased risk for rhabdomyolysis and other side effects: monitoring is required.	Reducing triglycerides. Avoid combination with HMG-CoA reductase inhibitors. Contraindications (1) advanced liver dysfunction; and (2) severe renal impairment
Cholesterol resorption reducing agents		
Ezetemibe (Ezetrol®) (10 mg)	Caution: Simultaneous treatment with HMG-CoA reductase inhibitors leads to an increased risk of myopathies and rhabdomyolysis and elevation of liver enzymes: close monitoring of liver function is required. No combination with fibrates: tolerability and effectiveness were not evaluated. Combination with Fenofibrate leads to an increased risk for cholelithiasis and gall bladder diseases. Caution with the simultaneous use of Cyclosporine: AUC of Ezetemibe rises, no data concerning changes in Cyclosporine-blood levels available. No clinical effects and interactions with other immunosuppressants have been observed to date. Monitoring of immunosuppressive agents is required[50].	Lowering of LDL cholesterol: (1) advanced liver diseases; and (2) persistent elevated liver enzymes Rare interactions (no induction of CYP450 enzymes)

AUC: Area under the curve; CYP: Cytochrome P; LDL: Low density lipoprotein; HIV: Human immunodeficiency virus; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; MMF: Mycophenolate mofetil; mTOR: Mammalian target of rapamycin.

GENERAL TREATMENT RECOMMENDATIONS FOR HLP IN LIVER TRANSPLANT RECIPIENTS

The evidence showing that reducing total cholesterol and LDL-C can prevent atherogenic end points is strong, based on results from multiple randomized controlled trials. Serum total cholesterol and LDL-C concentrations remain the primary targets of therapy. Integrating these findings with the ESC/EAS guidelines for life style changes suggest[11]: Reductions in serum LDL-C concentrations require dietary changes, especially reductions in dietary saturated fatty acids, which have the greatest impact on serum LDL-C concentrations. Dietary trans-fats and cholesterol should also be avoided, and dietary fiber increased. Excessive body weight should be reduced. Habitual physical activity should be increased. Serum triglyceride concentrations may be reduced by normalization of body weight, reduced alcohol intake (forbidden for LT recipients) and reduction of mono- and disaccharides, along with increased physical activity and reduced total dietary carbohydrates. Ingestion of n-3-polysaturated fats should be eliminated and replaced by mono- or polyunsaturated fats. Omega-3-fatty acids may also improve severe hypertriglyceridemia in transplant patients since no drug-drug-interactions have been described. The latter are available over the counter and are approved by the FDA for the treatment of patients with very high triglyceride serum concentrations (> 500 mg/dL)[46]. However, omega-3-fatty acids may increase serum concentrations of LDL-C and TC.

Serum HDL-C concentrations may be increased by reducing dietary trans-fats. Increased physical activity, reduced body weight and replacement of dietary carbohydrates by unsaturated fats can also increase serum HDL-C concentrations.

Depending on additional individual risk factors for cardiovascular events, in addition to LT, medical treatment of hypercholesterinemia should be considered. The ESC/EAS guidelines utilize the SCORE system to estimate the 10-year risk of a first fatal atherosclerotic event (heart attack, stroke, or other occlusive arterial disease, including sudden cardiac death). This 10-year risk of CVD death can be estimated using tables on the ESC/EAS homepage (http://www.escardio.org/Guidelines-&-Education/Clinical-Practice-Guidelines/Dyslipidaemias-Management-of). The current joint European Guidelines on CVD prevention in clinical practice also recommend the use of the SCORE system because it is based on large, representative European cohort data sets[47]. The 2013 ACC/AHA guidelines, which also describe the elevated risk for CVD in LT recipients CVD, recommend statin treatment. These guidelines have found a net benefit from statin treatment when 10-year atherosclerosis CVD risk was as low as 5%.

MANAGEMENT OF LIPID LOWERING AGENTS AND PHARMACOLOGICAL INTERACTIONS

If an LT recipient receiving immunosuppressive therapy has a risk profile indicating the use of lipid lowering agents such as statins, serum concentrations of creatine kinase (CK) and liver enzymes should measured one, two, four, eight and 12 wk after starting a lipid lowering treatment, as severe side effects, such as rhabdomyolysis, may occur. Subsequent blood controls may be performed every 3 mo or whenever a new drug is introduced. A tolerable elevation of creatine kinase has defined as an increase to five times the upper limit of normal on two occasions. How statins affect skeletal muscle is not clear. Myopathy often occurs in persons, such as LT recipients, taking multiple medications. Myalgia without CK elevation has been reported to occur in 5%-10% of patients. Patients taking statins should be instructed to promptly report unexpected muscle pain or weakness. However, patients complaining of myalgia without elevated serum CK concentrations can continue taking statins if their symptoms are tolerable. If the symptoms are not tolerable or are progressive, the drug should be discontinued[11].

Table 7 highlights additional important and frequently observed drug-drug interactions. Due to the high risk of rhabdomyolysis, combinations of lipid lowering agents should be avoided. Bile acid binding agents may impair the absorption of some co-medications. Therefore, comedications should be administered a least one hour before or four hours after taking bile acid binding agents[48].

CONCLUSION

HLP can include hypercholesterolemia and/or hypertriglyceridemia. High serum LDL-C is associated with the development of atherosclerosis whereas hypertriglyceridemia may have metabolic consequences. As hypertriglyceridemia originates at least partially from malnutrition and obesity, high serum triglyceride concentrations may be reduced by lifestyle modifications, which may also increase serum HDL-C concentrations. Dietary measures have little effect in reducing high serum LDL-C concentrations. Because many LT recipients are at risk for CVD, lipid lowering medication is frequently required in this patient cohort. Modification of immunosuppressive agents according to their side-effect profiles and the early initiation of statin treatment to achieve targeted serum LDL-C concentrations may have a positive impact on primary prevention of cardiovascular events in LT patients.

HLP in LT recipients may result from different etiologies, including immunosuppressive agents. Immunosuppression using mTOR inhibiting agents should be administered

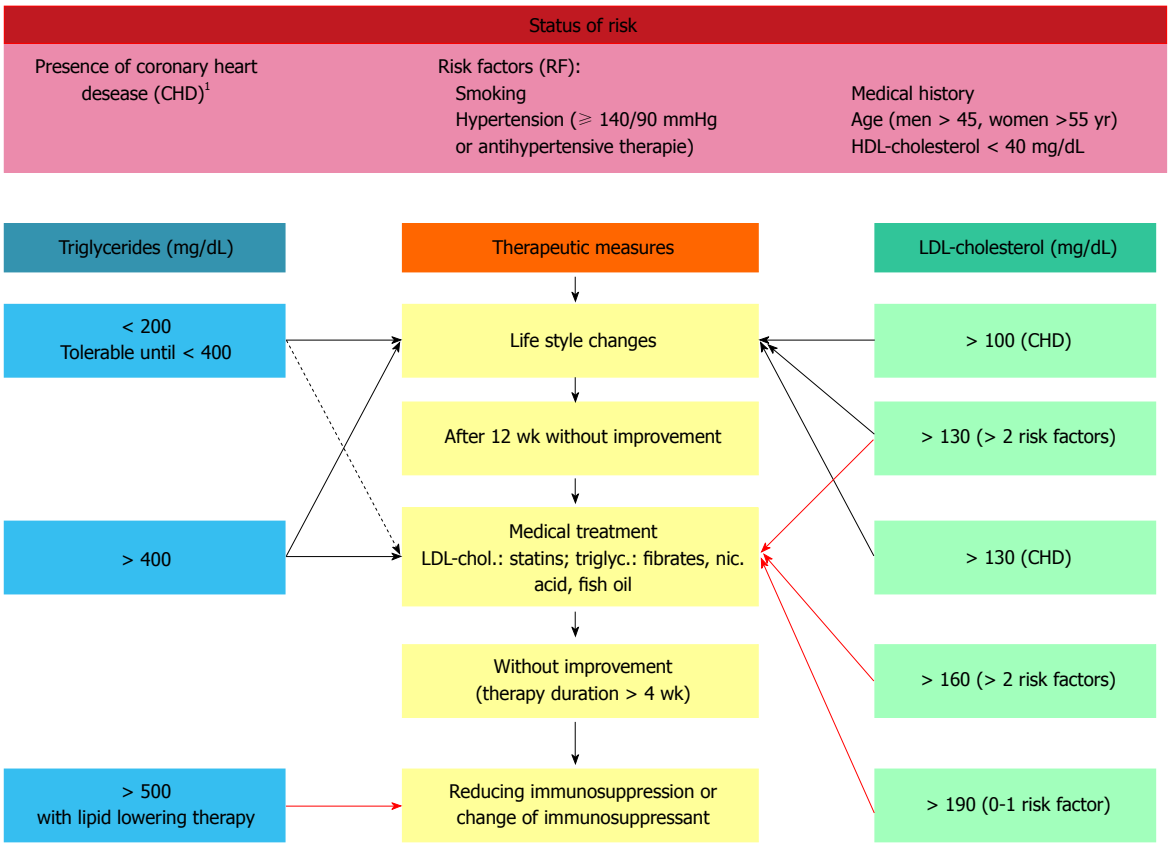

Figure 1 Algorithm for the diagnosis and treatment of hyperlipidemia in liver transplant recipients[49]. [1]Presence of cerebrovascular sclerosis or sclerosis of peripheral arteries. CHD: Coronary heart disease; LDL: Low density lipoprotein.

carefully to patients with known HLP, but should not be avoided generally. Serum lipid concentrations are not always impaired and may sometimes even improve during the use of mTOR inhibitors. LT recipients with serum triglyceride concentrations > 500 mg/dL and those with high serum LDL-C concentrations despite treatment with lipid lowering agents should not be administered mTOR inhibitors. Moreover, care should be taken in administering these pagents to patients with high serum LDL-C concentrations. mTOR inhibitors may also result in proteinuria (CAVE: nephrotic syndrome), which in turn increases serum lipid concentrations. Therefore, proteinuria should be monitored regularly in patients receiving these agents.

Patients should be regularly monitored for drug-drug interactions between lipid lowering and immunosuppressive agents. For example, combining calcineurin inhibitors, mTOR inhibitors and statins may increase the serum concentrations of these drugs. Due to increased risk of rhabdomyolysis, combinations of lipid lowering agents should be avoided in LT recipients. HLP in LT recipients receiving immunosuppressive agents is treatable and responds well to lipid lowering regimens. Transplant recipients should also be monitored for secondary causes of HLP, including hypothyroidism, diabetes and nephrotic syndrome. The latter is characterized by proteinuria exceeding 3.5 g/24 h combined with hypoalbuminemia, edema, hyperlipidemia and lipiduria. Excessive combined hyperlipidemia, mostly type V HLP, occurs in patients with nephrotic syndrome. Therefore, treatment of nephrotic syndrome is important in LT recipients.

We recommend that fasting lipid panel (total cholesterol, LDL-C, HDL-C and triglycerides) be measured in LT recipients before initiating statin therapy. Patients should be followed-up after one, two, four, eight, and 12 wk, and every three months therafter, to assess safety and efficacy. This is consistent with the ACC/AHA guidelines on the Treatment of Blood cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults[12]. Figure 1 provides an overview, based on these recommendations and our experiences in following up LT patients, on the diagnosis and possible therapeutic interventions in lipid management of LT recipients. As cardiovascular disease is the leading cause of long term mortality in LT recipients, additional prospective studies are warranted to confirm these findings and determine whether these risk-reduction strategies can attenuate the increased cardiovascular risk seen in this population.

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MINIREVIEWS

Liver transplantation for hepatocellular carcinoma beyond the Milan criteria: A review

Dong-Wei Xu, Ping Wan, Qiang Xia

Dong-Wei Xu, Ping Wan, Qiang Xia, Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Author contributions: Xu DW and Wan P equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing; and Xia Q coordinated the research and approved the final manuscript for publication.

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Correspondence to: Dr. Qiang Xia, Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Dongfang Road, NO. 1630, Shanghai 200127, China. xiaqiang@shsmu.edu.cn
Telephone: +86-21-68383775
Fax: +86-21-58737232

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Abstract

Liver transplantation (LT) has been accepted as an effective therapy for hepatocellular carcinoma (HCC). The Milan criteria (MC) are widely used across the world to select LT candidates in HCC patients. However, the MC may be too strict because a substantial subset of patients who have HCC exceed the MC and who would benefit from LT may be unnecessarily excluded from the waiting list. In recent years, many extended criteria beyond the MC were raised, which were proved to be able to yield similar outcomes compared with those patients meeting the MC. Because the simple use of tumor size and number was insufficient to indicate HCC biological features and to predict the risk of tumor recurrence, some biological markers such as Alpha-fetoprotein, Des-Gamma-carboxy prothrombin and the neutrophil-to-lymphocyte ratio were useful in selecting LT candidates in HCC patients beyond the MC. For patients with advanced HCC, downstaging therapy is an effective way to reduce the tumor stage to fulfill the MC by using liver-directed therapy such as transarterial chemoembolization, radiofrequency ablation and percutaneous ethanol injection. This article reviews the recent advances in LT for HCC beyond the MC.

Key words: Liver transplantation; Biological marker; Milan criteria; Hepatocellular carcinoma; Downstaging therapy; Adjuvant treatment

Core tip: The Milan criteria (MC) were widely used in selecting liver transplantation (LT) candidates in hepatocellular carcinoma (HCC) patients. Because a substantial subset of HCC patients exceeding the MC and who would benefit from LT may be unnecessarily excluded from the waiting list, many extended criteria beyond the MC were raised. To predict the risk of tumor recurrence, some biological markers were also useful in HCC patients beyond the MC. Downstaging and adjuvant therapies are effective ways to reduce the tumor stage and the risk of recurrence. This article reviews the recent advances in LT for HCC

beyond the MC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second cause of cancer-related death all over the world. Liver transplantation (LT) has been accepted as an effective therapy for HCC with decompensated liver cirrhosis. In the early stage of HCC treatment, however, the high tumor recurrence rate and poor survival outcomes after LT aroused attention[1-4]. Mazzaferro *et al*[5] in 1996 reported that patients with a single tumor ⩽ 5 cm in diameter, or no more than three tumors ⩽ 3 cm, displayed a favorable long-term prognosis. The 4-year overall and recurrence-free survival rates were 85% and 92%, respectively, which were comparable to LT recipients with benign diseases. The MC have been verified by several centers around the world since then and adopted by the United Network for Organ Sharing as the main basis for selecting patients with early-stage HCC for LT. However, many extended criteria beyond the MC were proposed recently because only a fraction of HCC patients are suitable for LT according to the MC. Because the simple use of tumor size and number were insufficient to precisely represent HCC biological features, some biological markers were used in many studies to evaluate the risk of tumor recurrence after LT for patients with HCC beyond the MC. Moreover, downstaging therapy to bring tumors to fulfill the MC has also become an alternative to treatment for patients with advanced HCC.

EXTENDED CRITERIA OF LT FOR HCC

Because the use of MC unnecessarily exclude a substantial subset of patients with HCC exceeding the MC who might benefit from LT from the transplant waiting list, several extended criteria beyond the MC have been reported recently, such as the Pittsburgh criteria, the University of California at San Francisco (UCSF) criteria, the up-to-7 criteria, among other criteria. Although these criteria greatly expanded the indication of LT for HCC, most studies showed that the newly added patients using extended criteria could achieve similar outcomes compared with patients within the MC[6-16].

The Pittsburgh criteria

To establish a more precise system to predict the prognosis of HCC patients undergoing LT, Marsh *et al*[15] investigated 307 transplant recipients with HCC and found that the depth of vascular invasion, tumor size, lobar distribution and lymph node status were independent predictors of tumor free survival, while tumor number did not affect the prognosis. On the basis of these results, they proposed the modified TNM criteria (also known as the Pittsburgh criteria), which significantly expanded the indication of LT for HCC. Chen's study[17] showed that the Pittsburgh Modified TNM Criteria were more reliable for prognostic prediction in HCC patients undergoing LT than the International Union Against Cancer (UICC) pTNM staging system. However, lymph node metastasis and tumor vascular invasion is difficult to diagnose before surgery in many cases, which limited its application.

The UCSF criteria

Yao *et al*[7] retrospectively analyzed 70 consecutive transplant recipients and proposed the UCSF criteria: solitary tumor ⩽ 6.5 cm or ⩽ 3 nodules with the largest lesion ⩽ 4.5 cm and a total tumor diameter ⩽ 8 cm (Table 1). The 1- and 5-year survival rates of the patients meeting the UCSF criteria were 90% and 75.2%, respectively, which is similar to the patients fulfilling the MC. Patients meeting UCSF criteria but exceeding the MC had a 2-year survival rate of 86%, and the UCSF criteria added the LT candidates by 51.5% without compromising the survival.

In recent years, many centers have testified to the value of the UCSF criteria. The University of California at Los Angeles Transplant Center[18] in 2007 reported 467 cases of transplant recipients and 5-year survival rates of patients meeting the MC and UCSF criteria were 79% and 64%, respectively, with no significant difference found between the use of the MC and UCSF criteria. However, in this series, the 5-year survival rate of patients exceeding UCSF criteria was less than 50%.

The up-to-7 criteria

In another attempt to expand the MC, Mazzaferro *et al*[8] proposed the up-to-7 criteria [the sum of the tumor number and the size of the largest tumor (in cm) was not larger than 7] on the basis of 1556 patients from 36 centers (Table 1). In this study, 283 patients without microvascular invasion but met the up-to-7 criteria achieved a 5-year overall survival of 71.2%. The model established in this study firstly stratified HCC patients in a continuum of outcome probabilities, and offered consistent data to estimate the outcome of LT recipients for HCC. However, the up-to-7 criteria did not apply tumor grading, etiology of cirrhosis, cause of death, response to pretransplant treatments or some genomic markers that may predict the outcomes of HCC patients. Moreover, the size and the number of tumors may be inaccurate when using imaging techniques, which may cause understaging or

Table 1 Selection criteria in different centers for hepatocellular carcinoma beyond the Milan criteria

Criteria	Year	Country	Sample size		Contents of criteria	Survival (5-yr)	
			DDLT	LDLT		OS	RFS
UCSF[7]	2001	America	70	0	Tumor ≤ 6.5 cm, or ≤ 3 nodules with the largest ≤ 4.5 cm and a total tumor ≤ 8 cm	75.2%	None
Up-to-7[8]	2009	Italy	1404	121	The sum of the tumor number and the size of the largest tumor no larger than 7 cm	71.2%	None
Tokyo[9]	2007	Japan	0	78	Tumors no larger than 5 cm and no more than 5 nodules	75.0%	94% (3-yr)
Hangzhou[12]	2008	China	195	0	Tumor ≤ 8 cm, or tumor > 8 cm, histopathologic grade I or II and preoperative AFP ≤ 400 ng/mL	78.3%	62.4%
Kyoto[6]	2007	Japan	0	125	Tumor ≤ 10 nodules, all ≤ 5 cm and a serum DCP level ≤ 400 mAU/mL	86.7%	None
Shanghai[14]	2009	China	1074	4	Tumor ≤ 9 cm, or ≤ 3 lesions with the largest ≤ 5 cm, tumor ≤ 9 cm without macrovascular and lymph node invasion and extrahepatic metastasis	78.1%	52.6%
Asan[11]	2008	South Korea	0	221	Tumor ≤ 5 cm in diameter, ≤ 6 in nodule number, and free of gross vascular invasion	81.6%	None

UCSF: University Of California San Francisco; LDLT: Living donor liver transplantation; DDLT: Deceased donor liver transplantation; OS: Overall survival; RFS: Relapse free survival.

overstaging.

The Tokyo criteria

The 5-5 rule (tumors no larger than 5 cm and no more than 5 nodules), the first well-defined criteria for potential candidates for living donor liver transplantation (LDLT), was proposed by University of Tokyo in 2007[9] (Table 1). In the study, 78 adult patients underwent LDLT were investigated, and the 3-year recurrence-free survival rate of patients fulfilling and exceeding the criteria was 94% and 50%, respectively. Compared with patients fulfilling the MC, patients within the 5-5 rule showed equivalent recurrence-free survival rates. Due to the short median follow-up period and the small patient number, however, a further large-scale study is necessary to verify the general application of the 5-5 rule.

The Hangzhou criteria

Nearly half of HCC cases in the world are from China. Data from the China Liver Transplant Registry (CLTR) showed that more than 23000 patients with HCC underwent LT before 2012[19]. The Hangzhou center, established by Chinese researchers, adopted the histopathologic grade and biological marker alpha fetoprotein (AFP) for selection of LT candidates. The Hangzhou criteria included a total tumor diameter less than or equal to 8 cm or a total tumor diameter more than 8 cm, with a histopathologic grade Ⅰ or Ⅱ and a preoperative AFP level less than or equal to 400 ng/mL (Table 1). Notably, a 5-year survival rate of 72.3% was achieved in HCC patients within the Hangzhou criteria. A recent study that reviewed 6012 HCC patients based on the CLTR data reported that the Hangzhou criteria added to the LT candidates by 51.5% compared with the use of the MC[19].

Other criteria from Asian countries

Kyushu University reported that patients with an HCC ≤ 5 cm and a serum Des-Gamma-carboxy prothrombin (DCP) level ≤ 300 mAU/mL could achieve a 5-year survival rate of 82.7%[13] (Table 2). At Kyoto University, a 5-year survival rate of 86.7% was achieved in patients with an HCC ≤ 10 nodules; all nodules were ≤ 5 cm in diameter and had a serum DCP level lower than 400 mAU/mL[6] (Table 2). The study from the Asan Medical Center in South Korea showed that the 5-year survival rate of patients with an HCC ≤ 5 cm in diameter, ≤ 6 in nodule number, and free of gross vascular invasion was 81.6%[11].

Furthermore, the Shanghai criteria include a solitary lesion ≤ 9 cm in diameter or no more than three lesions with the largest being ≤ 5 cm with a total tumor diameter ≤ 9 cm without macrovascular invasion, lymph node invasion and extrahepatic metastasis, and this criteria achieved a 5-year overall survival of 78.1%[14]. In another study conducted in Shanghai, a new promising grading system for HCC exceeding the MC was established by Wan *et al*[20]. Patients with grade I (maximum tumor size ≤ 10 cm, preoperative AFP ≤ 400 ng/mL and no vascular and extrahepatic invasions) could achieve similar survival outcomes compared with patients fulfilling the MC (Table 2).

BIOMARKERS USED TO PREDICT HCC RECURRENCE AFTER LT

Most of the extended criteria above are established based on tumor size and number, which are more objective and convenient for clinical application, but insufficient to precisely indicate HCC biological features and to predict the risk of tumor recurrence. Many

Table 2 Tumor markers and the cut-off of different selection criteria

Markers	Authors	Cut-off	Other content	Survival (5-yr)	
				OS	RFS
AFP	Zheng *et al*[12]	AFP ≤ 400	Hangzhou criteria	78.3%	62.4%
	Toso *et al*[27]	AFP ≤ 400	Total tumor volume ≤ 115 cm^3	None	None
	Wan *et al*[20]	AFP ≤ 400	Tumor ≤ 10 cm, no vascular and extrahepatic invasions	73.7%	74.4%
DCP	Ito *et al*[6]	DCP ≤ 400	Tumor size ≤ 10 cm	86.7%	None
	Soejima *et al*[13]	DCP ≤ 300	Tumor size ≤ 5 cm	None	93.8% (3-yr)
NLR	Xiao *et al*[43]	NLR < 4	None	61.5%	60.7%
AFP/CA199	Wan *et al*[30]	AFP ≤ 400, CA199 ≤ 400	None	74.6%	78.5%
NLR/CRP	Na *et al*[47]	NLR < 6.0 or CRP < 1.0	None	None	None
DCP/AFP	Todo *et al*[35]	DCP ≤ 100, AFP ≤ 200	Milan criteria	None	96.4%
	Shindoh *et al*[36]	AFP ≤ 250, DCP ≤ 450	Tokyo criteria	84.0%	96.8%

AFP: Alpha fetoprotein; DCP: Des-Gamma-carboxy prothrombin; NLR: Neutrophil-to-lymphocyte ratio; OS: Overall survival; RFS: Relapse free survival.

centers have demonstrated that vascular invasion and poor tumor differentiation are the most important factors affecting HCC recurrence[18,21,22]. Large or multiple HCCs were not always associated with poor biological behavior, indicating that tumor size and number could not completely predict vascular invasion and tumor grade. Although some studies have added the histopathologic characteristics of the tumor when evaluating the risk of tumor recurrence[7,12,14,15], the histopathologic results are usually difficult to obtain before LT. However, many biomarkers and their dynamic changes were demonstrated to be promising predictors for the prognosis of HCC patients after LT.

AFP

AFP is the most common prognostic maker that has been studied extensively in HCC. Several centers have identified the value of preoperative AFP concentrations in predicting prognosis after LT for HCC patients[10,12,23-25]. A recent study by Berry *et al*[26] also showed that preoperative AFP level could independently predict the prognosis of HCC patients after LT. In 2009, Toso *et al*[27] investigated 6478 recipients of the Scientific Registry of Transplant Recipients (SRTR) and proposed that the total tumor volume (TTV) and preoperative AFP level could independently predict patient survival. Recently, they reconfirmed the previously proposed viewpoint and expanded the criteria to HCC patients with TTV (≤ 115 cm^3)/AFP (≤ 400 ng/mL)[28]. A systematic review conducted by Hakeem *et al*[29] has shown that a preoperative AFP level > 1000 ng/mL was associated with poorer outcomes of recipients with HCC. A study by Wan *et al*[30] showed that the use of serum AFP and carbohydrate antigen 19-9 (CA19-9) level will greatly improve the prognostic prediction of HCC patients after LT. Xu *et al*[31] investigated the AFP data before and after LT in 97 patients and reported that post-transplant AFP levels that did not decrease to ≤ 20 ng/mL within 2 mo were indicative of higher risk of recurrence. Another study conducted by Hanouneh *et al*[32] confirmed patients beyond the MC with tumor growth < 1.61 cm^3/mo experienced less recurrence than those beyond the MC with tumor growth > 1.61 cm^3/mo.

DCP

DCP, also known as protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ), has also been used as an important HCC biological marker. Correlations between DCP levels and HCC microvascular invasion and metastasis have been reported in several studies[33,34]. In the transplantation field, centers from Japan first used the DCP levels in LT candidate selection[6,13]. Todo *et al*[35] conducted a study involving 49 centers of 653 patients and reported that recipients beyond the MC but with serum AFP levels ≤ 200 ng/mL and serum PIVKA-Ⅱ levels ≤ 100 mAU/mL had a 5-year disease-free survival rate of 84.3%. Recently, Shindoh *et al*[36] also confirmed the predictive value of pre-transplant AFP and DCP levels for post-transplant HCC recurrence. After investigating 124 patients who underwent LDLT for HCC, they proposed a new scoring system that was composed of AFP (≤ 250 ng/mL), DCP (≤ 450 mAU/mL) and the Tokyo criteria (≤ 5 tumors with each tumor ≤ 5 cm). Patients fulfilling all or two of the three factors (AFP, DCP and the Tokyo criteria) had better 5-year disease-free survival rates than patients fulfilling only one, or none of the three factors.

Neutrophil-to-lymphocyte ratio

Neutrophil-to-lymphocyte ratio (NLR), also known as an index of systemic inflammation, has been reported to be associated with the prognosis of HCC patients[37]. Several studies have highlighted the value of preoperative NLR in predicting outcome after LT for HCC[38-41]. A study conducted by Harimoto *et al*[42] showed that the 3-year survival rate after recurrence in patients with NLR < 4 (43.6%) was higher than patients with NLR ≥ 4 (0%). Xiao *et al*[43] determined that NLR ≥ 4 was the main predictor of tumor

recurrence in HCC patients after LT. A recent study, however, reported that NLR was not significantly associated with post-LT HCC recurrence. Another study also showed that increased NLR was associated with worse overall survival and recurrence-free survival[44].

C-reactive protein

Serum C-reactive protein (CRP), another inflammation maker, has also been shown to be related to the prognosis of HCC patients. Both An *et al*[45] and Kim *et al*[46] determined that, the high CRP level was an independent factor in predicting poor outcomes in HCC patients beyond the MC, but not in patients within the criteria. Na *et al*[47] investigated 224 patients and reported that the disease-free survival and overall survival in patients with NLR levels $\geqslant$ 6.0 or CRP levels $\geqslant$ 1.0 were significantly worse than those of patients with NLR levels < 6.0 or CRP levels < 1.0. Chung *et al*[48] also confirmed that the intra-operative decline of CRP was related to the occurrence of gross post-transplant outcomes. However, another study reported that the pre-transplant CRP level was not significantly associated with disease-free survival[36]. The clinical relevance and prognostic value of these inflammatory markers are still being debated, and further studies are needed to confirm their role in LT.

Fluorine-18-fluorodeoxyglucose

Fluorine-18-fluorodeoxyglucose (FDG) positron emission tomography (PET) has been verified to be effective in predicting the outcome of HCC patients after liver resection and detecting extrahepatic metastases and recurrent HCC[49-52]. In the transplantation field, Yang *et al*[53] first reported that the recurrence-free survival rate of PET (-) recipients was significantly higher than that of PET (+) recipients. Several centers successively highlighted the value of preoperative FDG-PET in the assessment of tumor aggressiveness and in the prediction of tumor recurrence after LT[54-57]. A recent study conducted by Kornberg *et al*[58] showed the 5-year recurrence-free survival rate of recipients exceeding the MC with PET (-) was comparable to that of recipients within MC (81% *vs* 86.2%), but it was significantly higher than that of recipients exceeding MC with PET (+) (n = 14, 21%, P = 0.002).

HCC DOWNSTAGING ADJUVANT THERAPY BEFORE LT

Downstaging has been proved to be an effective way for patients with advanced HCC to reduce the tumor stage to fulfill the MC and achieve a complete pathologic response (cPR) by undergoing neoadjuvant therapies such as transarterial chemoembolization (TACE), radiofrequency ablation (RFA) and percutaneous ethanol injection. A recent systemic review including 950 patients reported that more than 40% of patients successfully reduced HCC to within the MC[59]. Lei *et al*[60] investigated 72 patients with advanced HCC and reported that recipients fulfilling the MC or UCSF criteria after accepting successful preoperative downstaging therapy in LDLT can achieve similar outcomes. Yao *et al*[61] also confirmed that after successfully downstaging to within the MC, HCC recipients could achieve low HCC recurrence rates and excellent post-transplant survival which was comparable to those fulfilling the MC without downstaging therapy. Agopian *et al*[62] performed a retrospective review of 501 patients who received neoadjuvant therapies and found that compared with recipients without cPR, patients with cPR had significantly lower MELD scores and significantly superior 1-, 3-, and 5-year recurrence-free and disease-specific survival.

TACE has been widely used as an optional treatment for patients with unresectable HCC. The initial experience showed conflicting outcomes in treating HCC patients awaiting LT with TACE[63,64]. Recently more studies highlighted the importance of TACE in downstaging therapy and cPR[65-69]. In a retrospective report by Chapman *et al*[65], patients successfully downstaged by TACE alone achieved excellent midterm disease-free rates and overall survival. In another study, 77% T3N0M0 HCC patients successfully reduced their tumor stage to fulfill the MC by TACE[67]. Several studies regarded that TACE mediates its effect by inducing complete histological necrosis, with reported rates of cPR in 27% to 57% of patients with TACE[68,69].

RFA has also been verified as an effective treatment for HCC by many studies[70]. In the transplantation field, RFA was used as a bridge treatment to LT in many centers[71-73]. Yao *et al*[71] reported HCC patients exceeding the conventional criteria achieved an excellent survival rate after LT when combining RFA with other loco-regional therapies. Tsuchiya *et al*[74] recently reported that recurrence within 1 year after initial locally curative RFA therapy and AFP levels > 100 ng/mL were independently associated with earlier recurrence in patients exceeding the MC.

Although more and more centers have highlighted the value of various downstaging therapies, the post-transplant HCC recurrence rates are higher than patients fulfilling MC without pre-transplant therapies[59]. To further study the role of these downstaging therapies in patients with advanced HCC, more randomized data comparing these therapies are also necessary.

LDLT *VS* DDLT FOR HCC BEYOND THE MC

Currently, approximately 70% of LDLT recipients are from Asian countries due to the shortage of deceased donation, which was caused by many social and cultural reasons. There are several advantages

including reducing the pretransplantation waiting time of patients with HCC, alleviating the ischemia-reperfusion injury because of shortened ischemic time**,** providing a an optimal donor graft for those with end-stage liver disease and even a timely graft for patients with fulminant hepatic failure promote the development of the approach. Although LDLT is criticized for its higher rate of surgical complications post-transplantation(biliary complications, vascular complications) than DDLT[75,76], it has been generally recognized by most studies that LDLT could achieve a comparable long-term survival rate in adult patients compared with DDLT[77-79].

The criteria from western countries were mainly based on a single-center experience with DDLT. Centers from Japan[6,9,13] and South Korea[11], however, proposed appropriate criteria for patients undergoing LDLT. Shirabe *et al*[80] recently investigated 109 consecutive HCC recipients undergoing LDLT and reported that compared with the other expanded criteria (UCSF, Tokyo, Kyoto University and Up-to-seven criteria), the Kyushu University criteria were the most powerful predictive criteria for HCC recurrence after LT.

For patients with HCC exceeding the MC, whether recurrence and survival rate in LDLT are different from those in DDLT remains controversial. Woo *et al*[81] reviewed 37 patients exceeding the MC and reported that a tumor size > 6 cm, progressive disease after pre-transplant treatment and a tumor exposed to the liver surface may be useful for identifying those with high HCC recurrence potential after LDLT. A study from Bhangui *et al*[82] confirmed the advantage of shorter waiting time in LDLT; they also verified that patients exceeding the MC and UCSF criteria showed a trend toward worse outcomes with LDLT compared with those of DDLT. Recently, a study conducted in Hangzhou, however, showed that outcomes after LDLT are better than those after DDLT for HCC patients who did not meet the Hangzhou criteria[83].

For patients within the MC, the long-term survival rate was comparable between DDLT and LDLT. However, to further verify the value of LDLT in HCC recipients exceeding the MC, further case-controlled research with larger patient number needs to be conducted to propose more appropriate selection criteria.

POSTTRANSPLANT ADJUVANT TREATMENT FOR HCC

Sorafenib (SFN), an oral multi kinase inhibitor, has been approved for the treatment of unresectable HCC for years. Recently, many studies verified its value in reducing the risk of recurrence and treatment for recurrent HCC after LT. Huang *et al*[84] conducted a prospective randomized study for recipients with HCC exceeding the MC and reported that compared with capecitabine, SFN could reduce or delay tumor recurrence after LT and improve patient survival. In a case-control study designed in Taiwan, HCC patients exceeding the MC after OLT treated with adjuvant sorafenib had better disease-free and overall survival rate than those in the control group[85]. Other studies[86-88] also confirmed the role of SFN in the treatment of HCC recurrence.

Calcineurin inhibitors (CNIs) were widely used in LT. However, the short and long term adverse effects including renal dysfunction and a dose-dependent increase in the post-transplant risk of HCC recurrence drew attention[89-91]. The mammalian target of rapamycin inhibitors (mTORi), such as everolimus or sirolimus, might represent an alternative immunosuppressive agent; the antineoplastic effect of mTORi has also been confirmed by several studies[92]. Two meta-analyses have reported that compared with CNIs or SRL-free regimens, sirolimus is associated with significantly lower HCC recurrence rates after LT[93,94]. A more recent meta-analysis including 42 studies of 3666 patients confirmed that compared with CNIs, mTORi was associated with lower rates of HCC recurrence after LT[95]. Moreover, the rates of HCC recurrence in HCC patients within the MC were lower in mTORi (3.8% *vs* 9.2%, $P = 0.03$), but no difference was observed among patients who had HCC exceeding the MC (29.5% *vs* 29.2%, $P = 1.0$)[95].

CONCLUSION

As various therapies mature, the treatment of HCC has progressed greatly. Because a large number of patients who exceeded the MC were unnecessarily excluded according to the MC, several extended criteria beyond the MC were proposed to benefit these recipients. Moreover, many biological markers such as AFP, DCP, NLR and CRP have been verified to be closely associated with HCC patients' prognosis and the risk of post-transplant recurrence. Although downstaging tumors to fulfill the MC allows patients with advanced HCC to potentially have an opportunity for LT, the high tumor recurrence rate after LT reported in some studies has raised attention. Moreover, SFN and mTORi may be useful to reduce the risk of HCC recurrence and to treat recurrent HCC after LT. As for the selection of graft type, the outcomes of LDLT for patients beyond the MC remains controversial.

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ORIGINAL ARTICLE

Basic Study

Deficiency of platelet-derived growth factor receptor-α-positive cells in Hirschsprung's disease colon

Anne-Marie O'Donnell, David Coyle, Prem Puri

Anne-Marie O'Donnell, David Coyle, Prem Puri, National Children's Research Centre, Our Lady's Children's Hospital Crumlin, 12 Dublin, Ireland

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Correspondence to: Prem Puri, MS, FRCS, FRCS (Ed), FACS, FAAP (Hon.), DSc (Hon.), Professor, National Children's Research Centre, Our Lady's Children's Hospital Crumlin, 12 Dublin, Ireland. prem.puri@ucd.ie
Telephone: +353-1-4096420

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Abstract

AIM: To investigate whether the expression of platelet-derived growth factor receptor-α-positive (PDGFRα$^+$)-cells is altered in Hirschsprung's disease (HD).

METHODS: HD tissue specimens (n = 10) were collected at the time of pull-through surgery, while colonic control samples were obtained at the time of colostomy closure in patients with imperforate anus (n = 10). Immunolabelling of PDGFRα$^+$-cells was visualized using confocal microscopy to assess the distribution of these cells, while Western blot analysis was undertaken to quantify PDGFRα protein expression.

RESULTS: Confocal microscopy revealed PDGFRα$^+$-cells within the mucosa, myenteric plexus and smooth muscle in normal controls, with a marked reduction in PDGFRα$^+$-cells in the HD specimens. Western blotting revealed high levels of PDGFRα protein expression in normal controls, while there was a striking decrease in PDGFRα protein expression in the HD colon.

CONCLUSION: These findings suggest that the altered distribution of PDGFRα$^+$-cells in both the aganglionic and ganglionic HD bowel may contribute to the motility dysfunction in HD.

Key words: Platelet-derived growth factor receptor alpha; Hirschsprung's disease; Gastrointestinal motility; Aganglionosis; Myenteric plexus

Core tip: Hirschsprung's disease is a congenital condition characterised by an absence of ganglia in the distal colon. Platelet-derived growth factor receptor-α-positive (PDGFRα$^+$)-cells are a novel cell type recently found to be involved in gastrointestinal neurotransmission and smooth muscle contractility. Our study has revealed a striking decrease in PDGFRα$^+$-cell expression in Hirschsprung's disease colon compared to normal control colon. These results suggest an exciting new role for PDGFRα$^+$-cells in the pathophysiology of this complex condition.

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INTRODUCTION

Gastrointestinal smooth muscle contraction is controlled by co-ordinated interaction of three main cell types: enteric nerve cells, interstitial cells of Cajal (ICCs) and smooth muscle cells (SMCs). In recent years, a fourth cell type has been described as forming part of this complex network, namely platelet-derived growth factor receptor alpha-positive cells (PDGFRα$^+$-cells). These PDGFRα$^+$-cells were, for many years, known as "fibroblast-like cells" or "ICC-like" cells, as they resembled ICCs morphologically, but were c-kit negative[1]. More recently, enhanced green fluorescent protein (eGFP) labelling of these cells, as well as commercial availability of antibodies directed against PDGFRα, has enabled specific and reliable identification of this cell type[2]. PDGFRα$^+$-cells form discrete networks in the region of the myenteric plexus and within the circular and longitudinal muscle layers[3]. PDGFRα$^+$-cells express the small-conductance Ca^{2+}-activated K^+ channel (SK3), which is an important mediator of purinergic neurotransmission in gastrointestinal smooth muscle[3,4].

Platelet-derived growth factors (PDGFs) comprise four subtypes (A, B, C, and D), and their receptors, PDGFRs, have two subtypes, PDGFRα and PDGFRβ. It is known that PDGFs exist in epithelial cells, endothelial cells, and fibroblasts of adult mammals. They play an important role in cell proliferation, survival, and migration[5,6]. PDGFs are also present in the extracellular matrix and participate in tissue remodeling. It has been demonstrated that the PDGF/PDGFR signaling pathway plays a crucial role in embryonic development, especially in organogenesis, including alveogenesis, glomerulogenesis, angiogenesis and spermatogenesis[5]. This signaling pathway is also essential for interstitial cell and mesenchymal cell proliferation. Bonner[7] have previously shown that PDGF-A and the PDGFRα are important in gastrointestinal development, as mice lacking PDGF-A or PDGFRα develop abnormal gastrointestinal mucosal lining, which is also associated with a loss of PDGFRα-positive mesenchymal cells underlying the intestinal epithelium.

Hirschsprung's disease (HSCR) is a congenital gut motility disorder with an incidence of 1 in 5000 live births[8]. It is characterized by an absence of ganglia within the distal colon for varying distances[9]. Most cases present in the newborn period with a failure to pass meconium, abdominal distension and bilious vomiting. A disruption of neural crest cell migration during the early stages of embryonic development is thought to be the main cause of this condition, with neural crest cells failing to complete their cranio-caudal colonization of the gastrointestinal tract. In addition to a lack of ganglia, many studies have documented deficiencies in smooth muscle proteins, extracellular matrix molecules, ion channels and various other important molecules in HSCR colon[10]. Our group has previously reported a reduction of ICCs in HSCR colon[11].

In recent years, several animal studies have reported the expression of the PDGFRα$^+$-cells in the various regions of the gastrointestinal tract. However, there is little information available regarding PDGFRα$^+$-cells distribution in gastrointestinal diseases. We designed this study to test the hypothesis that PDGFRα$^+$-cell distribution is altered in Hirschsprung's disease.

MATERIALS AND METHODS

Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HD specimens from 10 patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic samples. Patients were aged 6 ± 3 mo old. No additional health issues existed in these patients. Colonic control samples included 10 specimens from patients who underwent colostomy closure following surgical correction of imperforate anus. Control samples were taken from patients who were 11 ± 4 mo old. None of the imperforate anus patients had HD. Tissue specimens were either snap-frozen in liquid nitrogen and stored at -80 ℃ for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at -80 ℃ until use.

Immunofluorescence staining and confocal microscopy

Frozen blocks of HD colon and control samples were sectioned transversely at a thickness of 10 μm, mounted on SuperFrost® Plus slides (VWR International, Leuven,

Belgium) and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma Aldrich Ltd, Arklow, Ireland) for 30 min to avoid non-specific absorption, sections were incubated with primary antibodies: Primary antibodies including: rabbit anti- PDGFRα (Abcam, Cambridge, United Kingdom), mouse anti-HuC/HuD (Molecular Probes), mouse anti-α-smooth muscle actin, mouse anti-c-kit mouse anti-TLR4, mouse anti-TLR5 (Abcam, Cambridge, United Kingdom), and mouse anti-P2YR1 (Abnova, Taiwan) all used at dilution 1:100, overnight at 4 ℃. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies (goat anti-rabbit Alexa Fluor® 488, dilution 1:200 and goat anti-mouse Alexa Fluor® 647, dilution 1:200, Abcam, Cambridge, United Kingdom) for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted and cover-slipped with Fluorescent Mounting Medium (DAKO Ltd, Cambridgeshire, United Kingdom). All sections were independently evaluated by two investigators with a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Protein extraction and Western blot

Specimens of HD colon and healthy control colon were homogenized in RIPA buffer (Radio Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland) containing 1% protease inhibitor cocktail (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). A total volume of 20 μL Laemmli sample buffer (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) containing 10 μg of protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis-Tris gels, Invitrogen, Carlsbad, United States) for electrophoretic separation. The electrophoresis was performed in MES SDS running buffer (Invitrogen, Carlsbad, United States). Proteins were then transferred to 0.45 μm nitrocellulose membrane (Millipore Corporation, Billerica, United States) by western blotting. Following western blotting, the membranes were blocked in 3% BS-0.05% Tween for 30 min before antibody detection. A primary antibody, rabbit anti-PDGFRα (Abcam, Cambridge, United Kingdom), dilution 1:1000, was used and incubation was performed overnight at 4 °C. Following extensive washing (four times in PBS-0.05% Tween) the membranes were incubated with the appropriate secondary antibody (goat anti-rabbit IgG, HRP-linked Antibody, dilution 1:10000, Abcam, Cambridge, United Kingdom) followed by washing (four times in PBS-0.05% Tween). Detection was performed with the ECL plus chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland). We used GAPDH (mouse anti-GAPDH, dilution 1:1000, Abcam, Cambridge, United Kingdom) as an additional loading control.

RESULTS

Immunofluorescence staining and confocal microscopy

Confocal microscopy revealed PDGFRα$^+$-cells within the mucosa, myenteric plexus and smooth muscle in colonic controls, with a marked reduction in PDGFRα$^+$-cells in the HD specimens (Figure 1). As other authors have reported, we noticed that PDGFRα$^+$-cells and their projections were located adjacent to and surrounding both enteric neurons and fibres, as well as alongside ICCs in the myenteric plexus and within the smooth muscle layers. In the colonic mucosa, we found PDGFRα$^+$-cells expressed the toll-like receptors 4 and 5, as well as the purinergic receptor, P2Y2R1, as has previously been reported in the murine colonic mucosa[2].

Western blot

Western blotting revealed high levels of PDGFRα protein expression in colonic controls, while there was a striking decrease in PDGFRα protein expression in both ganglionic and aganglionic regions of HD (Figure 2). Equal expression was observed in ganglionic *vs* aganglionic segments.

DISCUSSION

The location of PDGFRα$^+$-cells alongside enteric nerves, ICCs, their close proximity to nerve varicosities and the fact that they are coupled by gap junctions to SMCs, strongly suggest a role for these cells in enteric neurotransmission[12-14]. ICCs are the enteric pacemaker cells, which also function as mediators of neurotransmission, and are important regulators of GI motility. C-kit, which is structurally similar to PDGFRs, is mainly expressed in ICCs of the GI tract. C-kit and PDGFRα are both receptor tyrosine kinases. We postulate that in HD colon, the role of PDGFRα$^+$-cells in purinergic neurotransmission is disrupted leading to bowel dysmotility in both the ganglionic and aganglionic segments.

Many studies have investigated the expression of PDGFRα$^+$-cells in the gastrointestinal tract of various animals in recent years. Iino *et al*[1] were the first authors to examine PDGFRα$^+$-cells in the murine gastrointestinal tract. They observed these cells in the musculature in all regions of the gastrointestinal tract, closely associated with intramuscular ICCs and enteric nerve fibres. In the myenteric plexus, PDGFRα$^+$-cells were seen to form a cellular network with their ramified processes and encompassed myenteric ganglia. Numerous PDGFRα$^+$-cells were also observed in the sub-serosal plane and showed a multipolar shape[1]. They also analyzed the distribution of PDGFRα$^+$-cells in the ICC-deficient W(v)/W(v) mutant mice and

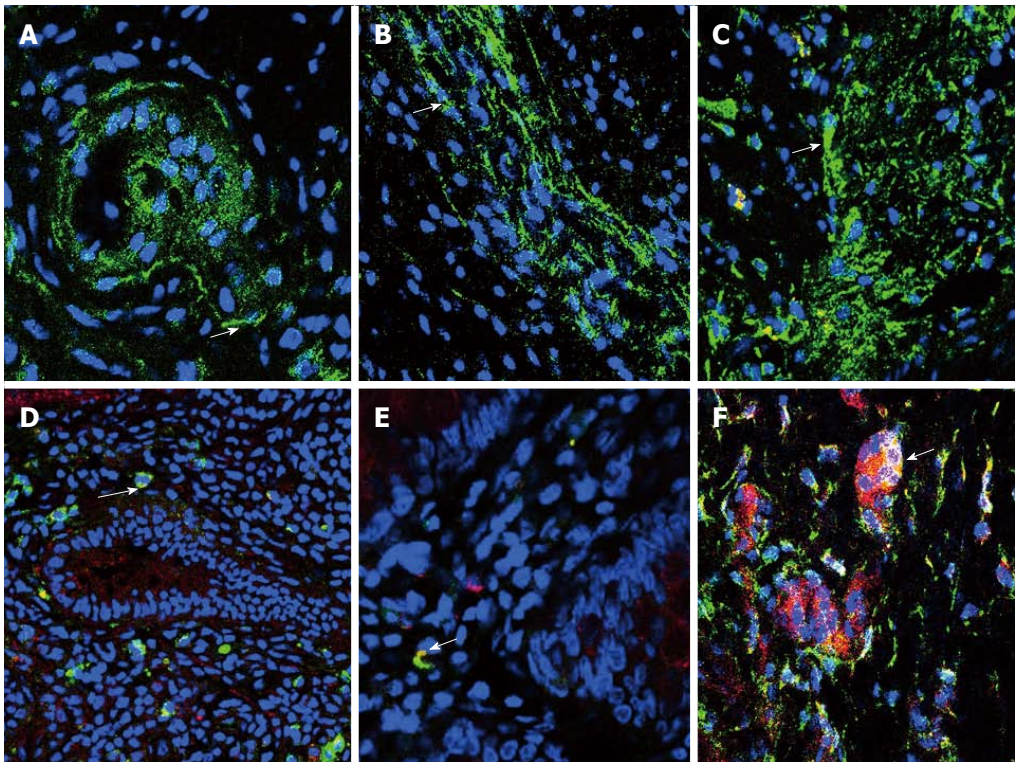

Figure 1 Immunofluorescent staining of platelet-derived growth factor receptor-α-positive-cells. Immunofluorescent staining of platelet-derived growth factor receptor-α-positive (PDGFRα+)-cells (green) in the myenteric plexus of normal control (A), aganglionic (B) and ganglionic (C) colon, arrow shows cell body. Nuclei were stained with DAPI (blue). Mucosal PDGFRα+-cells (green) were seen to co-express TLR4 (red) (D) (arrow), TLR5 (red) (E) (arrow) and P2RY1 (red) (F) (arrow). A-F: Magnification × 40.

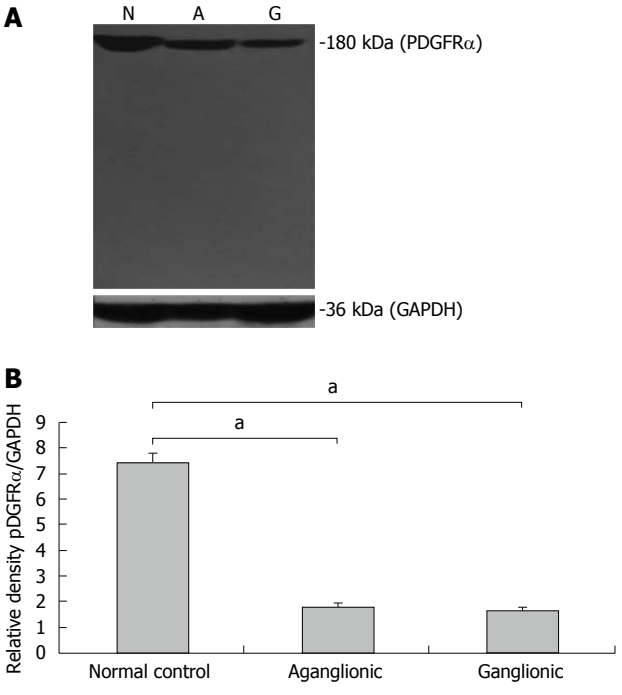

Figure 2 Western blot of platelet-derived growth factor receptor-α protein expression. A: Platelet-derived growth factor receptor-α (PDGFRα) protein expression was high in normal controls and markedly decreased in both ganglionic and aganglionic specimens. The loading control GAPDH was similarly expressed in normal controls, ganglionic and aganglionic specimens; B: Densitometry analysis showed the range of PDGFRα protein expression among the samples studied. ^aP values are significant *vs* normal control.

found that the expression pattern of PDGFRα$^+$-cells was the same as that in wild-type mice, suggesting no interdependence between ICCs and PDGFRα$^+$-cells[1]. The authors also reported different morphological features of PDGFRα$^+$-cells dependent on which region of the GI tract they assessed; for example PDGFRα$^+$-cells in the myenteric plexus of the gastric fundus had wider cell bodies than those in other GI regions, while PDGFRα$^+$-cells in the circular muscle layer of the small and large intestines had wider cell bodies and more cytoplasmic extensions than those of the stomach[1].

A later study by Cobine *et al*[12] examined the distribution of ICCs, PDGFRα$^+$-cells and nitric-oxide-synthase-positive neurons and nerve fibres in the internal anal sphincter of both wild-type mice and KitW/Wv mice, in which ICC numbers are greatly reduced in many regions of the GI tract. They found that PDGFRα$^+$-cells made up 18% of the total tissue volume within the circular muscle layer of the mouse internal anal sphincter, compared to ICC-IM which made up only 5% of tissue volume. They also noted PDGFRα$^+$-cells distributed within the submucosa and along the serosal and myenteric surfaces. They also noted a close morphological arrangement between inhibitory motor neurons, ICC-IM and PDGFRα(+)-IM, which suggested functional interaction between these cell types in the internal anal sphincter[12].

Kurahashi *et al*[13] were the first group to confirm

a functional role for PDGFRα$^+$ cells in gastrointestinal smooth muscle using transgenic mice with constitutive expression of enhanced green fluorescent protein (eGFP) in PDGFRα$^+$-cells, which allowed the authors to isolate and study the function of PDGFRα$^+$-cells. They found that PDGFRα$^+$-cells expressed appropriate receptors and effectors to receive and transduce purinergic neural signals[13]. In 2012, another study by Kurahashi *et al*[3] used immunohistochemical techniques to study the phenotype and intercellular relationships PDGFRα$^+$-cells widely distributed throughout the tunica muscularis of the human colon. They reported that PDGFRα$^+$-cells form discrete networks in the region of the myenteric plexus and within the circular and longitudinal muscle layers of the human colon. These cells were seen to be distinct from ICCs and were closely associated with varicose processes of neurons expressing the excitatory neurotransmitter substance P or the inhibitory neurotransmitter neuronal nitric oxide synthase. They also found that PDGFRα$^+$-cells express small conductance Ca(2$^+$)-activated K($^+$) channels (SK3), which are likely to mediate purinergic neural regulation of colonic muscles[3]. Our group has also recently confirmed SK3 channel expression is decreased in HD[4].

Furthermore, another recent study by Peri *et al*[15] confirmed that SK3 channels are strongly enriched in PDGFRα$^+$-cells and that the expression of these channels in this cell type far exceeded their expression in either ICCs or SMCs. Analysis showed that P2X receptor genes were expressed more highly in PDGFRα$^+$-cells than SMCs. PDGFRα$^+$-cells showed high expression of P2rx1, P2rx3, P2rx4, P2rx5, P2rx6, and particularly P2rx7. In addition, PDGFRα$^+$-cells showed high expression of P2ry1, P2ry2, P2ry12, and P2ry13. Therefore, PDGFRα$^+$-cells might be a target for extracellular ATP acting on ionotropic P2X receptors, as well as P2Y receptors which are activated by adenine, pyridine, and pyrimidine nucleotides[15]. Kurahashi *et al*[2], also revealed a unique population of PDGFRα$^+$-cells in the mucosal layer of colon. These authors found that sub-epithelial PDGFRα$^+$-cells in the mouse colonic mucosa expressed toll-like receptor genes, purinergic receptor genes, 5-hydroxytryptamine 4 receptor gene, and hedgehog signaling genes[14].

In our current study, we have verified that the mucosal PDGFRα$^+$-cells in the human colon also express TLR4, TLR5 and P2RY1, as has been observed in the mouse colonic mucosa, suggesting a role for these cells in the immune response and in purinergic neurotransmission. The results of our study have revealed a striking decrease in PDGFRα$^+$-cell expression in both ganglionic and aganglionic regions of HD compared to normal control colon. We have previously also shown a marked decrease in the expression of both ICCs and SK3 channels in HD colon compared to controls[11,16]. The reduced volume of PDGFRα$^+$-cell expression indicates a deficiency in inhibitory neurotransmission in HD bowel and may further our understanding of the mechanism by which aganglionic bowel remains in a state of tonic contraction. These results suggest an exciting new role for PDGFRα$^+$-cells in the pathophysiology of this complex condition.

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COMMENTS

Background

Gastrointestinal smooth muscle contraction has been thought to be controlled by co-ordinated interaction of three main cell types: enteric nerve cells, interstitial cells of Cajal and smooth muscle cells. In recent years, a fourth cell type has been described as forming part of this complex network, namely platelet-derived growth factor receptor-α-positive (PDGFRα$^+$)-cells.

Research frontiers

PDGFRα$^+$-cells have been studied in normal human colon, but as yet have not been analysed in human disease. Hirschsprung's disease is the most common congenital gut motility disorder. Although the characteristic pathological findings in HSCR are well understood (aganglionosis and nerve cell hypertrophy), the mechanism underlying motility disturbance is not completely understood.

Innovations and breakthroughs

PDGFRα$^+$-cells have been studied in normal human colon but, as yet, their expression in gastrointestinal disease has not been analysed. The authors report the first investigation of PDGFRα$^+$-cell expression in the colon of Hirschsprung's Disease, and show a deficiency of these cells in this condition.

Applications

In demonstrating reduced expression of PDGFRα$^+$-cells in Hirschsprung's Disease, this study provides the basis for future functional studies that may seek to determine their exact role in colonic dysmotility in HSCR and potentially in other gut motility disorders.

Terminology

PDGFRα$^+$-cells are a type of interstitial cell involved in neurotransmission in the gastrointestinal tract. Along with interstitial cells of Cajal, enteric neurons and smooth muscle cells, PDGFRα$^+$-cells help maintain peristalsis of the colon.

Peer-review

The authors have explored the expression of PDGFRα$^+$-cells in colon specimens of Hirschsprung's disease patients. They found, for the first time, a deficiency of PDGFRα$^+$-cells in both the aganglionic and ganglionic colon compared to normal controls. These results suggest a role for PDGFRα$^+$-cells in the pathophysiology of Hirschsprung's Disease.

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ORIGINAL ARTICLE

Basic Study

Auphen and dibutyryl cAMP suppress growth of hepatocellular carcinoma by regulating expression of aquaporins 3 and 9 *in vivo*

Rui Peng, Guang-Xi Zhao, Jing Li, Yu Zhang, Xi-Zhong Shen, Ji-Yao Wang, Jian-Yong Sun

Rui Peng, Guang-Xi Zhao, Jing Li, Yu Zhang, Xi-Zhong Shen, Ji-Yao Wang, Jian-Yong Sun, Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Author contributions: Peng R and Zhang Y performed the majority of experiments; Zhao GX and Li J provided vital reagents and analytical tools and were also involved in editing the manuscript; Shen XZ, Wang JY and Sun JY co-ordinated and provided the collection of all the human material in addition to providing financial support for this work; Peng R and Sun JY designed the study and wrote the manuscript.

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Correspondence to: Jian-Yong Sun, PhD, Department of Gastroenterology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China. sun_jianyong1986@163.com
Telephone: +86-10-64041990
Fax: +86-10-64041990

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Abstract

AIM: To investigate whether the regulation of aquaporin 3 (AQP3) and AQP9 induced by Auphen and dibutyryl cAMP (dbcAMP) inhibits hepatic tumorigenesis.

METHODS: Expression of AQP3 and AQP9 was detected by Western blot, immunohistochemistry (IHC), and RT-PCR in HCC samples and paired non-cancerous liver tissue samples from 30 hepatocellular carcinoma (HCC) patients. A xenograft tumor model was used *in vivo*. Nine nude mice were divided into control, Auphen-treated, and dbcAMP-treated groups (n = 3 for each group). AQP3 and AQP9 protein expression after induction of xenograft tumors was detected by IHC and mRNA by RT-PCR analysis. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and histological evaluation were used to detect apoptosis of tumor cells, and the concentration of serum α-fetoprotein (AFP) was measured using RT-PCR and an ELISA kit.

RESULTS: The volumes and weights of tumors decreased significantly in the Auphen- and dbcAMP-

treated mice compared with the control mice ($P < 0.01$). The levels of AQP3 were significantly lower in the Auphen treatment group, and levels of AQP9 were significantly higher in thedbcAMP treatment mice than in the control mice ($P < 0.01$). The reduction of AQP3 by Auphen and increase of AQP9 by dbcAMP in nude mice suppressed tumor growth of HCC, which resulted in reduced AFP levels in serum and tissues, and apoptosis of tumor cells in the Auphen- and dbcAMP-treated mice, when compared with control mice ($P < 0.01$). Compared with para-carcinoma tissues, AQP3 expression increased in tumor tissues whereas the expression of AQP9 decreased. By correlating clinicopathological and expression levels, we demonstrated that the expression of AQP3 and AQP9 was correlated with clinical progression of HCC and disease outcomes.

CONCLUSION: AQP3 increases in HCC while AQP9 decreases. Regulation of AQP3 and AQP9 expression by Auphen and dbcAMP inhibits the development and growth of HCC.

Key words: Hepatocellular carcinoma; Nude mice; Auphen; Dibutyryl cAMP; Aquaporin 3; Xenograft tumor model; Aquaporin 9

Core tip: This is the first study intending to evaluate the antioncogenic effects of Auphen and dibutyryl cAMP (dbcAMP) *in vivo* and investigate whether their underlying mechanism involves regulating aquaporin 3 (AQP3) and AQP9 expression. An in-depth description of AQP3 and AQP9 regulation by Auphen and dbcAMP will provide a better understanding of the mechanisms of hepatocarcinogenesis, which could be used in the development of novel therapeutic drugs. This work further confirms the significance of AQP-driven hepatocarcinogenesis, emphasizing the importance of both basic and clinical knowledge of the roles of aquaporins in hepatocellular carcinoma.

Peng R, Zhao GX, Li J, Zhang Y, Shen XZ, Wang JY, Sun JY. Auphen and dibutyryl cAMP suppress growth of hepatocellular carcinoma by regulating expression of aquaporins 3 and 9 *in vivo*. *World J Gastroenterol* 2016; 22(12): 3341-3354 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3341.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3341

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant cancer worldwide; however, the mechanism of hepatocarcinogenesis is unknown, and a reliable prognosis is still lacking[1,2]. Thus, novel treatment regimens that allow for the prevention and retardation of HCC still need to be identified. Aquaporins (AQPs) consist of 13 small, hydrophobic, integral, transmembrane, water channel proteins, which have an important role in the control of water movement, fluid transport, and cell migration[3]. AQPs are closely associated with cancer biological functions and have been identified in > 20 human cancer cell types[4]. AQP expression is positively correlated with tumor type, grade, proliferation, migration, angiogenesis, or tumor-associated edema[5-7], which can be considered a diagnostic and therapeutic target in anti-cancer treatment. Thus, analyzing the expression and distribution of AQPs in liver tumors is of great significance.

AQP3 and AQP9, in particular, are considered to be closely associated with cancer development because of their dramatically changed levels in various cancers, including HCC[8]. AQP3 and AQP5 are overexpressed in HCC, which are related to tumor grade, stage, metastasis and prognosis, and may be helpful in diagnosis of HCC when combined with serum α-feto-protein (AFP)[9]. AQP9 expression is reduced in HCC and mainly located in non-tumorigenic liver tissue[10]. Furthermore, AQP3 has been found to be involved in cell proliferation in many cell types such as those in the skin, colon, and cornea. Serna *et al*[11] found that AQP3 was positively associated with cell proliferative activity. Several *in vivo* and *in vitro* experiments have shown that AQP3 can promote cell proliferation and migration[12-15]. Some researchers suggest that AQP9 could be a novel target for drug therapy in liver cancer patients because its transport activities do not extend to charged neutral molecules, such as purine, pyrimidine, and urea, including permeability to 5-fluorouracil. Besides, Jablonski *et al*[16] found that decreased AQP9 expression in HCC can increase resistance of HCC cells to apoptotic stimulation, and AQP9 expression decreases with the degree of tumor cell differentiation. Thus, the targeted regulation of AQP3 and AQP9 may provide significant therapeutic benefits to HCC patients.

Recently, agents modulating the expression of AQPs have been reported, which contain heavy metals[17-20], quaternary ammonium salts[21-23], or mineral salts[24]. Although these agents are valuable in characterizing the effect of AQP regulation in cells, they are not suitable for clinical application because of their toxic side effects and poor selectivity. These modulators have various therapeutic traits, such as anticancer, antirheumatic, and antibiotic properties. Au(Ⅲ) compounds and isoelectronic and isostructural Pt(Ⅱ) compounds can be used as anti-tumor drugs[25-27]. An Au(Ⅲ) complex has been shown to have effective antiproliferative traits *in vitro* against various cancer cells with high cytotoxic potency and selectivity. It is possible that these properties arise from their possible inhibition of histone deacetylase[28]. Martins *et al*[29] reported that an Au(Ⅲ) complex was a selective and potent inhibitor of AQP3. In addition, Auphen showed antiproliferative traits in tumor cells *in vitro*[30-32]. Yamamoto *et al*[33]

Table 1 Clinicopathological data of the hepatocellular carcinoma cohort

Clinicopathologic parameters	Frequency	%
All cases	30	
Gender		
Male	23	76.7
Female	7	23.3
Age (yr)		
< 50	5	16.7
> 50	25	83.3
Tumor size (cm)		
< 5	8	26.7
> 5	22	73.3
Serum HBsAg		
Positive	24	80.0
Negative	6	20.0
Serum AFP (ng/mL)		
< 25	4	13.3
> 25	26	86.7
Cirrhosis		
Presence	27	90.0
Absence	3	10.0
UICC stage		
Ⅰ + Ⅱ	13	43.3
Ⅲ + Ⅳ	17	56.7
Metastasis/Recurrence		
Yes	18	60.0
No	12	40.0
Edmondson grade		
Low (Ⅰ/Ⅱ)	10	33.3
High (Ⅲ/Ⅳ)	20	66.7

found a protein kinase A (PKA) activator (dibutyryl cAMP; dbcAMP) and a PKA inhibitor (cycloheximide) can increase and reduce the expression of AQP9, respectively, thereby demonstrating that PKA-based approaches can increase AQP9 expression. There are currently no reports describing the effects of Auphen on AQP3 and dbcAMP on AQP9 in HCC *in vivo*. Therefore, we used nude mice subcutaneously xenografted with human HCC SMMC-7721 cells to study their effects.

In the present study, we assessed the anti-oncogenic effects of Auphen and dbcAMP *in vivo* and investigated whether their underlying mechanisms regulate AQP3 and AQP9 expression. We also analyzed the correlation between AQP3 and AQP9 expression and clinicopathologic features of HCC, which demonstrated that both AQP3 and AQP9 play an important role in HCC tumor development and clinical prognoses. Taken together, our results significantly contribute to the evaluation of the anti-oncogenic effects of Auphen and dbcAMP *in vivo*.

MATERIALS AND METHODS

Drugs

Auphen was synthesized according to a previously described method[31] and prepared at a concentration of 1 mmol/L by adding dimethylsulfoxide (DMSO) and normal saline (NS). dbcAMP was purchased from Calbiochem (San Diego, CA, United States) and prepared at a concentration of 3 mmol/L using NS. The purity of the complex was > 98% based on elemental analysis.

Patients and specimens

All patients were recruited between 2002 and 2012 at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). In accordance with the protocol approved by the Zhongshan Hospital Research Ethics Committee, all patients participating in this study provided informed consent. HCC specimens and paired normal liver tissues from 30 patients, and their clinicopathological information were obtained from the Liver Cancer Institute and Zhongshan Hospital (Table 1). The collected HCC tissues had no selection bias. All patients had: (1) a pathological diagnosis of HCC; (2) tumor stages diagnosed based on the 2002 TNM staging system of the Union for International Cancer Control; and (3) tumor differentiation determined using the Edmondson grading system. All patients were not being treated with any anti-tumor mediations before collecting the biopsy samples.

Immunohistochemistry

Endogenous peroxidase was blocked with 3% H_2O_2. Sections were treated with a primary antibody against AQP3 (1:50; Abcam, Cambridge, MA, United States) or AQP9 (1:50; Abcam), and were washed three times in phosphate-buffered saline. Then, sections were incubated with a biotinylated goat anti-rabbit IgG (1:250 dilution; Abcam). The reaction was visualized using a diaminobenzidine (DAB) substrate chromogen solution (Gene Technology, Shanghai, China). The sections were counterstained with Mayer's hematoxylin, and the slides were examined under an optical microscope.

Total RNA extraction and real-time PCR

Total RNA was prepared from tumor tissues using RNAiso Plus (TaKaRa, Tokyo, Japan), and reverse transcribed using PrimeScript RT Master Mix (TaKaRa) with the GeneAmp-PCR system 7500 (Applied Biosystems, Foster City, CA, United States). The cDNA obtained was amplified using SYBR Premix Ex Ta (TaKaRa) on the Master cycler ep realplex4 PCR system (Eppendorf, Hamburg, Germany) with the primers listed in Table 2. The cycling parameters were: 1 min at 95 ℃, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s. The mRNA expression levels were computed after normalization against β-actin mRNA levels using the $2^{-\Delta\Delta Ct}$ method. Each assay was performed in triplicate.

Xenograft tumor model in nude mice

SMMC-7721 cells (5×10^9 cells) were injected subcutaneously into each flank of nine male BALB/c nude mice (Shanghai Slac Laboratory Animal Co.

Table 2 Primer sequences used for the amplification of different genes by quantitative PCR

Gene	Forward primer sequence	Reverse primer sequence
AQP3	5'-CACAGCCGGCATCTTTGCTA-3'	5'-TGGCCAGCACACACACGATA-3'
AQP9	5'-CTTAACAATTCACAAGGCACTT-3'	5'-TCTCAGCCAGCTACTGATCTTC-3'
AFP	5'-ACCCTGGTGTTGGCCAGTGC-3'	5'-GCAGCGCTACACCCTGAGCT-3'
β-actin	5'-TCACCCACACTGTGCCCATCTACGA-3'	5'-CAGCGGAACCGCTCATTGCCAATGG-3'

Ltd., Shanghai, China) with an age of 4 wk and a weight of 18-20 g. Tumor diameters were measured in three dimensions with a Vernier caliper to diagnose tumorigenesis at 21 d. A nodal diameter up to 0.5 cm was defined as a tumor. The frequency of tumor production was 100% at 21 d after inoculation. The protocol for the treatment of animals was approved by the Ethics Committee of Zhongshan Hospital. When the tumor diameters measured at least 1-2 cm, the nude mice with tumors were divided into three groups with no selection bias (n = 3); group 1 was treated with a placebo; group 2 with Auphen at a dose of 1 mmol/L; and group 3 with dbcAMP at a dose of 3 mmol/L. Tumor volumes were measured with calipers using the formula: $(\text{width})^2 \times \text{length}/2$. Mice were followed for 2 wk. All mice were euthanized after 4 wk, and the tumors were removed and used for further analysis. The inhibition rate of tumor progression was calculated using the following formula: inhibition rate (%) = [NS treated tumor weight (g) - drug treated tumor weight (g)/NS treated tumor weight (g) × 100%]. Part of the tumor tissues were stored at -80 ℃. The remaining tissues were fixed in 10% formalin, embedded in paraffin, and used for real-time PCR.

ELISA for measurement of AFP in serum of nude mice

Blood collected from the tail vein under aseptic conditions was centrifuged at 2600 rpm at 10 ℃ for 10 min. The level of AFP was detected using an ELISA kit from HUMAN (GmbH, Wiesbaden, Germany). The ELISA method was based on the affinity of biotin to streptavidin immobilized on the surface of a microtiter well. A complex was formed by mixing the enzyme-antibody conjugate with the serum. The zymolyte was added following incubation and washing to develop a color. The strength of the color was directly proportional to the concentration of AFP in serum. An ELISA reader was used at 450 nm to detect the optical density of the reaction.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

After fixation with 10% formalin for 4 h, the tumor tissues were embedded in paraffin. Transferase-mediated dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (KGI Biotechnology, Nanjing, China). After being deparaffinized, the samples were mixed with 3% H_2O_2 for 10 min at room temperature, then incubated in a wet box with fluorescein dUTP for 1 h at 37 ℃. After treatment with horseradish peroxidase, the tissues were stained with DAB and counterstained with methyl green. The matched groups had the same treatment except for treatment with the fluorescein dUTP. A light microscope was used to visualize the nuclei of the tissue, which showed a brown color as a positive result.

Histological evaluation

Tissues were immobilized in formalin, embedded in paraffin, and cut into 4 μm sections. The sections were then stained with hematoxylin and eosin (HE) as previously described[34].

Statistical analysis

Fisher's exact test for nonparametric variables and Student's t-test (two-tailed) for parametric variables were used. Changes in animal survival were estimated by the Kaplan-Meier method and analyzed using Cox regression analysis and univariate analysis. Data are presented as the mean ± SD. Statistical analysis of data was performed with one-way analysis of variance among three groups using SPSS, version 19.0 software (SPSS, Armonk, NY, United States). A P-value < 0.05 was regarded as statistically significant.

RESULTS

Expression of AQP3 and AQP9 differs in HCC

We investigated AQP3 and AQP9 levels in HCC tumor samples and their coupled non-neoplastic counterparts. Representative mRNA images show that AQP3 (Figure 1A) was overexpressed whereas AQP9 (Figure 1B) was expressed at a low level in HCC tissues compared with their non-neoplastic counterparts, although the levels of AQP3 and AQP9 were similar in both tumors and noncancerous tissues (Figure 1A, samples 2 and 20; Figure 1B, samples 22 and 26). The results were confirmed by positive staining and protein expression analyses of AQP3 (Figure 2) and AQP9 (Figure 3). As shown by immunohistochemical analyses, HCC samples showed intense staining of AQP3 in the membrane and cytoplasm, and weak membrane staining of AQP9, while the noncancerous tissues presented mainly weak expression of AQP3 and strong expression of AQP9, indicating that AQP3 was

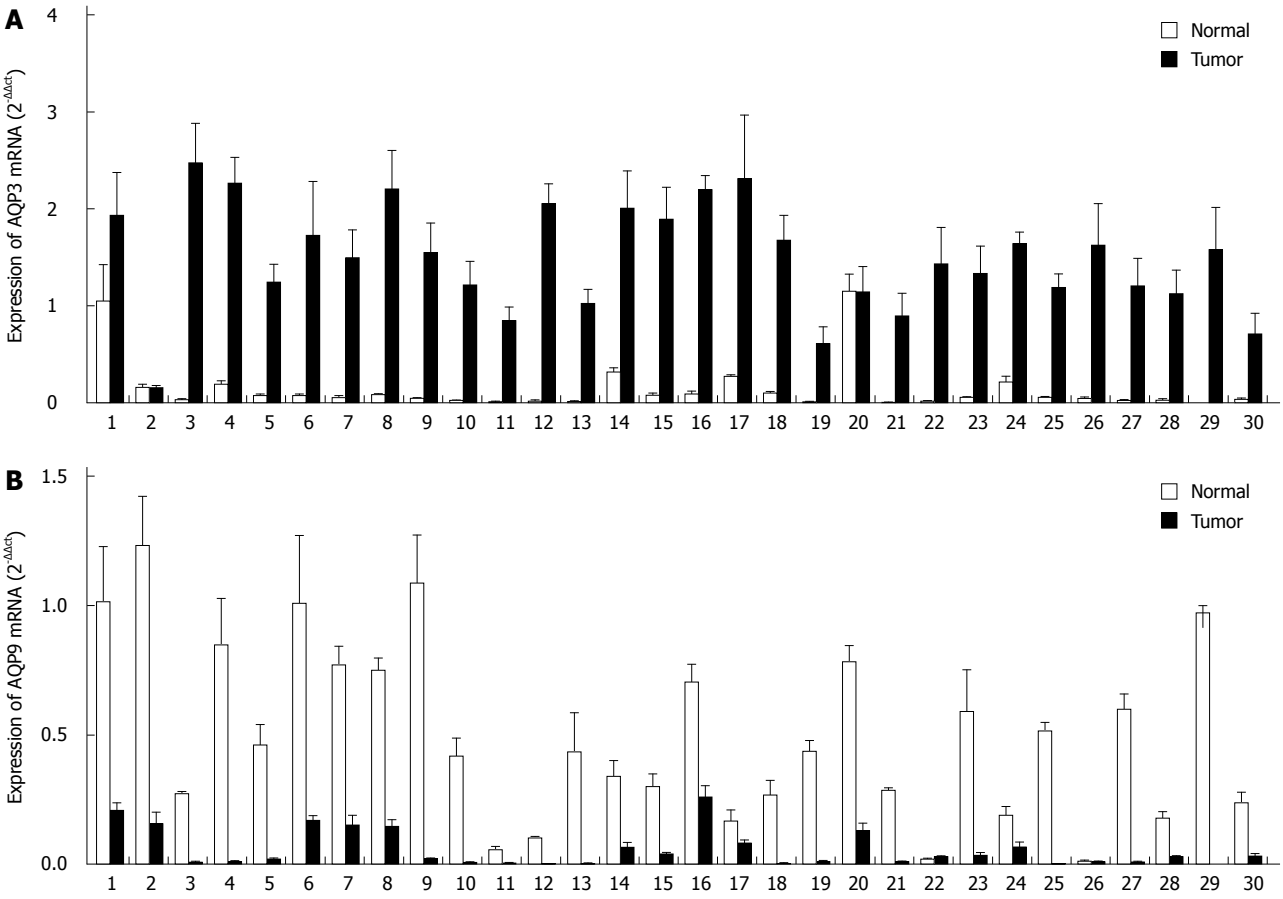

Figure 1 mRNA levels of AQP3 and AQP9 in hepatocellular carcinoma and normal liver tissues. A: mRNA levels of AQP3 in 30 paired HCC tissues and normal liver tissues measured by RT-PCR; B: mRNA levels of AQP9 in 30 paired HCC tissues and normal liver tissues measured by RT-PCR. HCC: Hepatocellular carcinoma; N: Normal liver tissues; T: HCC tissues.

overexpressed and AQP9 was reduced in HCC tissues.

Correlation of AQP3 and AQP9 expression with clinicopathologic traits and prognosis

To explore the relevance between AQP3 and AQP9 expression and clinicopathologic traits and prognosis, we analyzed the expression data in Table 3, and found that AQP3 and AQP9 expression correlated with liver neoplasm stage (P = 0. 029 and P = 0.003, respectively), metastasis (P = 0.026 and P = 0.031, respectively), andtumor differentiation (P = 0.016 and P = 0.047, respectively). Other clinical characteristics, including age, gender, tumor size, hepatic sclerosis, serum hepatitis B surface antigen (HBsAg), and serum AFP were not correlated with the expression of AQP3 or AQP9 (Table 3).

Correlation of AQP3 and AQP9 expression with overall survival

We explored whether AQP3 and AQP9 expression correlated with the clinical progression and prognosis of HCC, by examining patients' overall survival rates. The overall survival of the patients with low AQP3/high AQP9 was greater than that of the patients with high AQP3/low AQP9 (P = 0.045, P = 0.020) (Figure 4A and B), which was independent of whether they received HCC-tailored treatment or not. The results suggest that AQP3/AQP9 could be used for assessing the clinical prognosis of HCC. The parameters used to assess the influence on overall survival involved AQP3 and AQP9 expression, age, gender, tumor size, liver cirrhosis, serum HBsAg, serum AFP, tumor stage, metastasis, and tumor differentiation. The data according to the Cox proportional hazards test showed that AQP3 and AQP9 expression, tumor stage, metastasis, and tumor differentiation were independent prognostic parameters of survival (Table 4). Hence, the results show that AQP3 or AQP9 expression correlated with a poor prognosis in HCC patients.

Effect of Auphen and dbcAMP on expression of AQP3 and AQP9 in tumor-bearing nude mice

To obtain a better understanding of the regulation of AQP3 and AQP9 in HCC, we determined the effect of Auphen and dbcAMP on the expression of AQP3 and AQP9 *in vivo*. Expression of AQP3 in Auphen-treated tumors was lower than that in the control mice. Expression of AQP9 in dbcAMP-treated tumors was higher than that in the control mice. Decreased levels of AQP3 and increased levels of AQP9 were confirmed at both the mRNA level (Figure 5A and B), and the protein level by Western blot (Figure 5C and D), and

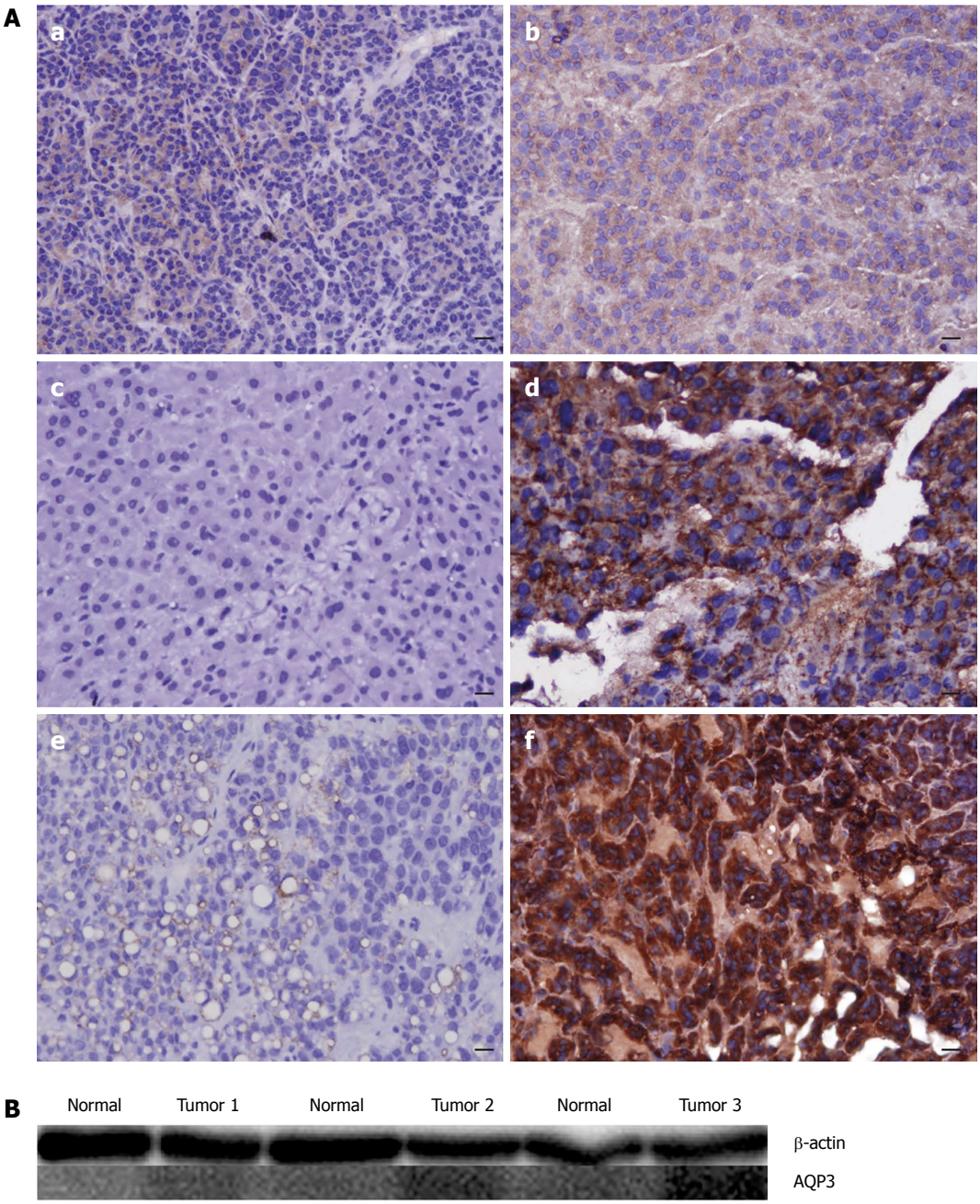

Figure 2 Levels of AQP3 in hepatocellular carcinoma of different differentiation. A: Analysis of AQP3 protein expression by immunohistochemistry. a: AQP3 expression decreased in normal liver tissues; b: AQP3 expression was weak in well-differentiated HCC samples; c: AQP3 expression decreased in normal liver tissues; d: AQP3 expression was moderate in moderately differentiated HCC samples; e: AQP3 expression decreased in normal liver samples; f: AQP3 expression was high in poorly differentiated HCC samples. B: The protein levels of AQP3 in HCC of different degrees of differentiation corresponded to their immunohistochemistry results. Tumor 1, well-differentiated HCC sample; tumor 2, moderately differentiated HCC sample; and tumor 3, poorly differentiated HCC sample. HCC: Hepatocellular carcinoma.

an immunohistochemical assay (Figure 5E).

Effect of regulation of AQP3 and AQP9 on hepatic tumor growth

To gain further insights into the roles of AQP3 and AQP9 in growth of HCC, we characterized the roles of Auphen and dbcAMP *in vivo*. The growth rates and volumes of Auphen- and dbcAMP-treated tumors were found to be lower than those in the controls. There was a marked decrease in tumor size in the Auphen- and dbcAMP-treated mice when compared with the control mice ($P < 0.01$; Figure 6), and the weights of tumors in the three groups were 0.06 ± 0.01, 0.07 ± 0.01, and 0.19 ± 0.03 g, respectively. There were significant differences between the Auphen- and dbcAMP-treated mice and the control mice ($P < 0.01$). The results suggest that low expression of AQP3 and high expression of AQP9 suppressed tumor growth of HCC *in vivo*. Our study clearly indicates that Auphen and dbcAMP reduced the serum AFP density in mice

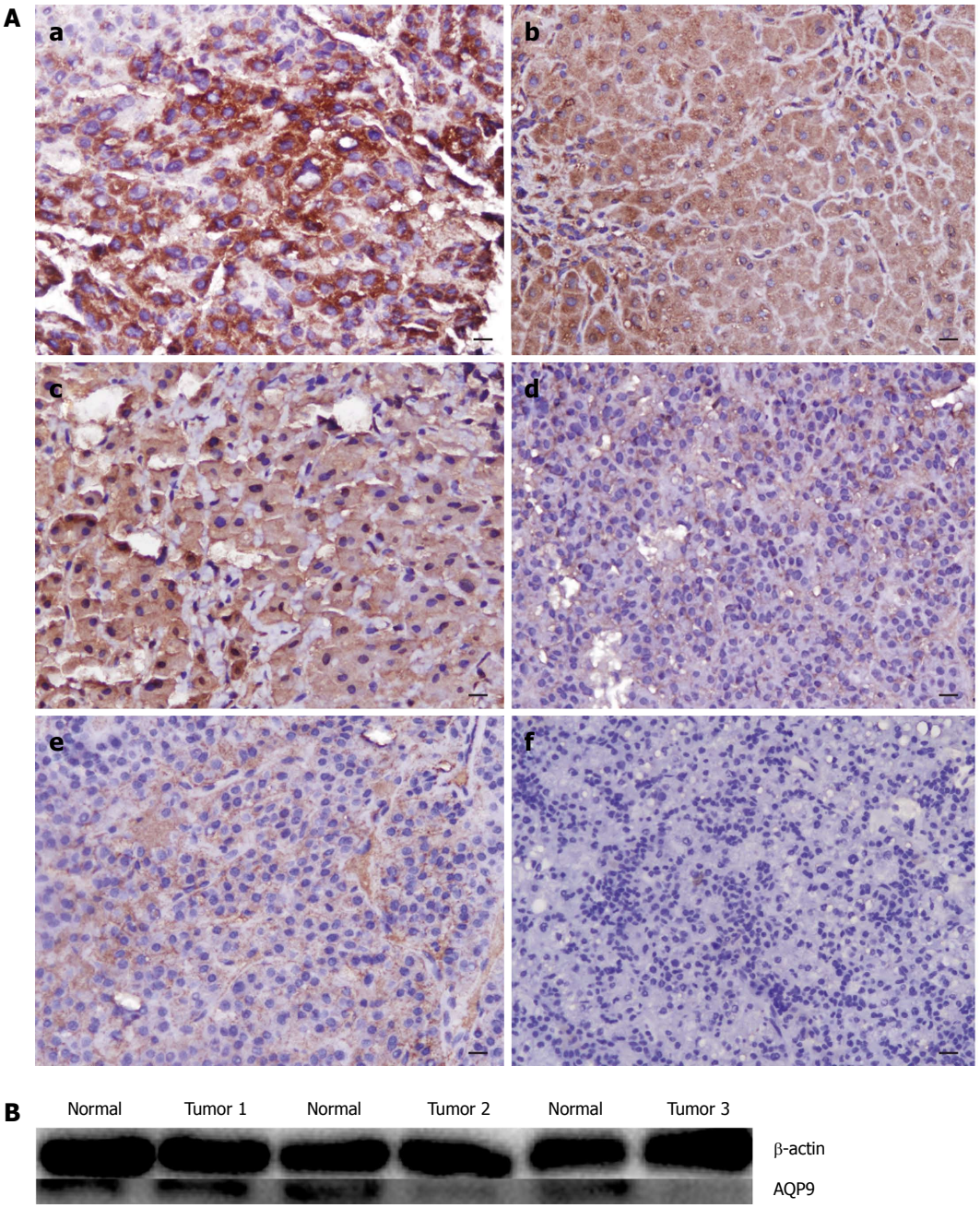

Figure 3 Levels of AQP9 in hepatocellular carcinoma of different differentiation. A: Analysis of AQP9 protein expression by immunohistochemistry. a: AQP9 expression was high in normal liver samples; b: AQP9 expression was weak in well-differentiated HCC samples; c: AQP9 expression was high in normal liver samples; d: AQP9 expression was moderate in moderately differentiated HCC samples; e: AQP9 expression was high in normal liver samples; f: AQP9 expression was low in poorly differentiated HCC samples (magnification × 200, bar = 50 μm). B: The protein levels of AQP9 in HCC of different differentiation corresponded to their immunohistochemistry results: Tumor 1, well-differentiated HCC sample; tumor 2, moderately differentiated HCC sample; and tumor 3, poorly differentiated HCC sample. HCC: Hepatocellular carcinoma.

compared with the control mice ($P < 0.01$; Figure 7A and B). The TUNEL assay revealed more apoptotic changes in tumor tissues in the Auphen and dbcAMP groups (Figure 7C). Light microscopy was employed to detect the results of HE staining (Figure 7D). The Auphen and dbcAMP groups showed more apoptotic cells, which were revealed as karyopyknosis, and had a cytoplasmic red color.

DISCUSSION

HCC is the fifth most fatal malignant tumor worldwide, with no effective therapy. Thus, it is particularly important to identify novel targets for more effective treatments for this disorder. AQPs are known to be related to carcinogenesis and cancer malignancy[35]. Hu *et al*[36] showed that AQPs had strong correlations with

Table 3 Correlation of AQP3 and AQP9 expression levels (low and high) to clinico-pathological data

Factors	AQP3		OR (95%CI)	*P* value	AQP9		OR (95%CI)	*P* value
	Low	High			Low	High		
Gender								
Male	4	19	0.526 (0.074-3.748)	0.603	16	1	7.111 (0.686-73.76)	0.138
Female	2	5			9	4		
Age (yr)								
< 50	1	4	1.000 (0.091-11.03)	1.000	3	2	0.205 (0.024-1.771)	0.183
> 50	5	20			22	3		
Tumor size (cm)								
< 5	3	5	3.800 (0.580-24.90)	0.300	6	2	0.474 (0.063-3.540)	0.589
> 5	3	19			19	3		
Serum HBsAg								
Positive	5	19	1.316 (0.124-13.98)	1.000	21	3	1.185 (0.166-8.475)	1.000
Negative	1	5			4	2		
Serum AFP (ng/mL)								
< 25	3	2	11.00 (1.271-95.23)	0.041	4	0	2.302 (0.107-49.54)	1.000
> 25	3	22			21	5		
Cirrhosis								
Presence	6	21	2.116 (0.096-46.56)	1.000	11	2	6.000 (0.780-46.17)	0.102
Absence	0	3			14	3		
UICC stage								
Ⅰ + Ⅱ	4	4	10.00 (1.341-74.55)	0.029	2	4	0.022 (0.002-0.300)	0.003
Ⅲ + Ⅳ	2	20			23	1		
Metastasis/recurrence								
Yes	1	17	0.082 (0.008-0.839)	0.026	17	1	12.67 (1.177-136.4)	0.031
No	5	7			8	4		
Edmondson grade								
Low (Ⅰ/Ⅱ)	3	3	14.00 (1.741-112.6)	0.016	7	4	0.097 (0.009-1.029)	0.047
High (Ⅲ/Ⅳ)	3	21			18	1		

Table 4 Cox regression analysis of patients with hepatocellular carcinoma

Variables	Univariate		*P* value
	HR	95%CI	
AQP3 expression (1 = low, 2 = high)	4.948	1.037-23.605	0.045
AQP9 expression (1 = low, 2 = high)	10.835	1.488-78.877	0.019
Gender (1 = Male, 2 = Female)	2.681	0.852-8.436	0.092
Age (1 < 50, 2 ⩾ 50)	2.579	0.332-20.049	0.365
Tumor size (1 < 5 cm, 2 ⩾ 5)	1.261	0.334-4.759	0.732
Serum HBsAg (1 = Positive, 2= Negative)	0.575	0.153-2.154	0.411
Serum AFP (1 < 25 ng/mL, 2 ⩾ 25 ng/mL)	1.102	0.293-4.146	0.886
Cirrhosis (1 = Presence, 2 = Absence)	1.401	0.373-5.268	0.618
UICC stage (1 = Ⅰ + Ⅱ, 2 = Ⅲ + Ⅳ)	0.115	0.027-0.485	0.003
Metastasis/Recurrence (1 = Yes, 2 = No)	0.228	0.065-0.804	0.021
Edmondson grade [1 = Low (Ⅰ/Ⅱ), 2 = High (Ⅲ/Ⅳ)]	0.560	0.175-1.791	0.328

tumor proliferation and metastasis. Also, it is suggested that AQPs are of great diagnostic and prognostic value in tumor tissues[37,38]. Modulation of AQP expression has a wide range of clinical applicability, as suggested by the results from mice with AQP gene deletions and from humans with functional mutations in AQPs[39].

More sophisticated molecular dynamics-based methods have been explored to simulate AQP regulation, involving the computation of water permeability modulus[40]. Nevertheless, these processes are computationally intensive, and water permeability modulus has no connection with inhibitory potency. Various chemotherapeutic drugs depend on high doses of toxic compounds to destroy cancer cells completely, and are frequently associated with serious side effects, tumor relapse, and progression to highly malignant states. Gold-based complexes have been reported to depress AQP3, with Auphen being the most efficient[29,41]. Auphen has a greater ability to depress glycerol permeation *via* AQP3 than water permeation. Computational modeling has shown that gold-containing inhibitors have a mutual action to control cancer cell migration, with Cys40 located in the extracellular domain of AQP3[29]. These advantageous properties of Auphen indicate its potential suitability for use in adoptive therapy against cancer. The PKA pathway is one of the main signal transduction pathways that regulate cell proliferation, mRNA expression, and enzyme activation, and dbcAMP increases the expression of AQP9 *via* the activation of PKA[33]. Lee *et al*[42] found that dbcAMP can inhibit cancer cell migration, thereby suggesting that cAMP-mediated PKA and CAMP (EPAC)-mediated Ras related protein 1 (RAP1) could be therapeutic targets.

Our previous studies have revealed that Auphen inhibited AQP3 and dbcAMP increased AQP9 in HCC cells in a dose-dependent manner *in vitro*. However, whether regulating AQP3 and AQP9 can be used as potential targets for HCC treatment has not been confirmed.

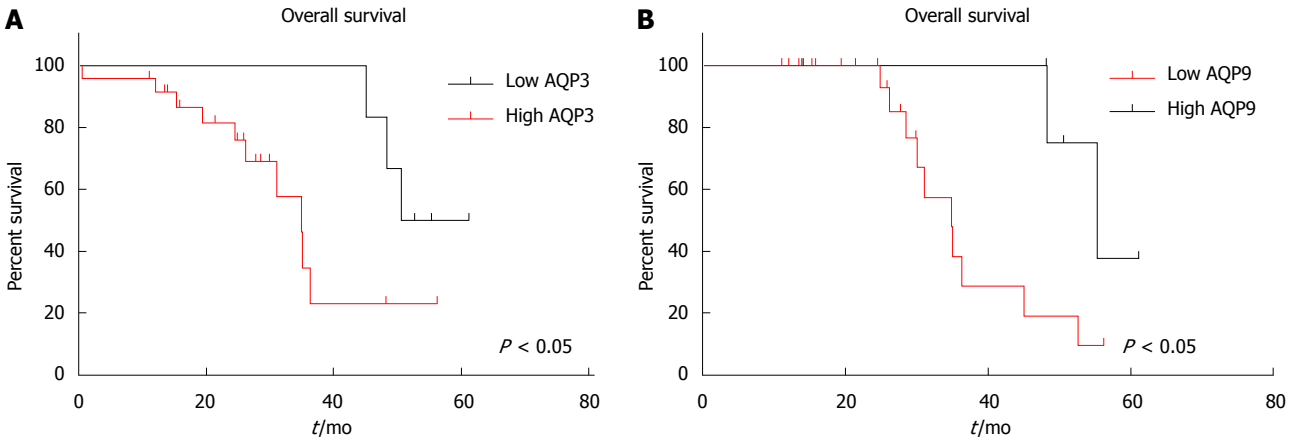

Figure 4 High AQP3 and low AQP9 expression predict worse survival in hepatocellular carcinoma patients. A and B: Survival of 30 hepatocellular carcinoma patients, including both untreated and HCC-treated patients using Kaplan-Maier analysis. High AQP3 and low AQP9 levels resulted in lower patient survival. HCC: Hepatocellular carcinoma.

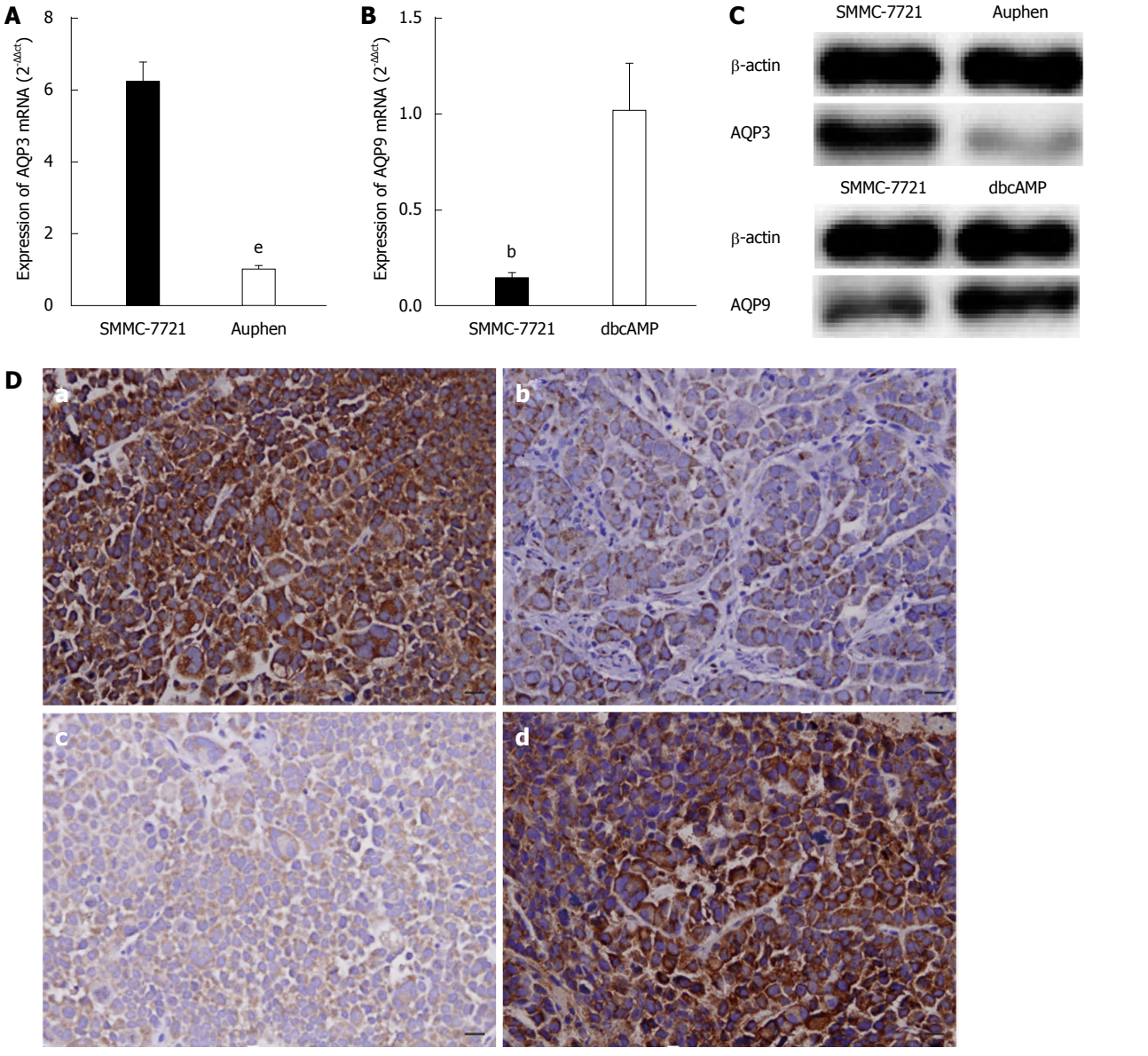

Figure 5 Effects of Auphen and dibutyryl cAMP on AQP3 and AQP9 expression. A: mRNA levels of AQP3 in tumors from nude mice; B: mRNA levels of AQP9 in tumors from nude mice; C: Protein levels of AQP3 and AQP9 in tumors from nude mice; D: Immunohistochemical analysis of a subcutaneous tumor: a: Analysis of AQP3 expression in the control group; b: Analysis of AQP3 expression in the Auphen group; c: Analysis of AQP9 expression in the control group; d: Analysis of AQP9 expression in the dbcAMP group. (magnification × 200, bar = 50 μm). All data represent the mean ± SD (n = 3). $^{b}P < 0.01$ *vs* the control; $^{e}P < 0.001$ *vs* the control. dbcAMP: Dibutyryl cAMP.

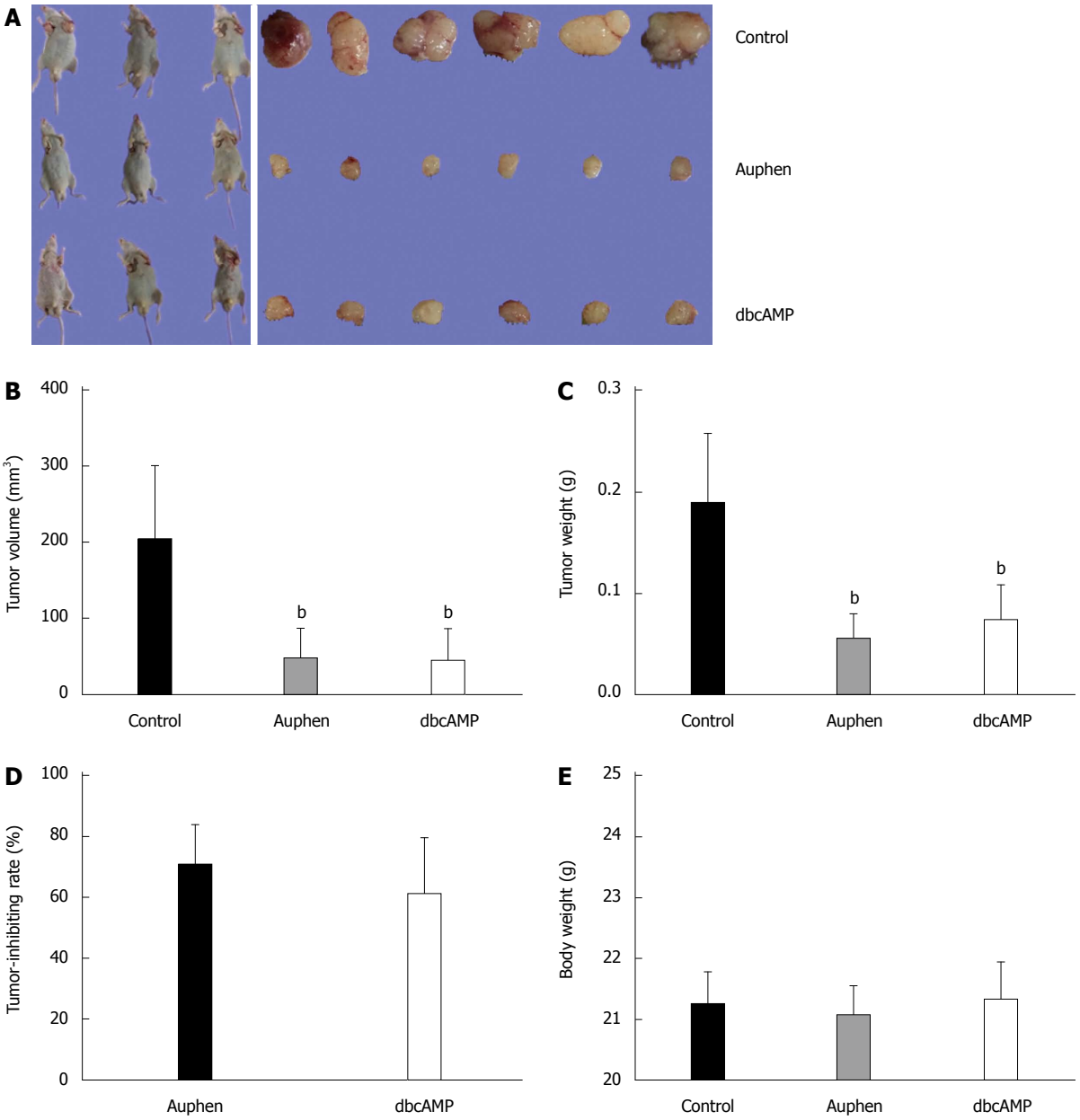

Figure 6 Auphen and dbcAMP suppress hepatocellular carcinoma tumor growth. A: Tumor volumes of control (NS), Auphen-treated (Auphen), and dbcAMP-treated (dbcAMP) groups; B: Tumor volumes of control, Auphen-treated, and dbcAMP-treated groups; C: Tumor weights of control, Auphen-treated, and dbcAMP-treated groups; D: Tumor suppression rates of Auphen-treated, and dbcAMP-treated groups; E: Body weights of control, Auphen-treated, and dbcAMP-treated groups. All data represent the mean ± SD (n = 3). $^{b}P < 0.01$ *vs* the control. dbcAMP: Dibutyryl cAMP.

Hence, in the present study, we characterized the functions of AQP3 and AQP9 *in vivo*.

As shown by the immunohistochemical assays, 90.7% of the HCC samples showed strong membrane and cytoplasmic staining for AQP3 and weak membrane staining for AQP9, while the noncancerous tissues presented mainly weak expression of AQP3 and strong expression of AQP9, indicating that AQP3 and AQP9 could play important roles in the development of HCC. In addition, we have confirmed that AQP3 and AQP9 expression correlated with liver neoplasm stage, metastasis, and tumor differentiation. There was no significant correlation between AQP3 and AQP9 expression and age, gender, tumor size, serum HBsAg, or serum AFP. Our results indicated that AQP3 and AQP9 expression is related to tumor biological characteristics, such as rapid tumor development. These findings suggest that clinicopathologic features together with expression of AQP3 and AQP9 in tumor tissues could be valuable in prognosis assessment and design of individual treatment strategies for HCC.

To understand the functions of AQP3 and AQP9, we tested whether regulating AQP3 and AQP9 by Auphen and dbcAMP suppressed tumor growth *in vivo*. Doses of 1 mmol/L Auphen and 3 mmol/L dbcAMP suppressed tumor growth, with decreases of tumor sizes and weights. The Auphen-treated group had lower AQP3 expression and the dbcAMP-treated group had higher

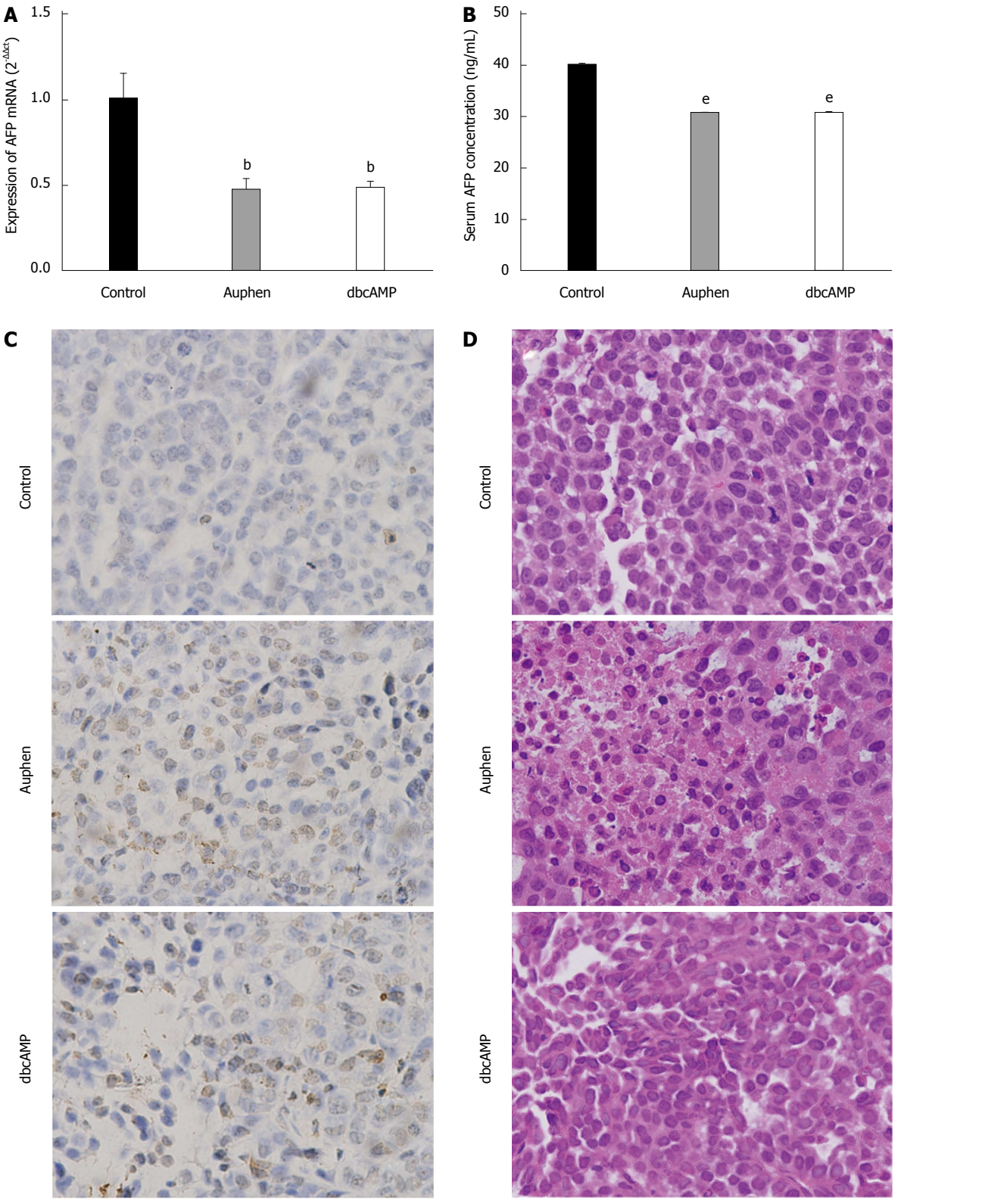

Figure 7 Effects of Auphen and dbcAMP on hepatocellular carcinoma *in vivo*. A: mRNA levels of α-fetoprotein (AFP) in tumors from nude mice; B: AFP concentration in blood of control (NS), Auphen-treated (Auphen), and dbcAMP-treated (dbcAMP) groups. All data represent the mean ± SD (n = 3). $^{b}P < 0.01$ *vs* the control; $^{e}P < 0.001$ *vs* the control; C: Testing of apoptosis by TUNEL assay in tumor samples of nude mice in different treated groups (magnification, × 400). The nuclei of positive cells were stained brown; D: Tumor samples from nude mice in different treated groups (HE, magnification, × 400). Pathological traits of positive cells were revealed as karyopyknosis, and had a cytoplasmic red color.

AQP9 expression than the control group. Our study also is the first confirmation of the tumorigenic abilities of AQP3 and AQP9 *in vivo*. Inhibiting AQP3 and increasing AQP9 expression showed increased suppression of

tumor growth in nude mice. AFP has previously been reported to be a target for diagnosing and monitoring HCC. Collectively, our findings demonstrate that Auphen and dbcAMP decreased AFP expression and secretion *in vivo*. TUNEL assays revealed more apoptotic changes in tumor tissues in the Auphen and dbcAMP groups. We assume that this was the result of the anti-tumor activities of Auphen and dbcAMP. All these studies confirmed our findings that AQP3 and AQP9 exerted oncogenic effects in HCC. Our study also showed no differences in body weight, appetite, or behavior between Auphen- and dbcAMP-treated mice and the control mice, indicating the efficiency and nontoxicity of Auphen and dbcAMP. Combined with previous *in vitro* results showing inhibition of hepatoma cell proliferation, we conclude that Auphen and dbcAMP have anti-cancer effects both *in vitro* and *in vivo*. The results also showed that the Auphen-treated group had smaller tumors than the dbcAMP-treated group. It may be that AQP3 has a more important function than AQP9 in the development of HCC, although this still needs more research.

In summary, our *in vivo* and *in vitro* results provide a useful platform to study tumor growth and possible anti-tumor treatments. In the present study, the association of clinicopathologic and the expression results suggest that overexpression of AQP3 and low expression of AQP9 correlate with clinical prognosis of HCC. Because of the small sample size in our study, the relationship between AQP3 and AQP9 expression and metastasis still needs more studies in larger cohorts. Studies are ongoing to develop targeted and effective delivery systems for administering AQP3 and AQP9 in HCC patients. These studies should further emphasize the significance of AQP3 and AQP9 in HCC development. Our findings are consistent with previous results indicating that regulation of AQP3 and AQP9 levels in HCC cells leads to a decrease in cell proliferation. In general, the results we obtained from the regulation and control of function tests *in vitro* will be important in the implementation of future studies to explore tumor cell behavior, *in vivo*, to gain additional understanding into the function of AQP3 and AQP9 in hepatocarcinogenesis.

In conclusion, combining human clinical data and *in vivo* experiments, our study strongly suggests AQP3 and AQP9 as key players inHCC development. Further research should verify AQP3 and AQP9 as diagnostic biomarkers for HCC occurrence. Furthermore, an in-depth description of AQP3 and AQP9 regulation by Auphen and dbcAMP will provide a better understanding of the mechanisms of hepatocarcinogenesis, which could be used in the development of novel therapeutic drugs. Finally, our work further confirms the significance of AQP-driven hepatocarcinogenesis, emphasizing the importance of both basic and clinical knowledge of the roles of AQPs in HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a highly malignant cancer worldwide; however, the mechanism of hepatocarcinogenesis is unknown, and a reliable prognosis is still lacking. Aquaporin (AQP) expression is positively correlated with tumor type, grade, proliferation, migration, angiogenesis, or tumor-associated edema, which can be considered a diagnostic and therapeutic target in anti-cancer treatment. AQP3 and AQP9, in particular, are considered to be closely associated with cancer development because of their dramatically changed levels in various cancers, including HCC. However, their influence on the development of HCC is poorly understood. This suggests that AQP3 and AQP9 may be promising targets for HCC therapy.

Research frontiers

Previous experiments have already proved that AQP3 was overexpressed and AQP9 was decreased in HCC compared to normal liver tissues. AQP3 and AQP9 are considered to be closely associated with cancer development. Auphen and dibutyryl cAMP (dbcAMP) can regulate the expression of AQP3 and AQP9. Moreover, Auphen and dbcAMP showed antiproliferative traits in tumor cells *in vitro*, which have high cytotoxic potency and selectivity. More sophisticated molecular dynamics-based methods have been explored to simulate AQP regulation. Nevertheless, these processes are computationally intensive, and water permeability modulus has no connection with inhibitory potency. Various chemotherapeutic drugs depend on high doses of toxic compounds to destroy cancer cells completely, and are frequently associated with serious side effects, tumor relapse, and progression to highly malignant states. The potency and specificity properties of Auphen and dbcAMP indicate their potential suitability for use in adoptive therapy against cancer.

Innovations and breakthroughs

This is the first study intending to evaluate the antioncogenic effects of Auphen and dbcAMP *in vivo* and investigate whether their underlying mechanism involves regulating AQP3 and AQP9 expression. The authors used Auphen and dbcAMP to determine the role of AQP3 and AQP9 during HCC development, and to explore the function and mechanism of targeting AQP3 and AQP9 in HCC therapy.

Applications

This study demonstrated that both AQP3 and AQP9 play a major role in HCC and are related to tumor development and clinical prognosis. In addition, these results contribute to the evaluation of the antioncogenic effects of Auphen and dbcAMP *in vivo*. An in-depth description of AQP3 and AQP9 regulation by Auphen and dbcAMP will provide a better understanding of the mechanisms of hepatocarcinogenesis, which could be used in the development of novel therapeutic drugs.

Peer-review

Authors demonstrated that Auphen and dbcAMP have antioncogenic effects in HCC and their underlying mechanism involves regulating AQP3 and AQP9 expression *in vivo*. This is a good paper with very interesting data.

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ORIGINAL ARTICLE

Basic Study

Ultrasound virtual endoscopy: Polyp detection and reliability of measurement in an *in vitro* study with pig intestine specimens

Jin-Ya Liu, Li-Da Chen, Hua-Song Cai, Jin-Yu Liang, Ming Xu, Yang Huang, Wei Li, Shi-Ting Feng, Xiao-Yan Xie, Ming-De Lu, Wei Wang

Jin-Ya Liu, Li-Da Chen, Jin-Yu Liang, Ming Xu, Yang Huang, Wei Li, Xiao-Yan Xie, Wei Wang, Ming-De Lu, Department of Medical Ultrasonics, Institute of Diagnostic and Interventional Ultrasound, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Shi-Ting Feng, Hua-Song Cai, Department of Radiology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Author contributions: Liu JY, Chen LD, Lu MD and Wang W designed the research; Liu JY, Chen LD, Feng ST, Liang JY, Xu M, Huang Y, Li W, Cai HS, Xie XY and Wang W performed the research; Liu JY, Chen LD, Wang W analyzed the data; Liu JY and Wang W wrote the paper.

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Correspondence to: Wei Wang, MD, PhD, Department of Medical Ultrasonics, Institute of Diagnostic and Interventional Ultrasound, The First Affiliated Hospital of Sun Yat-Sen University, Sun Yat-Sen University, No. 58, Zhongshan Road 2, Guangzhou 510080, Guangdong Province, China. wangw73@mail.sysu.edu.cn
Telephone: +86-20-87765183
Fax: +86-20-87765183

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Abstract

AIM: To present our initial experience regarding the feasibility of ultrasound virtual endoscopy (USVE) and its measurement reliability for polyp detection in an *in vitro* study using pig intestine specimens.

METHODS: Six porcine intestine specimens containing 30 synthetic polyps underwent USVE, computed tomography colonography (CTC) and optical colonoscopy (OC) for polyp detection. The polyp measurement defined as the maximum polyp diameter on two-dimensional (2D) multiplanar reformatted (MPR) planes was obtained by USVE, and the absolute measurement error was analyzed using the direct measurement as the reference standard.

RESULTS: USVE detected 29 (96.7%) of 30 polyps, remaining a 7-mm one missed. There was one false-positive finding. Twenty-six (89.7%) of 29 reconstructed

images were clearly depicted, while 29 (96.7%) of 30 polyps were displayed on CTC with one false-negative finding. In OC, all the polyps were detected. The intraclass correlation coefficient was 0.876 (95%CI: 0.745-0.940) for measurements obtained with USVE. The pooled absolute measurement errors ± the standard deviations of the depicted polyps with actual sizes ⩽ 5 mm, 6-9 mm, and ⩾ 10 mm were 1.9 ± 0.8 mm, 0.9 ± 1.2 mm, and 1.0 ± 1.4 mm, respectively.

CONCLUSION: USVE is reliable for polyp detection and measurement in *in vitro* study.

Key words: Three-dimensional ultrasound; *In vitro*; Virtual endoscopy; Intestinal polyps; Technical feasibility

Core tip: We present our initial experience regarding the feasibility of ultrasound virtual endoscopy (USVE) and its measurement reliability for polyp detection in an *in vitro* study using pig intestine specimens. USVE is a new technique that simulates views of computed tomography colonography (CTC). We found that USVE is an accurate screening method for simulated polyp detection and compares favorably to CTC and optical colonoscopy. As a dynamic, non-invasive, radiation-free, cost-effective method, USVE shows great promise for the screening and surveillance of colorectal cancers.

Liu JY, Chen LD, Cai HS, Liang JY, Xu M, Huang Y, Li W, Feng ST, Xie XY, Lu MD, Wang W. Ultrasound virtual endoscopy: Polyp detection and reliability of measurement in an *in vitro* study with pig intestine specimens. *World J Gastroenterol* 2016; 22(12): 3355-3362 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3355.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3355

INTRODUCTION

Colorectal cancer (CRC), which generally develops from benign adenomatous polyps, is a major cause of morbidity and mortality worldwide[1,2]. Evidence-based guidelines recommend CRC screening[3,4] because the early detection and removal of polyps has been shown to reduce both the incidence and the mortality of CRC[5,6]. Optical colonoscopy (OC) is the primary method employed for CRC screening and the removal of polyps. As a primary imaging test, computed tomography colonography (CTC) is performed in average-risk individuals, particularly when endoscopy is contraindicated or incomplete[4,7]. It is highly sensitive for CRC screening with a sensitivity of 96.1% according to a meta-analysis[8]. Unfortunately, these tests have some drawbacks, such as OC related invasiveness, procedure-related discomfort, the risk of bowel perforation, and CTC related ionizing radiation[9]. Ultrasound virtual endoscopy (USVE) is a new technique that simulates views of CTC. It allows for the reconstruction of inner bowel-surface structures from the dynamic three-dimensional (3D) ultrasound data sets. Based on a surface reconstruction algorithm by interactive settings of threshold values and surface displays, endoscopic views can be obtained within seconds. Similar ultrasound virtual endoscopy imaging has been used for detection of carotid atherosclerosis and portal vein thrombus[10-12]. But, USVE has never been used for polyp detection. It has several potential advantages for CRC screening over other modalities. USVE is dynamic, non-invasive, radiation-free, and cost-effective. Therefore, this new method shows great promise for the screening and surveillance of CRC.

The likelihood that a given polyp develops into malignancy or demonstrates high-grade dysplasia is directly related to its size[13-15]. This risk is estimated to be less than 1% for lesions 5-mm or smaller, whereas the risk increases to approximately 10%-25% for lesions 10 mm or larger. Polyps of 6-9 mm are also almost always benign. Advanced adenomas, defined as lesions 10 mm or larger, are the target of CRC screening and should be referred for polypectomy[16]. However, radiologists suggested 6-mm as the minimum size for reporting polyp lesions[17]. Hence, understanding of the polyp size measurement error by USVE is very important for accurate polyp matching in the diagnostic performance.

Currently, there is no published report regarding the role of USVE in polyp detection, and its sensitivity and specificity for the detection of polyps are unknown. In this ideal *in vitro* study, we investigated the detection rate of simulated polyps using USVE compared with CTC and OC. We estimated the conspicuity of the reconstructed endoscopic images. Additionally, we evaluated the reliability of the two-dimensional (2D) optimized polyp measurements of USVE using the direct measurement as the reference standard. The goal of this study was to report our initial experience regarding the feasibility of USVE and its measurement reliability in an *in vitro* study using porcine intestine specimens.

MATERIALS AND METHODS

Specimen preparation

Six porcine small intestine specimens, each approximately 50 cm long, were acquired from fresh pig intestines that were commercially available at an abattoir. Each specimen was cleansed to remove fecal matter, and no polyploid structures were found on the mucosal surface. Thirty simulated sessile polyps with maximum diameters of 4-13 mm were created from pork wrapped by other intestinal mucosa with sutures. The maximum diameter of the polyp was confirmed by means of physical measurement with a caliper and a millimeter marked ruler. Then, the polyps were sutured to the mucosa of the inverted specimens and randomly

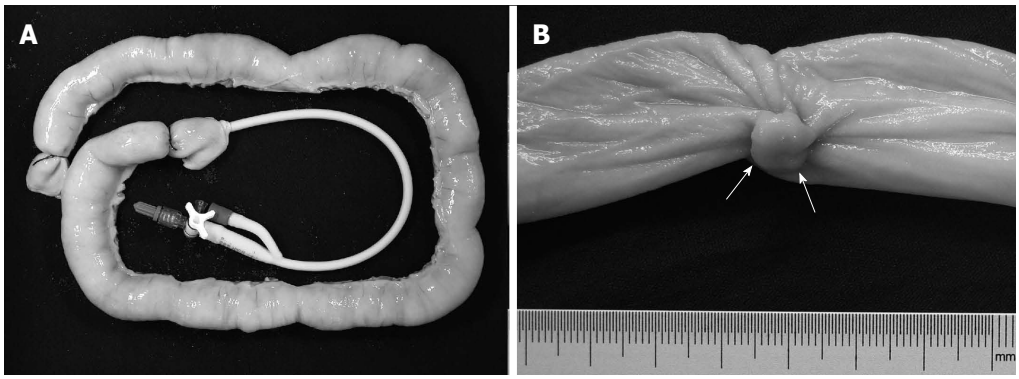

Figure 1 A pig intestine specimen and simulated polyps. A: A porcine intestine specimen with a flexure configuration was placed on a black sponge. One end was double-tied with sutures. A catheter used for intestinal distention during the USVE and CTC was placed through the other end. Optical colonoscopy was also performed through the latter end after removal of the catheter; B: A simulated polyp with a maximum diameter of 8 mm (arrows) was sutured to the inverted intestine specimen. USVE: Ultrasound virtual endoscopy; CTC: Computed tomography colonography.

placed with different distances between adjacent polyps. Either the number or the size of the polyps was randomized in each specimen. A detailed polyp map of the specimen was recorded. Next, each specimen was re-inverted, and the distal end was double-tied with sutures. An 18-F urinary catheter was inserted into the open proximal end, which was then closed with a double-tied suture (Figure 1). A 50-mL syringe with a 3-way stopcock was attached to the urinary catheter.

US virtual endoscopy

A 0.9% saline solution was introduced through the syringe, and the specimen was maximally distended to a diameter of approximately 3.5 cm. Then, it was placed in a plastic container containing water. The wall and the bottom of the container were covered with 2-mm-thick black sponges that helped absorb the ultrasound and reduce echo reflection.

The USVE was performed with an ultrasound scanner (Aplio 500, Toshiba, Otawa, Japan) equipped with a volume transducer (7-14 MHz) (PLT-1204MV, Toshiba Medical Systems). Imaging was performed by one radiologist (Wang W) who was unaware of the locations, numbers and sizes of the polyps in each specimen. After optimal 2D images were obtained, 3D scan mode was initiated, and volume data were acquired, including the following parameters: longitudinal scanning orientation (running along the longitudinal axis of the specimen); 3D frequency, 8 MHz under the difference mode; 3D gain, 70%-80%; dynamic range, 40-50 dB; depth (distance between the probe and the proximal colon wall), approximately 30 mm; focus, placed in the middle of the lumen; 3D Aplipure, on; and 3D scanning angle, 60^0. Each specimen was scanned from the distal to the proximal end, and 3D volume data were obtained once approximately every 5 cm because of the sweeping length of the PLT-1204MV volume transducer. Therefore, one specimen was artificially separated into several approximately 5-cm-long segmentations. Then, the data from the scanning were loaded to the Fly Thru workstation (Fly Thru 3.0, Toshiba Medical Systems), which was capable of producing 2D multiplanar reformatted (MPR) images and 3D endoluminal surface renderings (Figure 2, Video 1). Virtual endoscopic images were shown by setting optimal threshold values based on a surface reconstruction algorithm. To prevent holes in the intestinal wall and intraluminal artifact, a threshold value (0-150) was selected for all reconstructions with other defined settings as follows: transparency, 20; and filter, 3. All machine parameters remained unchanged between the examinations and were verified before the imaging.

Interpretation of the findings and measurements with USVE

Two independent readers (Chen LD and Xu M), who did not know the size ranges and numbers of polyps, reviewed all the ultrasound examinations and recorded the numbers, positions (distance between adjacent polyps) and sizes of the simulated polyps. To achieve a valid match between virtual endoscopy and specimen, a polyp had to appear with the same segment, same position and similar diameters.

Lesion size is best defined as the single largest diameter of the polyp head, excluding the stalk[17]. With electronic calipers, the two readers independently measured the polyp size on an optimized 2D MPR plane, on which the maximum polyp diameter was viewed. The measurements were determined to the nearest millimeter. For the same polyp, the mean measured size was the average of measurements from the two readers. For the analysis of measurement accuracy, the absolute measurement error referred to the difference between the actual size and the mean measured size. The standardized polyp size was defined as the mean measured optimized 2D diameter divided by the reference diameter, multiplied by 100%.

An overall ranking of image quality of the USVE protocol was performed. Both radiologists graded conspicuity and evaluated the images together to resolve any disagreements. The image conspicuity of

Table 1 Detection rate for the simulated polyps by ultrasound virtual endoscopy, computed tomography colonography and optical colonoscopy

Actual polyp size, mm	Referenced No. of polyps	OC		CTC		USVE	
		No. of detected polyps	Sensitivity	No. of detected polyps	Sensitivity	No. of detected polyps	Sensitivity
≤ 5	9	9	100%	8	88.9%	9	100%
6-9	15	15	100%	15	100%	14	93.3%
≥ 10	6	6	100%	6	100%	6	100%
All	30	30	100%	29	96.7%	29	96.7%

USVE: Ultrasound virtual endoscopy; CTC: Computed tomography colonography; OC: Optical colonoscopy.

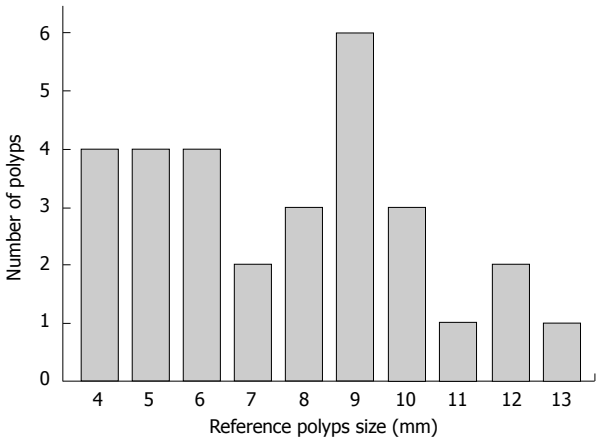

Figure 2 Polyp size and number.

each polyp was graded on a three-point scale[18,19]: grade 1 = not visible or very poorly depicted; grade 2 = poorly depicted, with some artifacts that but do not affect diagnosis; and grade 3 = clearly depicted, without artifacts.

CTC

After US examination, the six specimens were transferred to the CT suite. They were placed in a plastic container and then manually distended with room air using a syringe. Five liters of 93.7% soybean oil was poured into the container until the specimen was completely submerged[20]. To avoid flotation, the specimen was fastened to the bottom of the container by adhesion of plastic tape, before the oil was poured into the container.

Blinded to the locations, sizes and numbers of polyps, two independent radiologists (Feng ST and Cai HS), performed the CT examinations with a 64-detector row CT scanner (Aquillion 64, Toshiba, Japan). The images were obtained with these parameters: 120 kV; 200-250 mA; section thickness, 0.5 mm; beam collimation, 64 mm × 0.5 mm; reconstruction interval, 0.5 mm; reconstructed section thickness, 0.5 mm; beam pitch, 0.828; gantry rotation time, 0.4 second; and field of view, 5 cm. The scanning matrix was 512 × 512. Then, the data were loaded to a workstation (HP workstation XW8200, Vitrea 2, Version 3.7). The reconstructed virtual endoscopic images were read by the above radiologists, who synchronously recorded the locations, numbers and sizes of the simulated polyps.

OC

The OC of the six specimens was performed with a colonoscope (CF-H260AI, CV-260, Olympus, Tokyo, Japan) by two independent endoscopists (Liang JY and Xie XY) immediately after CT scanning. The oil mixture was removed. The gastroenterologists were unaware of the locations, numbers and sizes of the polyps in each specimen, but were aware of the presence of polyps. The colonoscope was introduced through the open proximal end after the urinary catheter was removed. The examination was started, and endoscopy views of the entire specimen were acquired. The same endoscopists recorded the locations, numbers and sizes of the simulated polyps.

Statistical analysis

The relationship between conspicuity grades and polyp sizes was examined using Pearson's Chi-square test (SPSS 18, IBM Corporation, New York, NY, United States). A *P* value of less than 0.05 was considered to indicate a statistically significant difference. The intraclass correlation coefficient (MedCalc version 10.2.0.0, Medalc Software, Mariakerke, Belgium) was applied to analyze the inter-observer agreement for polyp measurement.

RESULTS

Simulated polyps detection

The reference polyp size and the number of the 30 simulated polyps for evaluating the detectability are shown in Figure 3. The polyps were classified into three groups according to size (≤ 5 mm, 6-9 mm, and ≥ 10 mm)[21,22]. The detection rates for each group of the three modalities are shown in Table 1. With USVE, 29 (96.7%) of the 30 polyps were detected; only a 7-mm polyp was missed. There was one false-positive finding (identified as a 10-mm polyp). An initial analysis of the polyp conspicuity data revealed that no observation was assigned a grade of 1. Twenty-six (89.7%) of 29 polyps were clearly depicted (grade 3) (Figure 4), whereas the remaining 3 (10.1%) polyps were poorly

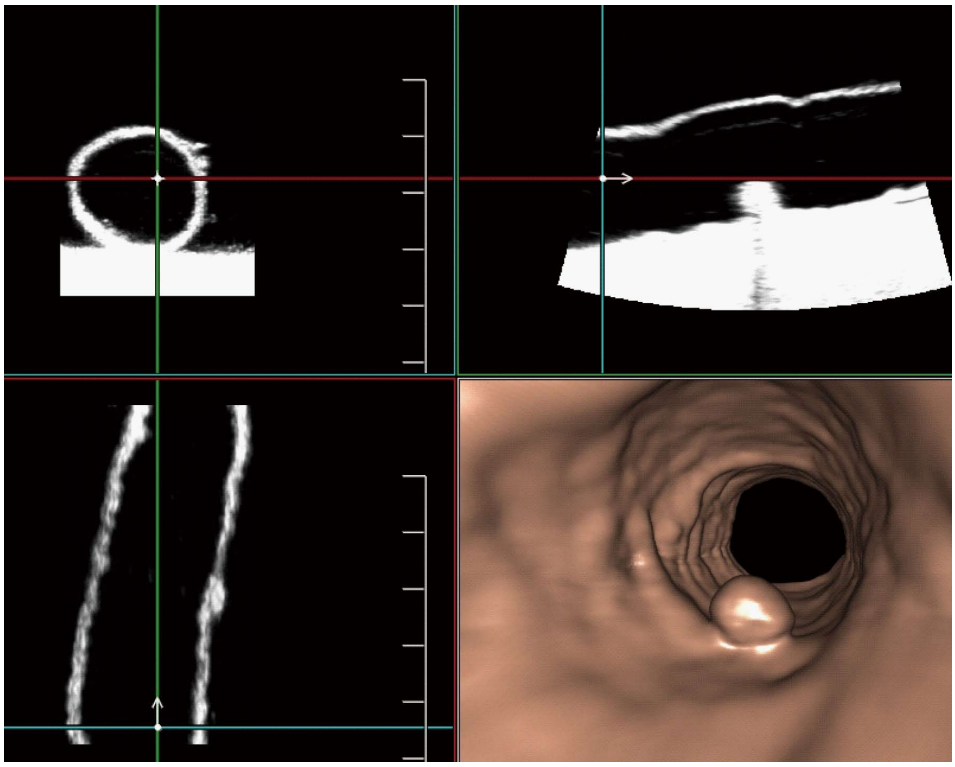

Figure 3 The 3D endoluminal reformatted image of ultrasound virtual endoscopy clearly depicting an 8-mm polyp. We could observe the polyp on the 2D transversal, sagittal and coronal ultrasonography simultaneously.

Table 2 Relationship between conspicuity grade and polyp size *n* (%)

Actual polyp size, mm	Conspicuity grade 2	Conspicuity grade 3	*P* value
≤ 5 (*n* = 9)	1 (11.1)	8 (88.9)	0.627
6-9 (*n* = 14)	2 (14.3)	12 (85.7)	
≥ 10 (*n* = 6)	0 (0)	6 (100)	
All (*n* = 29)	3 (10.3)	26 (89.7)	

depicted (grade 2). There was no significant interaction between polyp conspicuity grade and polyp size (*P* = 0.638) (Table 2).

Twenty-nine (96.7%) of the 30 polyps were depicted on the CTC. All of the polyps 6 mm or larger were detected. One 5-mm polyp, which was located behind a fold, was not prospectively detected by the observer on any of the images but was depicted in a retrospective review. There was one false-positive finding (identified as a 6-mm lesion) as well. All of the polyps were clearly detected by OC.

Polyp measurement

The intraclass correlation coefficient was 0.876 (95% confidence interval: 0.745, 0.940) for measurements obtained with USVE. The pooled absolute measurement errors ± standard deviations of the depicted polyps with actual sizes of 5-mm or smaller, 6-9-mm, and 10-mm or larger were 1.9 ± 0.8 mm, 0.9 ± 1.2 mm, and 1.0 ± 1.4 mm, respectively. The pooled standardized polyp size of the depicted polyps with actual sizes of 5-mm or smaller, 6-9-mm, and 10-mm or larger were 142.5% ± 18.7%, 111.2% ± 16.7%, and 109.2% ± 12.2%, respectively (Table 3).

Table 3 Mean error of the optimized two-dimensional multiplanar reformatted measurement and the pooled standardized polyp size with ultrasound virtual endoscopy

Actual polyp size, mm	Mean measurement error ± SD, mm	Pooled standardized polyp size ± SD, %
≤ 5 (*n* = 9)	1.9 ± 0.8	142.5 ± 18.7
6-9 (*n* = 14)	0.9 ± 1.2	111.2 ± 16.7
≥ 10 (*n* = 6)	1.0 ± 1.4	109.2 ± 12.2
≥ 6 (*n* = 20)	0.9 ± 1.3	110.5 ± 14.6

DISCUSSION

This study demonstrates, for the first time, that USVE is technically feasible in an *in vitro* intestinal specimen. We found that 96.7% (29/30) of the simulated polyps with different sizes ranging from 4 to 13 mm in diameter were depicted using USVE. Thus, USVE is an accurate screening method and compares favorably with CTC and OC for polyp detection. With USVE, the pooled error ± standard deviation and the pooled standardized polyp size of the optimized 2D MPR measurements were 0.9 ± 1.3 mm and 110.5% ± 14.6% for polyps ≥ 6 mm, respectively. These results indicate that USVE is reliable for polyp measurement.

Gastrointestinal tract ultrasonography is challenging.

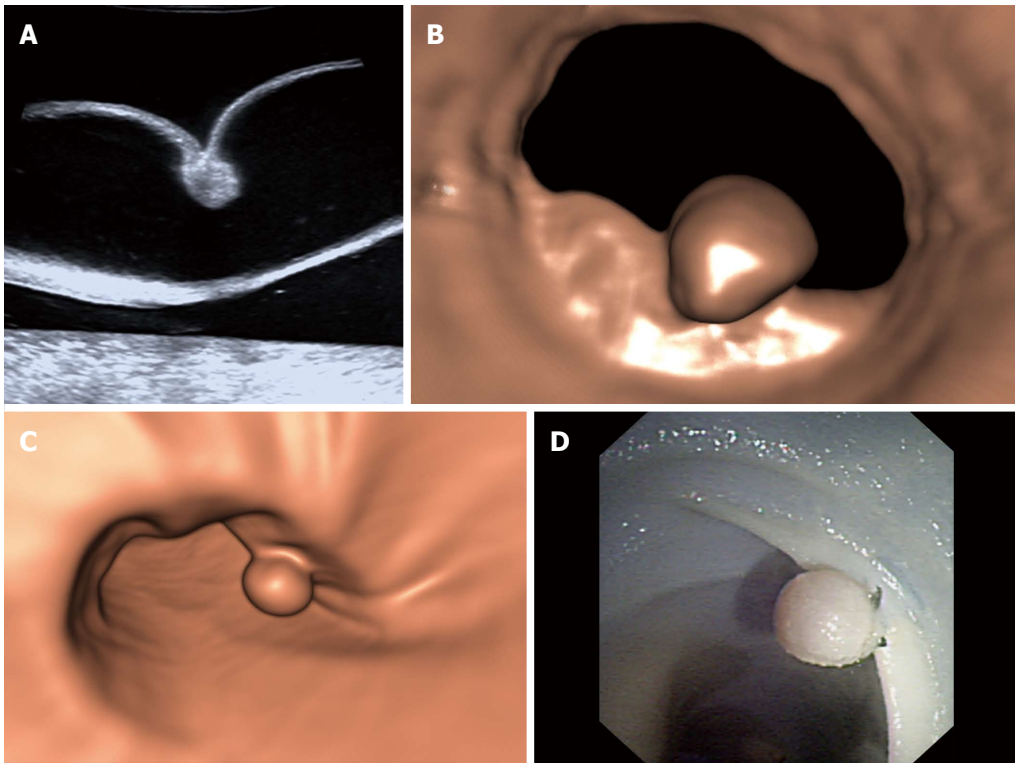

Figure 4 A 6-mm created polyp is clearly depicted on two-dimensional ultrasonography (A), ultrasound virtual endoscopy (B), computed tomography colonography (C) and optical colonoscopy (D).

Gas and fecal residue within the colonic lumen make visibility difficult. The value of abdominal ultrasound in the diagnosis of CRC has previously studied[23-26]. Following the standard bowel preparation, including colonic cleansing and oral administration of a solution for adequate luminal distention[19], transabdominal ultrasound is capable of acquiring 3D data of the colon which then generates virtual endoscopic imaging. Additionally, transabdominal ultrasound can observe the presence of lymphadenopathy, the extracolonic extension of a mass, and the presence of distant metastases.

In 2001, a study reported on ultrasound virtual endoscopic imaging[27], using a curved array probe or a linear array probe with a position-sensing sweeper device. A series of 2D images were manually scanned and then stored in a graphics workstation. In our study, however, we used a new 3D image processing system, called the Fly Thru workstation, and a 3D volume probe. The new method is simpler, more convenient, and has higher spatial resolution. In this ideal pig intestine specimen, USVE was capable of efficiently depicting polyps as small as 4-mm in diameter. In total, 89.7% of the reconstructed images were clearly depicted (Grade 3), which was approximately equal to the polyp conspicuity of CTC[18]. Furthermore, the detection rate of polyps $\geqslant$ 6-mm on USVE approached 100% with only sporadic failures, which was similar to the detection rate of *in vitro* CTC[28]. With the high resolution and high sensitivity of detecting polyps $\geqslant$ 6-mm, USVE is expected to be a new colon neoplasm screening and surveillance modality.

Neither of the observers found one of the four 7-mm polyps during the retrospective reviews of USVE. In contrast, a 10-mm polyp, which was located at the division of two adjacent scan areas, was depicted twice and was regarded as a false-positive finding by both observers. These missing depiction and false-positive findings resulted from the limited sweeping range of the 3D volume transducer. When performing USVE, this transducer scanned approximately 5-cm of the intestine specimen for each acquisition. Thus, as one specimen was artificially separated into several segmentations, the polyps located at the division of adjacent scan areas could be repeatedly depicted or even missed. Based on our preliminary experience, we conclude that the limited scanning range is a major limitation of USVE.

The achievement of precise polyp matching is critical for the sensitivity of USVE. According to previous *in vitro* studies of CTC, the optimized 2D MPR measurement was as accurate as the 3D endoluminal measurement[20,29]. In our study, we adapted the optimized 2D MPR measurement as the standard method of measurement. The mean absolute optimized 2D measurement errors and the mean standardized polyp sizes in the $\geqslant$ 6-mm group were generally consistent with values of CTC[20,29]. This important finding demonstrates that the optimized 2D MPR measurement of USVE is reliable for clinical practice.

This study has some limitations. First, we assessed

the technical accuracy of USVE performed under ideal conditions, which includes the absence of patient motion or peristaltic bowel activity, the clean, debris-free mucosal surface without folds, and maximal lumen distention. The study was not able to reflect an *in vivo* examination on human. The polyps, either their morphologic features or locations, may show more variability in patients. Also, polyp detection and measurement can be affected by colonic curvature, haustral folds, and even different degrees of colonic distention. Additionally, we did not attempt to create flat adenomas in our specimens. Second, polyp size was not measured on the 3D view because the system is currently not able to obtain 3D measurements. Typically, 3D polyp measurements are closer to the "truth" than 2D measurements as the maximum diameter of a polyp is straightforward with the former measurement. Although 2D measurements would optimize polyp diameter by comparing measurements on MPR planes, even using nonstandard oblique planes, this complex procedure would prolong the measurement[29].

In summary, USVE enables the reliable detection and measurement of small simulated mucosal polyps in our *in vitro* model. It is a good potential alternative for colorectal polyp detection. Further *in vivo* studies are required to determine its sensitivity and specificity for polyp detection in patients.

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COMMENTS

Background

Most colorectal cancers (CRC) arise from adenomatous polyps, and the early detection and removal of polyps resulted in reducing both the incidence and mortality of CRC. Optical colonoscopy (OC) is widely accepted as the best available method for CRC screening. Computed tomography colonography (CTC) has yielded promising results. However, these tests have some drawbacks such as invasiveness or ionizing radiation. Ultrasound virtual endoscopy (USVE), a novel non-invasive, radiation-free modality, shows great promise for the screening of CRC. Currently, the role of USVE in polyp detection has never been investigated, and its sensitivity and specificity are unknown.

Research frontiers

Virtual colonoscopy is an attractive alternative for CRC screening. According to some guidelines, CTC has been regarded as the leading imaging technique for CRC screening and the preferred test following incomplete OC.

Innovations and breakthroughs

This *in vitro* study has revealed, for the first time, the feasibility of USVE and its measurement reliability in pig intestine specimens.

Applications

USVE enables reliable detection and measurement of small simulated mucosal polyps in the *in vitro* specimens. It is a good alternative for colorectal polyp screening.

Terminology

USVE is a novel trans-abdominal ultrasonic technique that simulates views of CTC. It allows for the reconstruction of inner bowel-surface structures from dynamic three-dimensional ultrasound data sets.

Peer-review

This is an *in vitro* study that reports the feasibility of a new, non-invasive, radiation-free technique (USVE) in detecting and measuring polyps. The authors compared USVE with CTC and colonoscopy, as the reference standard, with interesting results. The authors performed a well-designed study. It is detailed, easy to read and the subject (with all of the important limitations of a preliminary approach) may be relevant in medicine in future. *In vivo* studies were encouraged.

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ORIGINAL ARTICLE

Basic Study

Tolvaptan regulates aquaporin-2 and fecal water in cirrhotic rats with ascites

Chao Chen, Ren-Pin Chen, Hai-Hua Lin, Wen-You Zhang, Xie-Lin Huang, Zhi-Ming Huang

Chao Chen, Ren-Pin Chen, Hai-Hua Lin, Wen-You Zhang, Xie-Lin Huang, Zhi-Ming Huang, Department of Gastroenterology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

Xie-Lin Huang, Ren-Ji College of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

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Correspondence to: Dr. Zhi-Ming Huang, Professor, Department of Gastroenterology, the First Affiliated Hospital of Wenzhou Medical University, Shangcai Village, Ouhai District, Wenzhou 325000, Zhejiang Province, China. wyyyhzm@126.com

Telephone: +86-577-55578033
Fax: +86-577-55578033

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Abstract

AIM: To investigate the role of tolvaptan in regulating aquaporin (AQP)-2 expression and fecal water content in cirrhotic rats with ascites.

METHODS: Cirrhosis with ascites was induced in rats by repetitive dorsal injection of CCl_4 for 14 wk. In total, 84 cirrhotic rats with ascites divided into three groups (vehicle, 3 mg/kg and 5 mg/kg tolvaptan), and then further divided into five subgroups (days 1, 2, 3, 4, and 5). Blood samples were obtained to measure vasopressin and sodium concentrations. Rats were killed and colonic mucosa was scraped for analysis of protein expression and AQP-2 transcriptional level. The whole layer was fixed for hematoxylin&eosin (HE) staining and feces were collected for determination of fecal water content.

CONCLUSION: Compared with vehicle, vasopressin decreased significantly in the tolvaptan groups from day 2 to a similar level in each treatment group. AQP-2 showed significant upregulation in cirrhotic rats with ascites compared with an untreated control group (100% ± 22.9% *vs* 22.2% ± 10.23%, $P < 0.01$). After administration of tolvaptan, AQP-2 expression began to decrease significantly from day 2 in each treatment group, but no significant difference was finally found between the treatment groups. Fecal water content in

the distal colon was increased by 5 mg/kg tolvaptan on day 1 (66.8% ± 9.3% *vs* 41.4% ± 6.3%, in the vehicle group, $P < 0.05$). Fecal water content returned to baseline at day 4 at the latest in both treatment groups, and did not correspond to the change in AQP-2 expression. HE staining of the colonic mucosa showed no mucosal damage related to tolvaptan.

CONCLUSION: Upregulation of AQP-2 in the distal colon is found in cirrhotic rats with ascites. Tolvaptan inhibits its expression and may decrease water reabsorption and induce diarrhea.

Key words: Tolvaptan; Aquaporin-2; Cirrhosis; Ascites

Core tip: Aquaporin (AQP)-2 is mainly expressed in the kidneys, although it has been detected in the distal colon. We confirmed its expression in the distal colon and its upregulation in cirrhotic rats with ascites. Tolvaptan is a highly potent and selective AQP-2 antagonist and is used to treat cirrhotic ascites and hyponatremia. It blocked AQP-2 expression in the distal colon after oral administration, and temporarily increased fecal water content. Fecal water content returned to baseline quickly. The results suggest that tolvaptan inhibited AQP-2 expression in the distal colon and may induce diarrhea to clear water.

Chen C, Chen RP, Lin HH, Zhang WY, Huang XL, Huang ZM. Tolvaptan regulates aquaporin-2 and fecal water in cirrhotic rats with ascites. *World J Gastroenterol* 2016; 22(12): 3363-3371 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3363.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3363

INTRODUCTION

Ascites is a serious complication often seen in patients with advanced liver cirrhosis with portal hypertension. The onset and development of cirrhotic ascites are multifactorial. Liver function deterioration, portal hypertension, and systemic vasodilation lead to activation of the renin-angiotensin-aldosterone system, sympathetic nervous system, and vasopressin[1]. Ascites and water retention are associated with increased mortality rates[2], and redistribution of liquid is a major component of the physiological and pathological changes.

The colon is a vital organ for regulating water and salt balance. A total of 6-8 L of digestive fluids is secreted into the intestinal tract every day, and mainly comes from plasma and tissue fluid, but little is excreted. Water and salt homeostasis are mainly regulated by the colonic epithelium. The aquaporin (AQP) family plays an important role in modulating absorption and secretion of water and electrolytes. The major AQPs in the colon include AQP-1, 2, 3, 4, and 8[3-5]. AQP-2 is mainly expressed in the kidneys, and was first discovered by Preston *et al*[6,7] in 1992. Since then, it has been found in human and rat colons. In the kidneys, activation of the vasopressin V2 receptor increases water reabsorption, both by short- and long-term regulation[1]. Whether AQP-2 regulates the water balance in the intestinal tract remains obscure.

Tolvaptan is a novel vaptan and is characterized as a highly effective and selective non-peptide V2 receptor antagonist. It binds to the V2 receptor and induces aquaresis in humans and rats after oral administration[8]. Tolvaptan increases free water clearance and shows its unique curative effect in kidney and cardiovascular diseases[9-11]. Tolvaptan has been shown to alleviate the syndrome of inappropriate antidiuretic hormone or ascites[12-14].

In our clinical cases, some patients with hyponatremia appeared to have diarrhea during aquaretic therapy. We suspected that they might respond to tolvaptan administration, and we designed the present animal study to investigate the correlation between tolvaptan and fecal water content.

MATERIALS AND METHODS

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University in 2013 (No. wydw2013-0071). Tolvaptan was a gift from Otsuka Pharmaceutical Company, Japan.

Modeling

The study was performed in 84 conscious adult male Sprague-Dawley rats with cirrhosis and ascites induced by dorsal subcutaneous injection of CCl_4 (1 mg/kg) dissolved in paraffin (1:1; v/v) twice weekly (Monday and Friday) for 14 wk. Rats weighing 200-250g were fed *ad libitum* with standard chow and filtrated water containing phenobarbital (0.3g/L) as drinking fluid. After 1 wk phenobarbital induction, CCl_4 treatment began. One hundred and thirty rats were submitted to the model protocol, but 46 of these could not be included in the study for the following reasons: 20 died during the protocol and 26 failed to develop ascites. After developing ascites, all rats were observed for one more week to judge the stability of the ascites. All the animals with ascites were kept in metabolic cages for 3 d before tolvaptan (dissolved in 1% hydroxypropyl methylcellulose) treatment.

Experimental protocols

In total, 84 cirrhotic rats with ascites were included in this protocol and randomly assigned to three groups (vehicle, 3 mg/kg and 5 mg/kg tolvaptan). Each group was further divided into five subgroups (1, 2, 3, 4, and 5 d). Tolvaptan was applied by oral gavage. Body weight was measured daily at 09:00 am prior to treatment.

Tissue preparation

At the end of the experiment, rats were anesthetized intraperitoneally with 10% chloral hydrate; blood samples were obtained by postcaval puncture, and the descending colon was removed rapidly, feces were collected, and the colon was washed with phosphate-buffered saline at 4 ℃ three times. Animals were then killed by cervical dislocation. Colonic mucosa was isolated from underlying serosa by scraping with a glass slide and placing it into liquid nitrogen and then storingat -80 ℃ for western blotting and real-time polymerase chain reaction (PCR); the whole layer was fixed in 4% paraformaldehyde for 24 h and prepared for hematoxylin&eosin (HE) staining. Blood was collected in centrifuge tubes containing EDTA (1.5 mg/mL) and proteinase inhibitor (Roche, Switzerland), and then kept on ice until centrifugation at 4 ℃ to obtain plasma. The plasma was stored at -20 ℃ for subsequent assay of plasma vasopressin and ion concentrations.

Hormone determination

Hormone levels were determined by avidin-biotin ELISA using commercially available kits, vasopressin ELISA kit (Westang Bio-Tech, Shanghai, China) for plasma vasopressin concentration. Na^+ concentration was determined using a potentiometric method (AU5800; Beckman Coulter, CA, United States).

Western blotting

Colonic mucosal epithelium was obtained by scraping with a glass slide, and homogenized in lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with a glass homogenizer. The successive process followed the instructions of the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific). The supernatant was collected and its protein concentration was analyzed by BCA kit (Tiangen Biotech, Beijing, China), and then diluted with loading buffer (FUDE Biological Technology, Hangzhou, China) to 4 μg/μL. Each mixture was boiled at 100 ℃ for 5 min and chilled on ice. The mixture was stored at -20 ℃ and equal volumes were loaded in SDS-PAGE, together with molecular markers. The protein was transferred from the gel to a PVDF membrane, and the membrane was blocked for 1h at room temperature using 5% bovine serum albumin. Membranes were incubated with AQP-2 antibody (Cell Signaling Technology, Danvers, MA, United States) overnight at 4 ℃. After washing the membrane with Tris-buffered saline containing 0.1% Triton X-100, horseradish peroxidase was added for color development. Images were obtained by darkroom development techniques for chemiluminescence by ECL Plus (Advansta, Menlo Park, CA, United States).

mRNA detection for target genes

Total RNA was isolated from the colonic mucosal epithelium samples using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Reverse transcription was carried out using a Fist-Strand cDNA Synthesis kit (Thermo Scientific) in a total volume of 20 μL. The resultant cDNA was amplified using a SYBR Green qPCR kit (Applied Biosystems, Carlsbad, CA, United States), and quantified using an ABI PRISM 7500 sequence detection system (Applied Biosystems). For PCR, the following sense and antisense primers were designed from rat AQP-2 cDNA sequence: sense, 5′-TGGGTTGCCATGTCTCCTTC-3′; and antisense, 5′-GCGTTGTTGTGGAGAGCATT-3′. The PCR system consisted of 1 μL cDNA, 0.5 μL each of specific primers (10 μmol/L), 5 μL 2 × SYBR Green Mix and 3 μL water in a total volume of 10 μL. The PCR cycling conditions for AQP-2 and GAPDH were as follows: an initial denaturation and activation at 95 ℃ for 10 min, followed by 40 amplification cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. mRNA expression of the target genes was standardized by reference gene GAPDH. The relative expression of each gene was calculated by the comparative Ct method. The $2^{-\Delta\Delta Ct}$ method was adopted to quantify the mRNA expression level between each group, and ΔΔCt was defined by the following equation: $\Delta\Delta Ct = [(Ct \text{ of AQP-2} - Ct \text{ of GAPDH})_{tolvaptan} - (Ct \text{ of AQP-2} - Ct \text{ of GAPDH})_{vehicle}]$.

HE staining

Colonic mucosal epithelium samples from cirrhotic rats with ascites were fixed overnight in 4% para-formaldehyde at 4 ℃ and routinely processed for paraffin embedding. Histological sections (5 μm) were then prepared and stained with HE. Images (× 400 magnification) of the distal colon were acquired with a biological imaging microscope (Olympus, Tokyo, Japan).

Fecal water content

Fecal samples were collected at the time of sacrifice, and dried in anoven at 60 ℃ for 24 h. The fecal water content was calculated based on the following equation: [wet weights (g)-dry weights (g)]/wet weights (g) × 100%.

Statistical analysis

The results were expressed as the mean ± SEM, and the analyses were performed using SPSS version 17.0 software. The Student *t* test was used for two-group comparisons. Differences were considered statistically significant at $P < 0.05$. All the statistical analyses were performed by a biomedical statistician.

RESULTS

In the pathological examination, all liver specimens from cirrhotic rats with ascites showed cirrhosis, but we failed to distinguish any significant differences between rats receiving tolvaptan or vehicle. HE staining of the

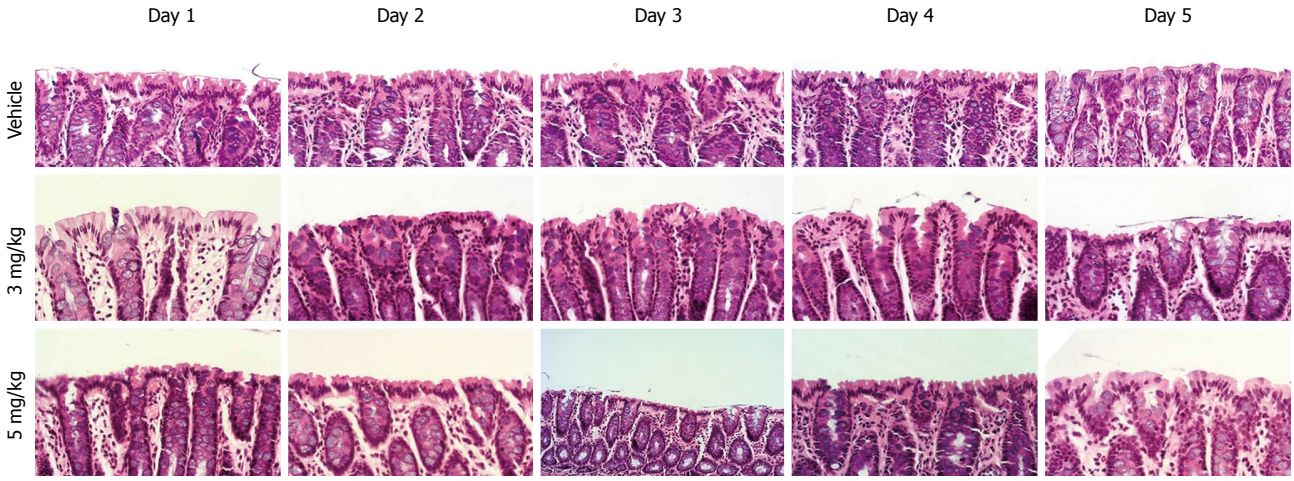

Figure 1 Hematoxylin and eosin staining of the distal colon of cirrhotic rats with ascites receiving different doses of tolvaptan for different periods (× 400). No mucosal damage was attributable to administration of tolvaptan.

Table 1 Body weight, vasopressin and serum sodium in baseline conditions in cirrhotic rats prior to receiving tolvaptan (3 mg/kg), tolvaptan (5 mg/kg) or vehicle

		1 d	2 d	3 d	4 d	5 d
Body weight (g)	Vehicle	486.7 ± 12.57 (5)	492.1 ± 10.12 (5)	472.8 ± 18.21 (5)	475.2 ± 9.21 (5)	482.8 ± 13.24 (5)
	Tolvaptan (3 mg/kg)	502.3 ± 11.91 (6)	487.3 ± 12.43 (6)	465.6 ± 16.59 (6)	465.6 ± 13.24 (6)	476.3 ± 16.00 (5)
	Tolvaptan (5 mg/kg)	493.4 ± 16.34 (6)	485.2 ± 20.21 (6)	490.1 ± 13.12 (6)	472.1 ± 12.12 (6)	490.1 ± 11.12 (6)
Vasopressin(pg/L)	Vehicle	13.25 ± 1.24 (5)	14.15 ± 1.41 (5)	15.44 ± 1.38 (5)	14.99 ± 0.94 (5)	13.05 ± 0.85 (5)
	Tolvaptan (3 mg/kg)	15.29 ± 0.93 (6)	13.37 ± 1.22 (6)	16.87 ± 0.73 (6)	15.16 ± 1.17 (6)	14.33 ± 0.99 (5)
	Tolvaptan (5 mg/kg)	14.85 ± 1.39 (6)	14.23 ± 0.81 (6)	15.12 ± 1.21 (6)	13.21 ± 1.21 (6)	15.23 ± 0.81 (6)
Serum sodium (mol/L)	Vehicle	141.2 ± 1.31 (5)	141.2 ± 1.11 (5)	139.2 ± 0.75 (5)	140.1 ± 0.92 (5)	140.2 ± 1.31 (5)
	Tolvaptan (3 mg/kg)	139.2 ± 1.27 (6)	141.3 ± 1.20 (6)	138.2 ± 1.85 (6)	140.1 ± 1.23 (6)	138.2 ± 1.87 (5)
	Tolvaptan (5 mg/kg)	141.2 ± 1.21 (6)	140.9 ± 1.12 (6)	142.2 ± 1.22 (6)	138.1 ± 2.21 (6)	139.1 ± 1.55 (6)

Data are mean ± SEM (number of rats); no significant difference was found between different doses of tolvaptan and vehicle.

colon tissue sections showed no mucosal damage to the colon attributable to the administration of tolvaptan (Figure 1).

Table 1 shows the effect of different doses of tolvaptan at different times on cirrhotic rats, and we chose the first 5 d to observe plasma vasopressin, sodium concentration, and body weight. No significant differences were observed in body weight and no change was identified for sodium and vasopressin concentrations based on different doses of tolvaptan and vehicle.

After administration of both doses of tolvaptan, there was a significant decrease in body weight on day 1, as well on successive days (Table 2). After drug treatment for 4-5 d, ascites was eliminated (confirmed by abdominal laparotomy when sacrificed). Vasopressin decreased from day 2, and the successive days.

AQP-2 expression in the distal colon was detected in each group. Two bands of AQP-2 protein were detected by western blotting. One band appeared at 23 kDa and the other at 39 kDa, representing the nonphosphorylated and phosphorylated forms, respectively. The total expression of the two bands was analyzed as the protein level in the distal colon (Figure 2). The protein expression level of AQP-2 after 1 d tolvaptan administration showed no significant difference compared to that with vehicle. Compared with vehicle, significant AQP-2 downregulation was found on day 2 (3 mg/kg: 100% ± 22.9% *vs* 54.7% ± 11.7%, $P < 0.05$; 5 mg/kg: 100% ± 22.9% *vs* 53.0% ± 9.4%, $P < 0.01$), and on successive days with both doses of tolvaptan. However, no difference was found in AQP-2 expression between the 3 mg/kg and 5 mg/kg groups. Transcriptional AQP-2 in the distal colon was also measured (Figure 3). Compared with vehicle, there was a significant reduction on day 1 with tolvaptan (3 mg/kg: 100 ± 16.3% *vs* 68.5 ± 10.0%, $P < 0.05$; 5 mg/kg: 100 ± 16.3% *vs* 34.1 ± 15.1%, $P < 0.01$), as well as on successive days. Expression of AQP-2 mRNA before and after administration of tolvaptan showed a consistent trend to reflect protein expression.

Fecal water content after tolvaptan administration increased on days 2 and 3 with both doses of tolvaptan, but only the higher dose caused an increase on day 1 (Figure 4). After 3 d of tolvaptan administration, the fecal water content returned to baseline.

Table 2 Body weight, vasopressin, and serum sodium in cirrhotic rats after tolvaptan (3 mg/kg), tolvaptan (5 mg/kg), or vehicle

		1 d	2 d	3 d	4 d	5 d
Body weight (g)	Vehicle	492.6 ± 11.22 (5)	498.5 ± 12.21 (5)	490.3 ± 16.21 (5)	500.1 ± 16.21 (5)	493.1 ± 11.12 (5)
	Tolvaptan (3 mg/kg)	482.2 ± 20.21 (5)	466.2 ± 10.19 (6)[a]	450.1 ± 18.21 (5)[a]	443.1 ± 20.12 (6)[a]	440.1 ± 20.12 (5)[a]
	Tolvaptan (5 mg/kg)	460.2 ± 12.31 (5)[a]	455.1 ± 16.12 (5)[a]	440.2 ± 23.12 (6)[a]	437.3 ± 22.21 (5)[a]	446.1 ± 21.21 (4)[a]
Vasopressin (pg/L)	Vehicle	12.97 ± 0.92 (5)	13.81 ± 1.30 (5)	15.91 ± 0.89 (5)	16.97 ± 1.38 (5)	14.02 ± 0.98 (5)
	Tolvaptan (3 mg/kg)	12.25 ± 0.82 (5)	11.41 ± 0.62 (6)	10.21 ± 0.91 (5)[a]	9.77 ± 1.21 (6)[a]	7.12 ± 1.32 (5)[b]
	Tolvaptan (5 mg/kg)	12.01 ± 1.21 (5)	10.12 ± 0.92 (5)[a]	8.12 ± 1.22 (6)[a]	7.01 ± 1.63 (5)[b]	7.33 ± 1.13 (4)[a]
Serum sodium (mol/L)	Vehicle	140.2 ± 1.25 (5)	140.2 ± 1.51 (5)	141.2 ± 0.65 (5)	141.1 ± 1.61 (5)	142.2 ± 0.81 (5)
	Tolvaptan (3 mg/kg)	143.2 ± 2.32 (5)	144.2 ± 1.17 (6)[a]	146.1 ± 1.13 (5)[a]	147.2 ± 2.11 (6)[b]	146.1 ± 2.47 (5)[a]
	Tolvaptan (5 mg/kg)	144.1 ± 1.22 (5)[a]	147.1 ± 2.51 (5)[a]	147.9 ± 2.21 (6)[a]	146.2 ± 1.71 (5)[a]	147.2 ± 1.12 (4)[b]

Data are mean ± SEM (number of rats); [a]$P < 0.05$, tolvaptan *vs* vehicle, respectively; [b]$P < 0.01$, tolvaptan *vs* vehicle, respectively.

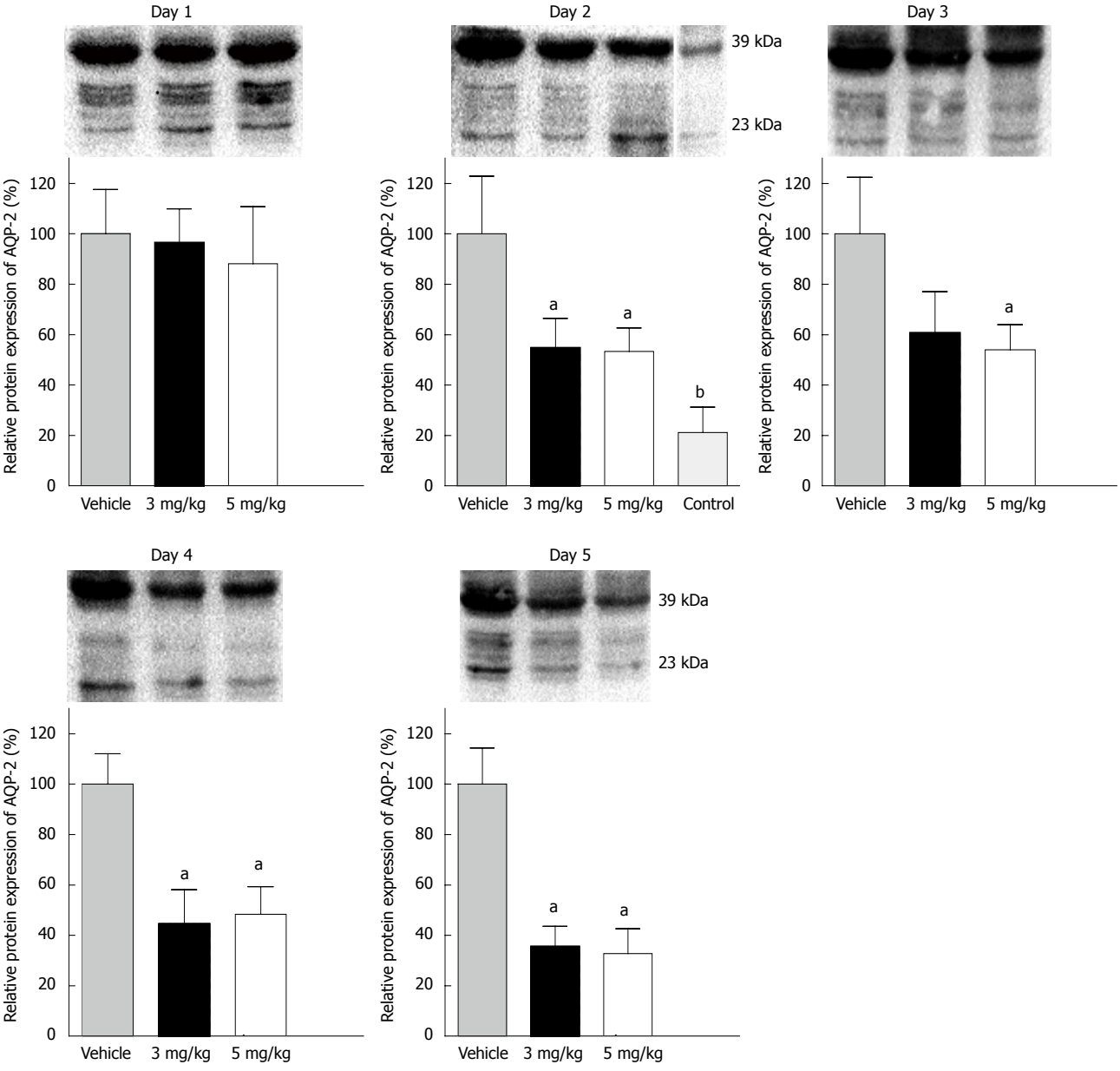

Figure 2 Phosphorylated (39 kDa) and nonphosphorylated (23 kDa) protein expression of AQP-2 in distal colon in cirrhotic rats with ascites treated by oral gavage of tolvaptan (3 or 5 mg/kg) or vehicle. After 1 d of both doses of tolvaptan, no significant difference was found between each group. Compared with vehicle, AQP-2 protein expression showed significant reductions on day 2 (3 mg/kg: 100% ± 22.9% *vs* 54.7% ± 11.7%, $P < 0.05$; 5 mg/kg: 100% ± 22.9% *vs* 53.0% ± 9.4%, $P < 0.01$) and on successive days. AQP-2 expression in a control group (no treatment) was also measured, and was significantly lower than that on day 2 compared with vehicle (22.2% ± 10.23% *vs* 100% ± 22.9%, $P < 0.01$). No significant difference was found between the cirrhotic groups treated with different dosesof tolvaptan. Data are mean ± SEM; [a]$P < 0.05$, tolvaptan *vs* vehicle; [b]$P < 0.01$, tolvaptan *vs* vehicle. AQP: Aquaporin.

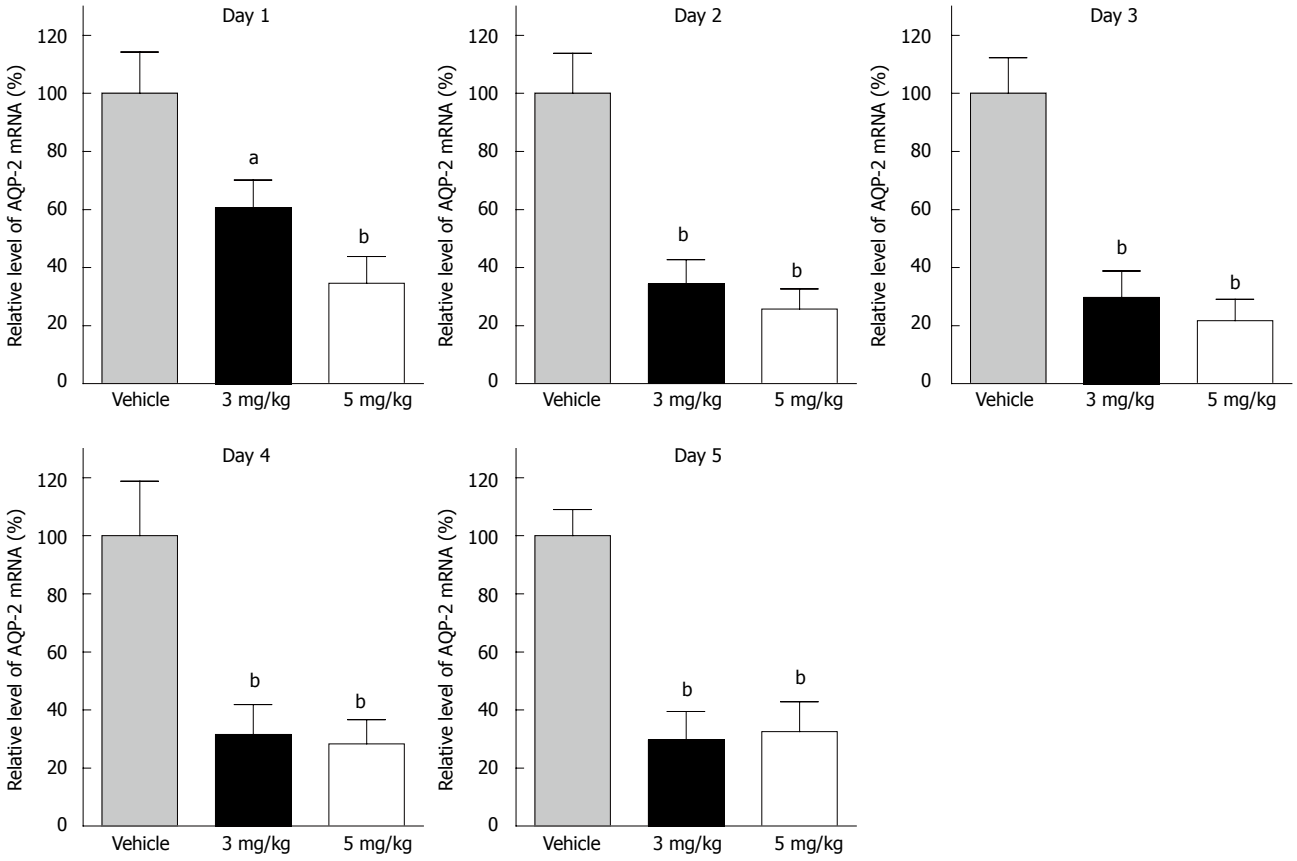

Figure 3 Relative mRNA expression of AQP-2 in the distal colon in the tolvaptan and vehicle groups. AQP-2 transcription in the distal colon in cirrhotic rats with ascites treated with different doses of tolvaptan (3 and 5 mg/kg) was measured. Compared with vehicle, a significant difference was detected on day 1 (3 mg/kg: 100% ± 16.3% *vs* 68.5% ± 10.0%, $P < 0.05$; 5 mg/kg: 100% ± 16.3% *vs* 34.1% ± 15.1%, $P < 0.01$) and on successive days. There was no significant difference between the respective tolvaptan groups. Data are mean ± SEM; $^{a}P < 0.05$, tolvaptan *vs* vehicle; $^{b}P < 0.01$, tolvaptan *vs* vehicle.

DISCUSSION

The effect of tolvaptan on AQP-2 in the distal colon has been reported in rats in order to explore the roles of vasopressin and aldosterone in physiological adaption[15,16]. In our study, different doses of tolvaptan were administered for different times to investigate the colonic expression of AQP-2 in cirrhotic rats with ascites, and to explore the role of tolvaptan in regulation of fecal water content. The findings presented here indicated that AQP-2 expression in the distal colon increased in cirrhotic ratswith ascites, and it was downregulated by oral administration of tolvaptan. Furthermore, the fecal water content increased temporarily during tolvaptan therapy.

Every AQP protein consists of six transmembrane domains, and the AQP family selectively transports water, glycerin, and other compounds through the membrane. It is generally accepted in the kidneys that transmembrane free water is regulated by water channel, namely AQP-2. Both water deprivation[17,18] and deamino-*arg8-vasopressin* (*dAVP*)[19,20] infusion increases AQP-2 expression in the kidney. AQP-2 was first observed in the distal colon in dehydrated rats by Gallardo *et al*[3] in 2001. They suggested that the colonic expression of AQP-2 in apical membranes of rats helps regulate transepithelial water transport from the intestinal side to the vascular side. This mechanism was strongly involved in water absorption and fecal dehydration.

In our study, colonic expression of AQP-2 was confirmed and we found that it was upregulated in cirrhotic rats with ascites. Ascites is a state of water retention, which is a combined effect of endocrine and hemodynamic systems. We attributed the increase in AQP-2 expression to the development of ascites. A high hemodynamic state accelerates hypothalamus secretion of excessive vasopressin[21], and deterioration of liver function reduces the vasopressin and aldosterone metabolism. Both of these contribute to the high levels of vasopressin and aldosterone in the bloodstream. Excessive vasopressin stimulates V2 receptors and activates adenylyl cyclase, which increases the concentration of cAMP in the cytoplasm, thus facilitating phosphorylation of AQP-2. The accumulation of AQP-2 is induced by vesicle trafficking from storage vesicles to apical surface membranes within several tens of minutes (short-term regulation)[22-25], and in the long-term, upregulation of AQP-2 in response to vasopressin plays a major role[26,27].

Tolvaptan is a highly effective and selective non-peptide V2 receptor antagonist. It was reported that

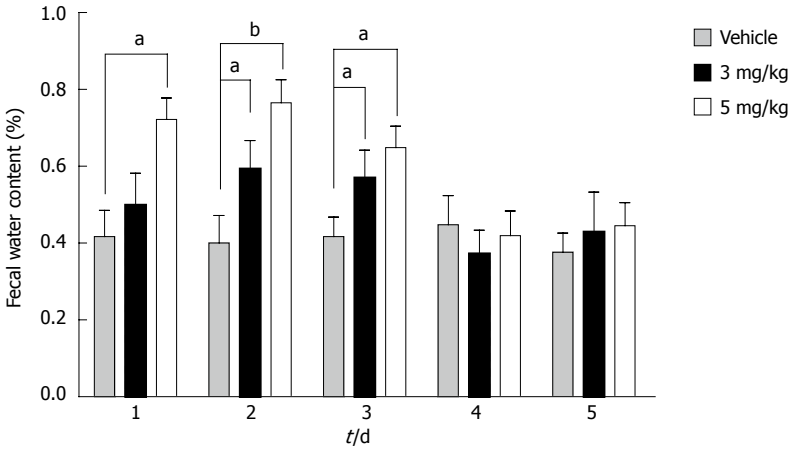

Figure 4 Fecal water content in the distal colon of cirrhotic rats with ascites. Compared with vehicle, the fecal water content increased from day 1 in the 5 mg/kg tolvaptan group (41.4% ± 6.3% *vs* 66.8% ± 9.3%, $P < 0.05$), and from day 2 in the 3 mg/kg group (40.0% ± 6.0% *vs* 59.2% ± 10.3%, $P < 0.05$). However, it returned to baseline after 3 d administration of both doses of tolvaptan. No significant difference was found between different doses of tolvaptan. Data are mean ± SEM; $^{a}P < 0.05$, tolvaptan *vs* vehicle; $^{b}P < 0.01$, tolvaptan *vs* vehicle.

oral tolvaptan (1 and 3 mg/kg) promoted a marked diuretic effect in dimethylnitrosamine-induced cirrhotic rats with ascites[28]. Cristia *et al*[16] reported that 3 mg/kg tolvaptan significantly inhibited AQP-2 expression in the distal colon. In our preliminary experiment, we selected different doses of tolvaptan and found that 1 mg/kg showed aquaresis but had no significant effect on colonic AQP-2 expression. So, we tried higher doses of 3 mg/kg and 5 mg/kg. Ascites was eliminated at the end of the protocol in most of the rats receiving tolvaptan therapy. Serum sodium increased and no hypernatremia was present. Some rats died of dehydration. We confirmed elevation of vasopressin in cirrhotic rats[21] and different doses of tolvaptan decreased it to a similar level. It seems that in the cirrhotic ascites model, elevation of vasopressin is mainly induced by a non-osmotic mechanism, because hyponatremia should inhibit vasopressin excretion in healthy individuals. After the circulation improved, vasopressin levels decreased.

As in the kidneys, the effect of vasopressinon AQP-2 regulation in the colon is a response to V2 receptor activation. Our results confirmed that the reduction of colonic expression of AQP-2 was a direct response to oral tolvaptan administration. In the present study, both doses of tolvaptan affected AQP-2 regulation but different doses eventually resulted in similar expression levels in the distal colon.

It is reported that the colon could be a target for action by vasopressin, as it has been shown that vasopressin stimulates Na^+ and water absorption[15]. Water is removed from luminal feces by the surface mucosa and the crypts[29]. Cristià *et al*[16] suggested that the stimulation of myofibroblast growth and the increase in AQP-2 expression are consistent with the antidiuretic role of tolvaptan in the distal colon. The studies above discussed the colonic microscopic function and electrochemical changes of tolvaptan, but the biological function has not been explored.

We showed that the fecal water content increased and we attributed it to tolvaptan administration. It is known that mucosal damage in the colon causes diarrhea[30,31]. We were not sure whether a higher dose of tolvaptan would cause mucosal damage. So, we performed HE staining of the colon tissue sections, and no mucosal damage was found that was attributable to administration of tolvaptan. However, after a few days of therapy, the fecal water content returned to normal (4-5 d). This phenomenon did not correspond to the alteration of AQP-2 expression in the distal colon. As tolvaptan was the only variable in this experiment, we suggest there would be compensatory mechanisms of water balance.

Vasopressin-dependent water flow occurs via AQP-2 from the intestinal tract, and outflow to the intercellular space via AQP-3 and/or AQP-4. However, it appears that neither AQP-3 nor AQP-4 is regulated by vasopressin in the distal colon[32,33]. It has been suggested that AQP-4 has little or no effect on colonic fluid secretion or fecal dehydration. However, AQP-3 is the most dominantly expressed AQP in the colon and plays an important role in the absorption of water[34,35], and its abundance appears to be regulated on a long-term basis in a manner similar to the long-term regulation of AQP-2. When exposed to an environment of possible water overload[36], AQP-3 could serve as a water channel to reabsorb water from the enteric cavity. When AQP-3 was specifically blocked by $HgCl_2$ or $CuSO_4$, diarrhea was induced[37]. We speculate that AQP-3 upregulation may be one of the mechanisms to balance water absorption in the distal colon after tolvaptan administration.

Our experiment explored fecal water content in response to oral tolvaptan administration in the therapy of cirrhotic ascites. Tolvaptan reduces water reabsorption and may promote clearance of redundant water. This mechanism could induce diarrhea in some

conditions. However, the precise mechanism needs to be further explored. Besides long-term regulation, short-term regulation should also be investigated in the distal colon and its biological function should be confirmed.

COMMENTS

Background

Aquaporin (AQP)-2 is one of the AQP family and marked free-water clearance is induced when its effects are blocked. Tolvaptan is a potent and selective antagonist, and, can be used to treat cirrhotic ascites and hyponatremia. AQP-2 is found in the distal colon but its biological function has not been established. This study was designed to determine the colonic expression of AQP-2 and to investigate the correlation between tolvaptan administration and fecal water content.

Research frontiers

AQP-2 is a member of the AQP family, and helps to absorb water in the kidneys. It is also found in the colon and is involved in water and electrolyte transportation. Tolvaptan is a specific antagonist of AQP-2 and has widespread clinical application.

Innovations and breakthroughs

This is the first study to explore colonic AQP-2 expression in cirrhotic rats with ascites, and tolvaptan inhibited its expression and increased fecal water content.

Applications

This study suggests that tolvaptan may be useful in upregulating fecal water content and indicates a way to clear water.

Peer-review

It is an interesting study with appropriate methodology and the results are clear and of great importance.

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ORIGINAL ARTICLE

Case Control Study

Association between polymorphisms of *APE1* and *OGG1* and risk of colorectal cancer in Taiwan

Ching-Yu Lai, Ling-Ling Hsieh, Reiping Tang, Regina M Santella, Chung Rong Chang-Chieh, Chih-Ching Yeh

Ching-Yu Lai, Chih-Ching Yeh, School of Public Health, College of Public Health and Nutrition, Taipei Medical University, Taipei 11031, Taiwan

Ling-Ling Hsieh, Department of Public Health, Chang Gung University, Taoyuan 333, Taiwan

Reiping Tang, Chung Rong Chang-Chieh, Colorectal Section, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Regina M Santella, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY 10032, United States

Chih-Ching Yeh, Department of Public Health, College of Public Health, China Medical University, Taichung 404, Taiwan

Author contributions: Lai CY, Hsieh LL, and Yeh CC designed the study, analyzed and interpreted the data, and drafted the manuscript; Tang R and Chang-Chieh CR enrolled participants; Santella RM conceived the study, participated in the experimental assays, and critically revised the manuscript; all authors have read and approved the final manuscript.

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Informed consent statement: All patients provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party directly or indirectly related to the study topic.

Data sharing statement: Technical appendix, statistical code, and data set are available with the corresponding author at ccyeh@tmu.edu.tw.

Correspondence to: Chih-Ching Yeh, PhD, Professor, School of Public Health, College of Public Health and Nutrition, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan. ccyeh@tmu.edu.tw
Telephone: +886-2-27361661-6534
Fax: +886-2-27384831

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Abstract

AIM: To evaluate the effects of *OGG1* (Ser326Cys, 11657A/G, and Arg154His) and *APE1* (Asp148Glu, and T-656G) polymorphisms on colorectal cancer (CRC) risk.

METHODS: We enrolled 727 cases newly diagnosed with colorectal adenocarcinoma and 736 age- and sex-matched healthy controls from a medical center in Taiwan. Genomic DNA isolated from the buffy coat was used for genotyping through polymerase chain reaction. Unconditional logistic regressions were used for calculating ORs and 95%CIs to determine the association between the genetic polymorphisms and CRC risk. Haplotype frequencies were estimated using PHASE software. Moreover, stratification analyses on

the basis of sex, age at diagnosis, and tumor subsite and stage were performed.

RESULTS: The CRC risk was higher in patients with the *OGG1* 326Ser/Cys + Cys/Cys genotype (OR = 1.38, 95%CI: 1.03-1.85, *P* = 0.030), particularly high in patients with stage Ⅲ + Ⅳ cancer (OR = 1.48, 95%CI: 1.03-2.13) compared with patients with the Ser/Ser genotype. In addition, *OGG1* 11657G allele carriers had a 41% reduced CRC risk among stage 0-Ⅱ patients (OR = 0.59, 95%CI: 0.35-0.98). The CRC risk was significantly higher among females with the *APE1* Glu allele (OR = 1.41, 95%CI: 1.02-1.96). The *APE1* 148Glu/-656G haplotype was also associated with a significant CRC risk in females (OR = 1.36, 95%CI: 1.03-1.78).

CONCLUSION: *OGG1* and *APE1* polymorphisms are associated with stage- and sex-specific risk of CRC in the Taiwanese population.

Key words: *APE1*; *OGG1*; Taiwan; Colorectal cancer; Polymorphisms

Core tip: The associations between base excision repair DNA polymorphisms and colorectal cancer (CRC) risk is controversial. The present study examined the effects of *OGG1* and *APE1* polymorphisms on the CRC risk by using a large-scale sample of 727 CRC cases and 736 healthy controls. Results demonstrated that *OGG1* 326Cys and *APE1* 148Glu alleles were significantly associated with an increased CRC risk in patients with stage Ⅲ + Ⅳ cancer (OR = 1.48) and females (OR = 1.41). Females carrying the *APE1* 148Glu/-656G haplotype also exhibited a 36% increased CRC risk. These findings suggest that OGG1 and APE1 polymorphisms are associated with stage- and sex-specific risk of CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer and third leading cause of cancer-related deaths in Taiwan[1]. Studies have indicated that both endogenous and exogenous oxidative damage resulting from reactive oxygen species (ROS) generation participates in all stages of cancer[2,3]. Several ROS can cause oxidative DNA damage, including single- or double-strand breakages, base modifications, deoxyribose modifications, and DNA cross-links[4]. DNA repair enzymes are critical in preventing drug resistance and protecting the genome against ROS carcinogenesis[2]. More than 100 proteins are involved in the DNA repair system through different pathways, including base excision repair (BER), nucleotide excision repair, double-strand break repair, and mismatch repair[5].

The BER gene family is activated through internal oxidative stress and DNA damage and involves 8-oxoguanine glycosylase 1 (*OGG1*) and apurinic-apyrimidinic endonuclease 1 (*APE1*)[6]. *OGG1* is located at 3p26.2 and encodes the major repair enzyme for directly removing 8-oxo-guanine (8-oxoG) from the damaged DNA[7]. In addition to catalyzing 8-oxoG excision from DNA, *OGG1* can incise at abasic sites through an apurinic and apyrimidinic (AP) lyase activity[8]. Studies have suggested low in DNA repair efficiency in patients with cancer. Most studies focused on the common single nucleotide polymorphism (SNP) C1245G that results in the substitution of serine with cysteine at codon 326 (Ser326Cys, rs1052133)[5,9-12]. The 326Cys protein, whose enzyme activity is lower than that of Ser, might be associated with cancer risk[5,13]. Two other *OGG1* SNPs, 11657A/G and Arg154His (rs 56053615), located in the downstream region have also been associated with cancer risk[14-16].

Human *APE1* is located on chromosome 14q11.2-q12 and comprises five exons spanning 2.21 kb[17]. *APE1* is crucial in initiating the BER of basic sites in DNA hydrolyzing the phosphodiester backbone 5' to an AP site and recruiting DNA polymerase β and DNA ligase Ⅲ[18,19]. Known allelic variants of *APE1* include an amino acid change from aspartic acid to glutamic acid (Asp148Glu, rs 1130409) in exon 5, which may be associated with hypersensitivity to ionizing radiation and cancer risk[20-22]. Moreover, a T to G SNP observed in the promoter region (T-656G, rs1760944) was associated with a 57% reduced risk of lung cancer[18].

Numerous studies have examined the association of *OGG1* and *APE1* polymorphisms with the CRC risk, but the results are controversial[5,7,12,23-25]. Therefore, in this study, we evaluated the association of genetic polymorphisms of *OGG1* (Ser326Cys, 11657A/G, and Arg154His) and *APE1* (Asp148Glu, and T-656G) with the CRC risk in a hospital-based case-control study in Taiwan. In addition, because age at diagnosis, sex, and tumor site and stage may modify the aforementioned association[23,26-29], we used stratification analysis for investigating whether these factors affect the association between *OGG1* and *APE1* polymorphisms and CRC risk.

MATERIALS AND METHODS

Participants

Participant characteristics have been previously des-

cribed[26,30]. Briefly, cases newly diagnosed with colorectal adenocarcinoma were enrolled from Chang Gung Memorial Hospital between January 1995 and January 1999. Age- and sex-matched healthy controls were enrolled from the Physical Check-Up Department during the same period. After excluding patients diagnosed with hereditary colorectal diseases, other related malignancies, or a history of cancer, 727 cases (94%, 727/776) and 736 controls (98%, 736/747) were included in this study. The Institutional Review Board of Chang Gung Memorial Hospital approved the study protocol, and all participants provided written informed consent.

Questionnaire

Trained nurses conducted standardized interviews of all participants in the hospital. Data on socio-demographic characteristics and risk factors for CRC, namely physical activity in the past year, cigarette smoking history, alcohol and coffee use, medical history, and food intake 5 years preceding the interview, were obtained from a structured questionnaire; simultaneously 10 mL of venous blood was collected.

Genotyping Assays

Genotyping assays for examining *OGG1* Ser326Cys and 11657A/G polymorphisms were performed on the genomic DNA extracted from the buffy coat by using the template-directed dye-terminator incorporation assay with fluorescence polarization detection[31]. DNA was amplified in a 10-μL final volume containing 2 pmol/L of each primer, 25 ng of genomic DNA, 10 mmol/L of $MgCl_2$, 2.5 mmol/L of dNTPs, and 0.5 U of Taq DNA polymerase (Roche, Mannheim, Germany) in the buffer provided by the manufacturer. PCR reactions were performed in a Mastercycler (Eppendorf, Hamburg, Germany). The Ser326Cys polymorphism was amplified using the following primers: Forward, 5′-TCCACCTCCCAACACTGTCACTA-3′ and Reverse, 5′-TCACCTGCTTCCCTACCACT-3′. The PCR program involved a 2-min denaturing step at 92 ℃, 10 cycles of 10 s at 92 ℃, 20 s at 62 ℃, and 30 s at 68 ℃, and followed by 30 cycles of 10 s at 92 ℃, 20 s at 60 ℃, and 30 s at 68 ℃. The 11657A/G polymorphism was amplified using the following primers: Forward, 5′-GGCAATCAGAGATGGTTAGA-3′, and Reverse, 5′-TGGCATTAAATCAAGCACTA-3′. The PCR program involved a 5-min denaturing step at 94 ℃ followed by 35 cycles of 30 s at 94 ℃, 45 s at 58 ℃, and 60 s at 72 ℃.

After amplification, the residual primers and dNTPs were degraded in a 10 μL final volume of 1 U of shrimp alkaline phosphatase (Roche) and 1 U of exonuclease I (USB, Cleveland, OH, United States) for 45 min at 37 ℃, followed by 15 min at 95 ℃ for enzymes inactivations.

The template-directed dye-terminator incorporation assay was performed using the AcycloPrime™-FP SNP detection kit (PerkinElmer Life Sciences, Boston, MA, United States). The AcycloPrime-FP reaction was conducted in a 10-μL mixture containing 2 μL of amplified and processed PCR product, 9 pmol/L of probe, 0.5 U of the appropriate AcycloTerminator kit, 0.025 μL of AcycloPol in the buffer provided by the manufacturer. The PCR program involved a 2-min denaturing step at 95 ℃ followed by 39 cycles of 15 s at 95 ℃ and 30 s at 55 ℃. The forward probes 5′-AGTGCCGACCTGCGCCAAT-3′ and 5′-CCAGGA-AGGACAAGGCTCA-3′ were used for Ser326Cys and 11657A/G polymorphisms, respectively. Fluorescence polarization was measured using the PerkinElmer Victor^2 reader (PerkinElmer) connected with data analysis and allele-calling software. All genotyping assays included three known genotypes and a negative control, and the laboratory analyser was blinded to the case-control status of the participants.

Genotyping for assessing the *OGG1* Arg154His polymorphism was modified from a previously reported method using primer extension and denaturing high-performance liquid chromatography (PE-DHPLC)[26]. Briefly, forward (5′-AGCAGGTACCTCTCCTACC-3′) and reverse (5′-AGGTCCAAAAGCCTGGCAC-3′) primers were used for PCR amplification. Reactions were run in 25 μL volumes using an amplification protocol involving an initial denaturation step at 95 ℃ for 5 min, followed by 34 cycles of 95 ℃ for 1 min and 60 ℃ for 30 s 72 ℃ for 30 s, and 72 ℃ for 5 min. After amplification, a 5 μL PCR product was degraded using 0.64 U of exonuclease I and 0.08 U of shrimp alkaline phosphatase for 40 min at 37 ℃. Furthermore, the product was amplified in a 25 μL final volume containing 1 mmol/L of dNTP and ddATP, 10 μmol/L of primer (5′-CCTCCAACAACAACATCGCCC-3′) and 0.5 U of Thermo Sequenase™ DNA polymerase (Amersham, Cleveland, OH, United States) in the buffer. Reactions were conducted as follows: an initial denaturation step of 95 ℃ for 75 s; 49 cycles of 95 ℃ for 15 s, 43 ℃ for 15 s, and 60 ℃ for 100 s, and an extension step of 96 ℃ for 30 s. The extended products were analyzed through DHPLC with a linear acetonitrile gradient in a triethylamine acetate buffer.

The *APE1* Asp148Glu polymorphism was determined using a 5′-nuclease assay with allele-specific TaqMan probes including 148Asp probe (5′-VIC-AATTCTGTTTCATTTCTATAGGCGAGGAG-GAGCATGATCAGGAAGGCCGGG-TAMRA-3′) and 148Glu probe (5′-FAM-AATTCTGTTTCATTTCTATAGGCGA-TGAGGAGCATGATCAGGAAGGCCGGG-TAMRA-3′). TaqMan SNP genotyping assay kits were purchased from Applied Biosystems (Foster City, CA, United States). Genotyping was performed using an allelic discrimination assay in the ABI 7500 FAST Real-Time PCR system (Applied Biosystems), and genotypes were distinguished using automated ABI 7500 software v2.0 (Applied Biosystems). Reactions were run in 10 μL volumes by using an amplification protocol with an

Table 1 Age, sex, and genotype frequencies of the cases and controls *n* (%)

Variable	Cases *n* = 727	Controls *n* = 736	*P* value[1]
Age (mean ± SD), yr	60.3 ± 12.8	60.7 ± 13.0	0.639
Gender			0.751
Male	410 (56.4)	409 (55.6)	
Female	317 (43.6)	327 (44.4)	
Colon/rectum, *n*	352/375		
OGG1 Ser326Cys			0.021
Ser/Ser	93 (13.0)	125 (17.1)	
Ser/Cys	363 (50.8)	324 (44.4)	
Cys/Cys	258 (36.1)	281 (38.5)	
Missing	13	6	
OGG1 11657A/G			0.318
A/A	648 (91.9)	654 (90.1)	
A/G	55 (7.8)	72 (9.9)	
G/G	2 (0.3)	0 (0.0)	
Missing	22	10	
OGG1 Arg154His			0.158
Arg/Arg	720 (100.0)	720 (99.7)	
Arg/His	0 (0.0)	2 (0.3)	
His/His	0 (0.0)	0 (0.0)	
Missing	7	14	
APE1 Asp148Glu			0.567
Asp/Asp	236 (33.0)	249 (34.3)	
Asp/Glu	349 (48.8)	361 (49.7)	
Glu/Glu	130 (18.2)	117 (16.1)	
Missing	12	9	
APE1 T-656G			0.876
T/T	217 (30.1)	211 (28.9)	
T/G	368 (51.0)	380 (52.0)	
G/G	136 (18.9)	140 (19.2)	
Missing	6	5	

[1]Continuous variables were tested by Student's *t*-test, and categorical variables were tested by χ^2 or Mantel-Haenszel χ^2 test.

initial denaturation step of 60 ℃ for 1 min and 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min.

The genotyping method for assessing the *APE1* T-656G polymorphism was modified from a previously reported method using a high resolution melting assay in the ABI 7500 FAST Real-Time PCR system (Applied Biosystems). Genotypes were distinguished using automated High Resolution Melting software v2.0. Forward (5'-CACAGCACATTGTGTGACACTGA-3') and reverse (5'-AGCCCTCTCCACTGTTTTTTTCC-3') primers used for the PCR reactions were run in 20 μL volumes by using an amplification protocol with a holding step of 95 ℃ for 10 min followed by 36 cycles of 95 ℃ for 15 s and 60 ℃ for 20 s. The melting curve stage was maintained at 95 ℃ for 15 s, 50 ℃ for 1 min, 95 ℃ for 15 s, and 60 ℃ for 15 s.

Statistical analysis

We compared the different distributions for each categorical variable measured using the chi-squared test, and continuous variables were measured using the Student's *t*-test. Allelic frequencies were examined using the χ^2 test for concordance with those expected in Hardy-Weinberg equilibrium. We used unconditional logistic regression for estimating the OR and 95%CI with the factors (age and sex) matched in the model[32]. Stratified analyses were conducted for evaluating the differences between sex, age at diagnosis (≤ 60 and > 60 years old), tumor subsites (colon and rectum), and tumor stages (stage 0 + Ⅰ + Ⅱ and Ⅲ + Ⅳ). Haplotype frequencies were estimated on the basis of a Bayesian algorithm by using PHASE 2.1. All analyses were performed using the SAS statistical package (Version 9.2 for Windows; SAS Institute, Inc., Cary, NC, United States) and all statistical tests were two-sided. The powers were estimated using Quanto.

RESULTS

Age, sex, and *OGG1* and *APE1* genotypes of all participants are summarized in Table 1. Patients with CRC had a mean age of 60.3 years, and 410 (56.4%) males and 352 (48.4%) experienced colon cancer. The mean age and sex distribution was comparable between the patients with CRC and controls. The variant allelic frequencies for *OGG1* Ser326Cys, *OGG1* 11657A/G, *OGG1* Arg154His, *APE1* Asp148Glu, and *APE1* T-656G among the controls were, 60.7%, 5.0%, 0.14%, 40.9%, and 45.1%, respectively. All genotype frequencies were in Hardy-Weinberg equilibrium. The distribution of the *OGG1* Ser326Cys genotype was significantly different between the patients and controls (P = 0.021). However, other genotypic distributions did not significantly differ among the patients and controls. Because of the low frequency of sequence variants of *OGG1* Arg154His (His allele, 0.14%), this polymorphism was excluded from further analyses.

Table 2 shows the CRC risks associated with *OGG1* and *APE1* polymorphisms. Compared with the Ser/Ser genotype, the *OGG1* Ser326Cys heterozygous genotype was significantly associated with the CRC risk (OR = 1.51; 95%CI: 1.11-2.05, P = 0.009). However, other *OGG1* Ser326Cys genotypes were not associated with the CRC risk. The CRC risks associated with the heterozygous or homozygous genotypes of all the studied polymorphisms were similar; therefore, we used dominant models for determining the effects of polymorphisms on the CRC risk. Carriers of the *OGG1* 326Cys allele exhibited a significantly increased CRC risk (OR = 1.38; 95%CI: 1.03-1.85, P = 0.030). No significant associations were observed between other variant alleles and the CRC risk. The dominant genetic models of the studied polymorphisms were used for further stratification analysis.

Stratified analyses by sex, age at diagnosis, tumor subsites, and stage for the CRC risk associated with the *OGG1* polymorphisms are shown in Table 3. Data stratified by the tumor stage demonstrated that compared with the Ser/Ser genotype, the *OGG1* Ser326Cys Ser/Cys + Cys/Cys genotype was associated with an increased

Table 2 Distribution of *OGG1* and *APE1* polymorphisms between cases and controls *n* (%)

Genotype	Cases	Controls	OR (95%CI)[1]	*P* value
OGG1 Ser326Cys				
Ser/Ser	93 (13.0)	125 (17.1)	1.00 (ref)	
Ser/Cys	363 (50.8)	324 (44.4)	1.51 (1.11-2.05)	0.009
Cys/Cys	258 (36.1)	281 (38.5)	1.23 (0.90-1.69)	0.198
Ser/Cys + Cys/Cys	621 (87.0)	605 (82.9)	1.38 (1.03-1.85)	0.030
OGG1 11657A/G				
A/A	648 (91.9)	654 (90.1)	1.00 (ref)	
A/G	55 (7.8)	72 (9.9)	0.77 (0.53-1.12)	0.168
G/G	2 (0.3)	0 (0.0)	-	
A/G + G/G	57 (8.1)	72 (9.9)	0.80 (0.56-1.15)	0.230
APE1 Asp148Glu				
Asp/Asp	236 (33.0)	249 (34.3)	1.00 (ref)	
Asp/Glu	349 (48.8)	361 (49.7)	1.02 (0.81-1.29)	0.864
Glu/Glu	130 (18.2)	117 (16.1)	1.17 (0.86-1.60)	0.308
Asp/Glu + Glu/Glu	479 (67.0)	478 (65.8)	1.06 (0.85-1.32)	0.615
APE1 T-656G				
T/T	217 (30.1)	211 (28.9)	1.00 (ref)	
T/G	368 (51.0)	380 (52.0)	0.94 (0.74-1.20)	0.630
G/G	136 (18.9)	140 (19.2)	0.95 (0.70-1.28)	0.731
T/G + G/G	504 (69.9)	520 (71.1)	0.95 (0.75-1.18)	0.621

[1]ORs and 95%CIs were estimated from unconditional logistic regressions after controlling for age and sex.

risk of CRC in patients with stage Ⅲ + Ⅳ tumors (OR = 1.48; 95%CI: 1.03-2.13). Conversely, the *OGG1* 11657A/G A/G + G/G genotype was associated with a decreased risk of CRC in patients with stage 0 + Ⅰ + Ⅱ tumors (OR = 0.59; 95%CI: 0.35-0.98). However, the *OGG1* polymorphisms associated with the CRC risk were not significantly modified by sex, age at diagnosis, and tumor subsite.

Table 4 presents the results of a similar stratification analysis for *APE1* polymorphisms. Among females, compared with genotypes containing the Asp/Asp genotype, those containing the *APE1* 148Glu allele (Asp/Glu + Glu/Glu) were associated with an increased CRC risk (OR = 1.41, 95%CI: 1.02-1.96). However, this sex-specific CRC risk was not observed for the *APE1* T-656G polymorphism. In addition, no *APE1* polymorphism was significantly associated with the CRC risk among subgroups of age at diagnosis, tumor subsite, and tumor stage.

To assess the combined influence of these polymorphisms, we conducted haplotype analysis for *OGG1* Ser326 and 111657A/G as well as for *APE1* Asp148Glu and T-656G (Table 5). Linkage analysis between each pairwise combination revealed significantly weak linkage disequilibrium (all $P < 0.001$). The r^2 values for the Ser326 and 111657A/G pair and Asp148Glu and T-656G pair were 0.06 and 0.04, respectively. Compared with the most common Asp/T haplotype in females, the *APE1* Glu/G haplotype was significantly associated with the CRC risk (OR = 1.36; 95%CI: 1.03-1.78). No significant associations were observed for the *OGG1* haplotypes and CRC risk (data not shown).

DISCUSSION

We observed a significant association between the *OGG1* Ser326Cys polymorphism and CRC risk. The Ser/Cys + Cys/Cys genotypes were associated with an increased CRC risk. Furthermore, this harmful effect of the 326Cys allele was more marked in patients with late-stage (Ⅲ + Ⅳ) CRC. Conversely, carrying the *OGG1* 111657G allele was beneficial for patients with early stage (0 + Ⅰ + Ⅱ) CRC. In addition, the CRC risk was high in females carrying the *APE1* 148Glu allele (Asp/Glu + Glu/Glu) or the 148Glu/-656G haplotype. No significant associations were observed between the *APE1* T-656G polymorphism and CRC risk.

OGG1 is a crucial DNA-repair gene involved in the BER pathway that can recognize and excise several lesions from the damaged DNA[5]. Mutations that alter the amino acid sequence affect the association of *OGG1* with other proteins, resulting in different DNA-repair activities[10,13,24]. Obtulowicz *et al*[10] reported that the *OGG1* repair capacity reduces with an increases in the Cys allele. Hill *et al*[13] observed that the 326Cys allele excised 8-oxoG from a duplex DNA and cleaved abasic sites 2- to 6-fold lower rates than did the 326Ser allele. Therefore, patients with the *OGG1* 326Cys allele have a lower DNA-repair activity and might be at a higher risk of cancer.

Our results are consistent with those of previous studies that reported a positive association between the *OGG1* 326Cys allele and CRC risk[12,33]. Moreno *et al*[12] observed that the *OGG1* 326Cys allele was associated with an increased risk of CRC (OR = 2.3; 95%CI: 1.1-5.0). Kim *et al*[33] demonstrated that the OR for colon cancer with frequent meat intake increased from 1.72 to 4.31 in carriers of the Cys/Cys genotype, whereas it was not increased in those of the Ser/Ser or Ser/Cys genotype. However, no significant association has been reported between the Ser326Cys polymorphism and CRC risk[5,34].

Functional studies have suggested that the *APE1* 148Glu allele altered endonuclease and DNA-binding activity, reduced the ability to communicate with other BER proteins, and increased mitotic delay after exposure to ionizing radiation[35,36]. Studies have also reported that the *APE1* -656G allele influenced the transcriptional activity, thus resulting in lung cancer risk[18,37]. Although we did not observe any significant independent associations between other DNA-repair genes and CRC risk, the risk slightly increased for the *APE1* 148Glu allele and decreased for the *OGG1* 11657G and *APE1* -656G alleles. Our findings were not completely consistent with those of some previous reports. Kasahara *et al*[25] reported that the *APE1* 148Glu allele significantly increased the CRC risk (OR = 2.33; 95%CI: 1.21-4.48). Zhou *et al*[20] evaluated 37 case-control studies and observed that the cancer risk was significantly low in participants with the *APE1* -656G allele. However, Li *et al*[23] and Moreno *et al*[12] have reported that the *APE1* Asp148Glu polymorphism

Table 3 Odds ratio and 95%CI of two *OGG1* single nucleotide polymorphisms for colorectal cancer stratified by sex, age at diagnosis, and tumor site and stage

	OGG1 Ser326Cys				OGG1 11657A/G			
	Ser/Ser		Ser/Cys + Cys/Cys		A/A		A/G + G/G	
	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]
Gender								
Male	50/67	1.00 (ref)	350/340	1.38 (0.93-2.05)	364/358	1.00 (ref)	31/47	0.65 (0.40-1.05)
Female	43/58	1.00 (ref)	271/265	1.38 (0.90-2.12)	284/296	1.00 (ref)	26/25	1.09 (0.61-1.93)
Age at diagnosis (yr)								
≤ 60	44/54	1.00 (ref)	275/264	1.28 (0.83-1.97)	292/283	1.00 (ref)	23/32	0.70 (0.40-1.22)
> 60	49/71	1.00 (ref)	346/341	1.47 (0.99-2.17)	356/371	1.00 (ref)	34/40	0.89 (0.55-1.43)
Tumor site								
Colon	45/125	1.00 (ref)	299/605	1.39 (0.96-2.01)	316/654	1.00 (ref)	23/72	0.67 (0.41-1.09)
Rectum	48/125	1.00 (ref)	322/605	1.39 (0.97-1.99)	332/654	1.00 (ref)	34/72	0.93 (0.61-1.43)
Stage								
0, Ⅰ, Ⅱ	46/125	1.00 (ref)	283/605	1.28 (0.88-1.84)	307/654	1.00 (ref)	20/72	0.59 (0.35-0.98)[a]
Ⅲ, Ⅳ	47/125	1.00 (ref)	338/605	1.48 (1.03-2.13)[a]	341/654	1.00 (ref)	37/72	0.99 (0.65-1.51)

[1]Number of cases/number of controls; [2]Adjustment for age and sex (except in gender stratification, where only sex was adjusted for). The [a]$P < 0.05$ is the comparison between CRC patients in stage 0, Ⅰ, Ⅱ and controls in calculating OR for A/G + G/G genotype *vs* A/A genotype.

Table 4 Odds ratio and 95%CI of two *APE1* single nucleotide polymorphisms for colorectal cancer stratified by sex, age at diagnosis, and tumor site and stage

	APE1 Asp148Glu				APE1 T-656G			
	Asp/Asp		Asp/Glu + Glu/Glu		T/T		T/G + G/G	
	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]	No.[1]	OR (95%CI)[2]
Gender								
Male	137/122	1.00 (ref)	264/283	0.83 (0.62-1.12)	128/111	1.00 (ref)	277/296	0.81 (0.60-1.10)
Female	99/127	1.00 (ref)	215/195	1.41 (1.02-1.96)[a]	89/100	1.00 (ref)	227/224	1.14 (0.81-1.60)
Age at diagnosis (yr)								
≤ 60	105/110	1.00 (ref)	213/205	1.09 (0.78-1.51)	108/99	1.00 (ref)	215/219	0.90 (0.65-1.26)
> 60	131/139	1.00 (ref)	266/273	1.03 (0.77-1.38)	109/112	1.00 (ref)	289/301	0.99 (0.73-1.35)
Tumor site								
Colon	114/249	1.00 (ref)	231/478	1.06 (0.81-1.40)	105/211	1.00 (ref)	244/520	0.94 (0.71-1.25)
Rectum	122/249	1.00 (ref)	248/478	1.05 (0.81-1.38)	112/211	1.00 (ref)	260/520	0.95 (0.72-1.24)
Stage								
0, Ⅰ, Ⅱ	106/249	1.00 (ref)	226/478	1.11 (0.84-1.47)	107/211	1.00 (ref)	226/520	0.85 (0.64-1.12)
Ⅲ, Ⅳ	130/249	1.00 (ref)	253/478	1.02 (0.79-1.33)	110/211	1.00 (ref)	278/520	1.03 (0.79-1.36)

[1]Number of cases/number of controls; [2]Adjustment for age and sex (except in gender stratification, where only sex was adjusted for). The [a]$P < 0.05$ is the comparison between CRC cases and controls among female in calculating OR for Asp/Glu + Glu/Glu genotype *vs* Asp/Asp genotype.

was unassociated with CRC.

We observed that the *OGG1* Ser326Cys polymorphism was significantly associated with an increased risk of CRC in patients with stage Ⅲ + Ⅳ tumors, whereas the *OGG1* 11657A/G polymorphism was associated with a decreased risk of CRC in patients with stage 0 + Ⅰ + Ⅱ tumors. This finding suggests that the *OGG1* 11657A/G polymorphism contributed to tumor initiation and that the *OGG1* Ser326Cys polymorphism may play a role in tumor progression. The exact mechanism underlying specific *OGG1* polymorphisms involved in different tumor stages remains unclear. Further studies are required to delineate the association.

Similar to the findings of earlier studies, our investigation demonstrated no association between the *OGG1* polymorphisms and CRC risk stratified by sex and tumor sites. Pardini *et al*[38] and Kasahara *et al*[25] have shown that the *OGG1* Ser326Cys polymorphism was not significantly associated with colon or rectal cancer, respectively. Our study was the first to evaluate the sex-specific risk of CRC associated with the *OGG1* polymorphisms, but no significant association was observed. Moreno *et al*[12] observed that the increased CRC risk associated with the 326Cys allele was marked among younger participants, but our study failed to find such an association.

We observed an elevated CRC risk associated with the *APE1* 148Glu allele in females, which was inconsistent with results of previous studies. Pardini *et al*[38] reported that compared with the Asp/Asp genotype, the Asp/Glu + Glu/Glu genotype was unassociated with the CRC risk. By contrast, they observed that the Asp/Asp genotype was associated with an increased risk of colon cancer (OR = 1.50; 95%CI: 1.01-2.22). In a study of a Japanese population, the *APE1* Glu allele

Table 5 Odds ratio and 95%CI of *APE1* haplotypes for colorectal cancer stratified by sex, age at diagnosis, and tumor site and stage

	APE1 Asp148Glu/T-656G							
	Asp/T		Asp/G		Glu/T		Glu/G	
	Ca/Co (%)[1]	OR (95%CI)[2]	Ca/Co (%)[1]	OR (95%CI)[2]	Ca/Co (%)[1]	OR (95%CI)[2]	Ca/Co (%)[1]	OR (95%CI)[2]
All	41.1/41.5	1.00 (ref)	16.3/17.7	0.93 (0.75-1.15)	14.5/13.4	1.09 (0.87-1.37)	28.1/27.5	1.03 (0.86-1.24)
Gender								
Male	42.5/40.0	1.00 (ref)	16.0/16.8	0.87 (0.66-1.16)	14.5/14.6	0.91 (0.67-1.22)	27.1/29.7	0.84 (0.66-1.06)
Female	39.3/44.6	1.00 (ref)	16.7/18.9	1.00 (0.73-1.36)	14.5/11.8	1.39 (0.98-1.97)	29.5/24.7	1.36 (1.03-1.78)[a]
Age at diagnosis (yr)								
≤ 60	42.1/42.7	1.00 (ref)	17.1/17.2	1.01 (0.74-1.38)	15.7/14.8	1.08 (0.77-1.50)	25.0/25.3	1.00 (0.76-1.32)
> 60	40.3/40.5	1.00 (ref)	15.6/18.1	0.87 (0.65-1.15)	13.5/12.3	1.10 (0.81-1.50)	30.6/29.1	1.06 (0.84-1.34)
Tumor site								
Colon	40.4/41.5	1.00 (ref)	16.7/17.7	0.96 (0.74-1.25)	15.5/13.4	1.20 (0.91-1.58)	27.4/27.5	1.03 (0.82-1.28)
Rectum	41.7/41.5	1.00 (ref)	16.0/17.7	0.90 (0.69-1.16)	13.5/13.4	0.99 (0.75-1.31)	28.8/27.5	1.04 (0.84-1.29)
Stage								
0, Ⅰ, Ⅱ	41.1/41.5	1.00 (ref)	15.8/17.7	0.90 (0.69-1.17)	14.9/13.4	1.14 (0.86-1.51)	28.2/27.5	1.02 (0.82-1.28)
Ⅲ, Ⅳ	41.1/41.5	1.00 (ref)	16.7/17.7	0.95 (0.74-1.23)	14.1/13.4	1.06 (0.81-1.39)	28.1/27.5	1.04 (0.84-1.29)

[1]Percentage of cases/percentage of controls; [2]Adjustment for age and sex (except in gender stratification, where only sex was adjusted for). The [a]$P < 0.05$ is the comparison between CRC cases and controls among female in calculating OR for Glu/G haplotype *vs* Asp/T haplotype.

carriers exhibited a significantly increased risk of colon cancer (OR = 3.04; 95%CI: 1.38-671)[25]. Li *et al*[23] observed that the *APE1* Asp148Glu polymorphism was unassociated with CRC even when stratified by sex, age at diagnosis, and tumor site. A meta-analysis of 27 case-control studies showed that the *APE1* Glu allele was associated with an increased CRC risk (OR = 1.23; 95%CI: 1.05-1.43) but not in the Asian population (OR = 1.03; 95%CI: 0.92-1.16)[39]. Furthermore, we also observed that the *APE1* 148Glu/-656G haplotype was associated with a significantly increased risk of CRC in females, which is inconsistent with a previous report. Pan *et al*[28] observed that carriers of the *APE1* 148Asp/-656T haplotype were at a higher risk of lung cancer than those of the 148Glu/-656G haplotype. The exact mechanism underlying this sex-specific risk of CRC associated with the *APE1* Asp148Glu polymorphism or Asp148Glu/T-656G haplotype remain unclear.

Our study has potential limitations. First, we did not examine OGG1 or APE1 mRNA expression levels nor did we measure the 8-oxoG levels for analyzing their correlations. Second, the case-control study design has inherent limitations. The controls are hospital based, but we consider that the genotype of DNA-repair genes do not affect the possibility of a participant being selected. The small sample size is another limitation. The power to detect a significant CRC risk for the *OGG1* 326Cys carriers (OR = 1.38) is approximately 60%. Therefore, an additional larger study is necessary to clarify the causality.

In conclusion, we reported a significant association between *OGG1* and *APE1* polymorphisms and the CRC risk in the Taiwanese population, particularly in patients with *OGG1* Ser326Cys in stage Ⅲ + Ⅳ tumors, *OGG1* 11657A/G in stage 0 + Ⅰ + Ⅱ tumors, and *APE1* Asp148Glu in females. Our results suggest that genetic variants in the DNA-repair pathway genes modulate the risk of sporadic CRC in this population; however, additional studies are required for verifying the findings.

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COMMENTS

Background

Colorectal cancer (CRC) is the second most commonly diagnosed cancers and third leading cause of cancer-related deaths in Taiwan. Oxidative damages resulting from reactive oxygen species (ROS) generation participate in the development and occurrence of CRC. DNA-repair enzymes are critical in maintaining genomic stability and minimizing damage accumulation from ROS. The expression of the base excision repair (*BER*) gene family is activated by internal oxidative stress and DNA damage. The BER pathway involves 8-oxoguanine glycosylase 1 (OGG1) and apurinic-apyrimidinic endonuclease 1 (APE1).

Research frontiers

Molecular epidemiological studies have suggested that polymorphisms in DNA repair pathways affect the DNA repair capacity to renovate the damaged DNA and may predispose participants to CRC risk. Studies have shown that polymorphisms of *OGG1* (Ser326Cys, 11657A/G, and Arg154His) and *APE1* (Asp148Glu, and T-656G) were associated with enzyme activities. However, the results are controversial.

Innovations and breakthroughs

This research recruited 727 cases with newly diagnosed colorectal adenocarcinoma and 736 age- and sex-matched healthy controls from a medical center in Taiwan. The authors not only confirmed the association of *OGG1* and *APE1* polymorphisms with the CRC risk but also suggest that genetic variants in DNA-repair pathway genes modulate the risk of sporadic CRC in the Taiwanese population.

Applications

These findings revealed a significant association between *OGG1* and *APE1*

polymorphisms and the CRC risk in Taiwanese Patients, particularly in *OGG1* Ser326Cys in stage Ⅲ + Ⅳ cases, *OGG1* 11657A/G in stage 0 + Ⅰ + Ⅱ cases, and *APE1* Asp148Glu in females. These results might facilitate identifying Taiwanese patients at a high risk of CRC.

Terminology

Single nucleotide polymorphism (SNP), also known as simple nucleotide polymorphism, is a DNA sequence variation prevalent in populations, in which a single nucleotide differs between members of a biological species or paired chromosomes. SNP in some genes may affect mRNA or protein expression resulting in an increase or decrease in the risk of certain diseases.

Peer-review

This manuscript is well established to investigate the association between *BER* gene polymorphisms and CRC susceptibility. The language and logic is well expressed, the structure and methods of this research were reasonable and completed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Contrast-enhanced harmonic endoscopic ultrasonography for assessment of lymph node metastases in pancreatobiliary carcinoma

Takeshi Miyata, Masayuki Kitano, Shunsuke Omoto, Kumpei Kadosaka, Ken Kamata, Hajime Imai, Hiroki Sakamoto, Naoshi Nisida, Yogesh Harwani, Takamichi Murakami, Yoshifumi Takeyama, Yasutaka Chiba, Masatoshi Kudo

Takeshi Miyata, Masayuki Kitano, Shunsuke Omoto, Kumpei Kadosaka, Ken Kamata, Hajime Imai, Hiroki Sakamoto, Naoshi Nisida, Yogesh Harwani, Masatoshi Kudo, Department of Gastroenterology and Hepatology, Kinki University Faculty of Medicine, Osaka-sayama 589-8511, Japan

Takamichi Murakami, Department of Radiology, Kinki University Faculty of Medicine, Osaka-sayama 589-8511, Japan

Yoshifumi Takeyama, Department of Surgery, Kinki University Faculty of Medicine, Osaka-sayama 589-8511, Japan

Yasutaka Chiba, Division of Biostatistics, Clinical Research Center, Kinki University Faculty of Medicine, Osaka-sayama 589-8511, Japan

Author contributions: Miyata T wrote the manuscript; Miyata T, Kitano M, Imai H, Sakamoto H and Kudo M drafted the study conception and design; Miyata T, Kadosaka K, Harwani Y, Murakami T and Takeyama Y critically revised the article for important intellectual content; Kitano M and Sakamoto H performed the endosonography studies; Omoto S, Kamata K, Nisida N and Takeyama Y performed the pathological evaluations; Kadosaka K and Chiba Y provided statistical analysis; Imai H, Murakami T and Kudo M read images; and Harwani Y critically revised English grammar.

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Correspondence to: Masayuki Kitano, MD, PhD, Department of Gastroenterology and Hepatology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-sayama 589-8511, Japan. m-kitano@med.kindai.ac.jp

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Abstract

AIM: To assess the usefulness of contrast-enhanced harmonic endoscopic ultrasonography (CH-EUS) for lymph node metastasis in pancreatobiliary carcinoma.

METHODS: All patients suspected of pancreatobiliary carcinoma with visible lymph nodes after standard EUS between June, 2009 and January, 2012 were enrolled.

In the primary analysis, patients with successful EUS-fine needle aspiration (FNA) were included. The lymph nodes were assessed by several standard EUS variables (short and long axis lengths, shape, edge characteristic and echogenicity), color Doppler EUS variable [central intranodal blood vessel (CIV) presence] and CH-EUS variable (heterogeneous/homogeneous enhancement patterns). The diagnostic accuracy relative to EUS-FNA was calculated. In the second analysis, N-stage diagnostic accuracy of CH-EUS was compared with EUS-FNA in patients who underwent surgical resection.

RESULTS: One hundred and nine patients (143 lymph nodes) fulfilled the criteria. The short axis cut-off $\geqslant$ 13 mm predicted malignancy with a sensitivity and specificity of 72% and 85%, respectively. These values were 72% and 63% for the long axis cut-off $\geqslant$ 20 mm, 62% and 75% for the round shape variable, 81% and 30% for the sharp edge variable, 66% and 61% for the hypoechogenicity variable, 70% and 72% for the CIV-absent variable, and 83% and 91% for the heterogeneous CH-EUS-enhancement variable, respectively. CH-EUS was more accurate than standard and color Doppler EUS, except the short axis cut-off. Notably, three patients excluded because of EUS-FNA failure were correctly N-staged by CH-EUS.

CONCLUSION: CH-EUS complements standard and color Doppler EUS and EUS-FNA for assessment of lymph node metastases.

Key words: Contrast-enhanced harmonic endoscopic ultrasonography; Sensitivity and specificity; Lymph node; Pancreatobiliary carcinoma; Endoscopic ultrasonography-fine needle aspiration

Core tip: Diagnosis of malignant intra-abdominal lymph nodes is often challenging for endoscopists and radiologists. In the present study, the diagnostic accuracy for differentiating malignant from benign lymph nodes of standard endoscopic ultrasonography (EUS), color Doppler EUS, and contrast-enhanced harmonic (CH)-EUS relative to EUS-fine needle aspiration (FNA) was assessed. A secondary objective of the present study was to assess the N-stage diagnostic accuracy of CH-EUS and EUS-FNA in patients who underwent surgical resection. In conclusion, CH-EUS was more accurate than standard and color Doppler EUS, except the short axis cut-off. Notably, three patients excluded because of EUS-FNA failure were correctly N-staged by CH-EUS.

Miyata T, Kitano M, Omoto S, Kadosaka K, Kamata K, Imai H, Sakamoto H, Nisida N, Harwani Y, Murakami T, Takeyama Y, Chiba Y, Kudo M. Contrast-enhanced harmonic endoscopic ultrasonography for assessment of lymph node metastases in pancreatobiliary carcinoma. *World J Gastroenterol* 2016; 22(12): 3381-3391 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3381.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3381

INTRODUCTION

Accurate staging by using the tumor, node and metastasis (TNM) classification system is the most important variable for determining the optimal treatment of pancreatobiliary carcinomas. In particular, since the lymph node stage relates not only to the choice of treatment but also to the prognosis, it is essential that the techniques used for N-staging are reliable[1,2]. However, diagnosis of malignant intra-abdominal lymph nodes is often challenging for endoscopists and radiologists[3]. Several studies report that although endoscopic ultrasonography (EUS) (which has good spatial resolution) is useful for the differential diagnosis of malignant and benign lymph nodes, its diagnostic accuracy remains unsatisfactory[4-6]. By contrast, a cyto-pathological diagnosis *via* EUS-fine needle aspiration (FNA) is highly accurate. However, an accurate noninvasive evaluating method[7] is needed for cases in which a lymph node cannot be accessed for EUS-FNA or EUS-FNA does not obtain adequate material for analysis[8]. In addition, noninvasive methods could facilitate EUS-FNA by identifying the target lymph node for EUS-FNA, namely, the lymph node that is most suspicious of malignancy and whose sampling will shape treatment decisions. One such noninvasive evaluation method is vascular imaging. Although color Doppler imaging can evaluate the vasculature in lymph nodes, it has several limitations, including blooming, overpainting and motion artifacts. It is also difficult to evaluate perfusion by using color Doppler imaging. This problem was recently overcome by a revolution in US technology, namely, the invention of US contrast agents that, when combined with contrast harmonic imaging, make it possible to depict the microvasculature in real time[9]. Recently, EUS was equipped with this novel perfusion imaging technique, thus yielding contrast-enhanced harmonic EUS (CH-EUS)[10,11].

In the present study, the diagnostic accuracy for differentiating malignant from benign lymph nodes of standard EUS, color Doppler EUS, and CH-EUS relative to EUS-FNA was assessed. For this, all patients with standard EUS-detected pancreatobiliary carcinomas with apparently visible intra-abdominal lymph nodes who underwent all four procedures during the study period were recruited prospectively and followed up. The CH-EUS variable that was analyzed was the detection of the microvasculature in visible lymph node(s); this was expressed as heterogeneous/homogeneous enhancement. A secondary objective of the present study was to assess the N-stage diagnostic accuracy of CH-EUS and EUS-FNA in patients who

underwent surgical resection.

MATERIALS AND METHODS

Patients and study design

All consecutive patients who were suspected of having pancreatobiliary diseases due to CT, MRI, or transabdominal US results and who then underwent standard EUS between June, 2009 and January, 2012 in a tertiary care referral center in Japan were recruited prospectively (Figure 1). All patients also underwent color Doppler EUS, CH-EUS, and EUS-FNA immediately after the standard EUS procedure. The primary objective of this study was to compare the diagnostic accuracy of standard EUS, color Doppler EUS and CH-EUS in terms of the ability to differentiate malignant nodes from benign nodes. For this primary retrospective analysis, only the patients from whom adequate and accurate EUS-FNA samples were retrieved and who were followed up for at least 12 mo after the standard EUS were included. The patients where a diagnosis was obtained by specimen histology rather than EUS-FNA because of EUS-FNA failure (sample inadequacy or lymph node inaccessibility) were excluded from this analysis because it was sometimes difficult to ensure that the lymph nodes harvested from surgical specimens were the same as those that were identified by imaging.

The study also had a secondary aim, namely, to compare the accuracy of CH-EUS and EUS-FNA in terms of N-stage diagnosis in all of the patients in the original cohort who underwent surgical resection.

The study was approved by the Institutional Review Board of Kinki University Faculty of Medicine. All patients provided informed consent with regard to the procedures and participation in the study.

Equipment

An echoendoscope developed for CH-EUS (Olympus GF-UCT260; Olympus Medical Systems, Tokyo, Japan) was used. An ALOKA ProSound SSD α-10 (Aloka Co Ltd, Tokyo, Japan) was used for US imaging. For CH-EUS, the extended pure harmonic detection mode was used. This mode selectively depicts signals from the microbubbles by simultaneously filtering the harmonic component and synthesizing the phase-shift signals. The preset variables were established for EUS and CH-EUS previously[10,11]. The transmitting frequency and mechanical indices were set at 4.7 MHz and 0.3, respectively. The frame rate was set at 10-15 frame per second. The focus point was set at the distal portion of the target lymph node.

US contrast

Sonazoid (Daiichi-Sankyo, Tokyo, Japan; GE Healthcare, Milwaukee, Wis) was used as the US contrast agent. This second generation US contrast agent is composed of perfluorobutane microbubbles with a median diameter of 2-3 μm[12]. Sonazoid was reconstituted with 2 mL of sterile water for injection. A dose of 0.015 mL/kg body weight was used.

Standard EUS, color Doppler EUS and CH-EUS

During the EUS analyses, the patients were sedated by midazolam and propofol. Standard EUS, color Doppler EUS, and CH-EUS were performed by two endosonographers (Kitano M and Sakamoto H). One was responsible for the endoscopic manipulation and scanning and the other for operating the US image scanner. Both endosonographers (who were qualified by the Japan Gastroenterological Endoscopy Society) have had experience with CH-EUS for more than 10 years: both have performed more than 1000 CH-EUS procedures. Each examination was performed by using the same protocol. Thus, after a pancreatobiliary carcinoma was observed, the trans-gastric or trans-duodenal approach was used to search for intra-abdominal lymph node(s). If an apparently visible lymph node was detected, standard EUS was used to evaluate the size (*i.e.*, the short and long axis lengths), shape (round or oval), edge characteristics (sharp or fuzzy), and echogenicity (hypo or hyper) of the lymph node. Thereafter, the imaging modality was changed to color Doppler EUS, which was used to determine whether a central intranodal blood vessel (CIV) was present in the lymph node.

Subsequently, the specific mode for CH-EUS (extended pure harmonic detection mode) was selected and a bolus injection of Sonazoid was administered at a speed of 1 mL/s through a 22-gauge cannula that was placed in the antecubital vein. This was followed by a 10-mL saline solution flush to ensure that all contrast was administered into the circulation system. If there were multiple apparently visible lymph nodes, each was separately assessed by injecting US contrast agent, performing CH-EUS, and then conducting EUS-FNA. These multiple CH-EUS procedures were performed at intervals of at least 10 min, which was found to be sufficient for the US contrast from the preceding CH-EUS procedure to be washed out from all lymph nodes. All movie clips were stored on the hard disk of the scanner for offline analysis.

Image analyses

All standard EUS, color Doppler EUS and CH-EUS variables were measured independently in a blinded fashion by two readers (Kudo M and Imai H). Both have had experience with CH-EUS for more than 8 years: both have read the data of more than 500 CH-EUS procedures. The two readers evaluated the movie clips of the lymph nodes. They were told that the movie clips that they were evaluating were standard EUS/color Doppler/CH-EUS analyses of lymph nodes. However, they were blinded to all CT, MRI, transabdominal US, and standard EUS findings of the primary lesions.

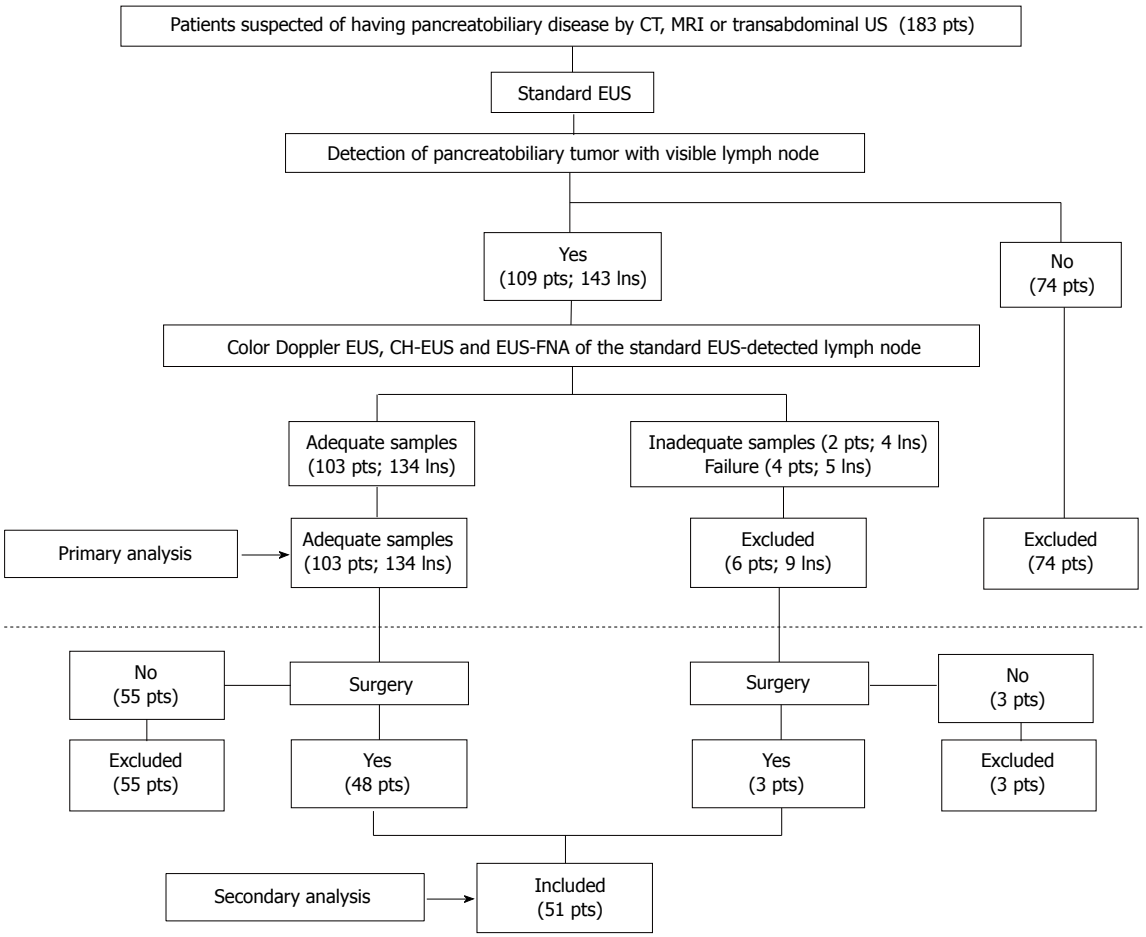

Figure 1 Schematic depiction of patient selection and exclusion criteria. pts: Patients; lns: Lymph nodes. CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; FNA: Fine needle aspiration.

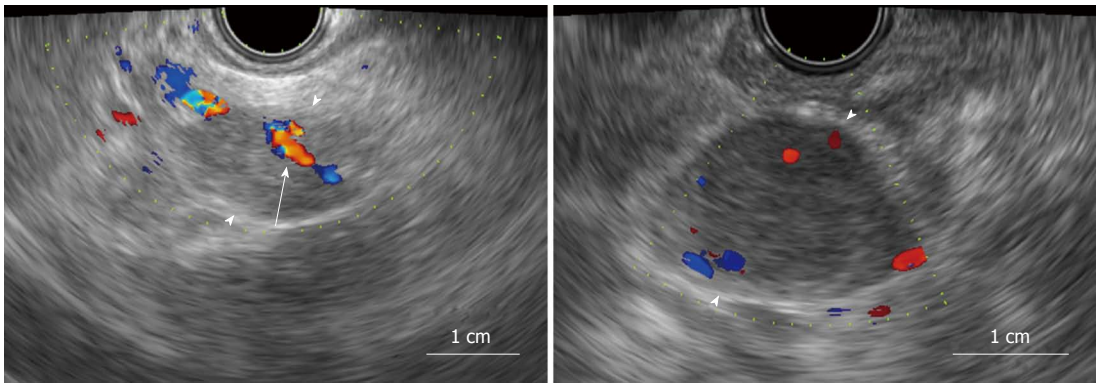

Figure 2 Typical examples of lymph nodes with (A) and without (B) a central intranodal blood vessel on color Doppler enhanced harmonic endoscopic ultrasonography. An apparently visible lymph node was detected in both A and B (arrowheads). A shows a tubular structure that was ⩾ 1 mm in diameter and was located toward the center of the lymph node and demonstrated blood flow on color Doppler enhanced harmonic endoscopic ultrasonography (arrow).

Receiver-operating characteristics (ROC) analysis was used to identify the standard EUS-detected short and long axis cut-off values that would optimize diagnosis of the lymph nodes. Based on a previous report[13], the readers predicted that the lymph nodes were malignant if they had a round shape and/or a sharp edge and/or exhibited hypoechogenicity on standard EUS. The color Doppler EUS images were assessed to determine whether CIV was present[4]. CIV was defined as a tubular structure with a well-defined smooth hyperechoic wall that was ⩾ 1 mm in diameter, located toward the center of the lymph node, and demonstrated blood flow on color Doppler EUS (Figure 2A). Based on a previous report[4], the readers predicted that the lymph nodes were malignant if a CIV was absent (Figure 2B). The CH-EUS images were assessed to determine the enhancement patterns, which were classified as being heterogeneous or

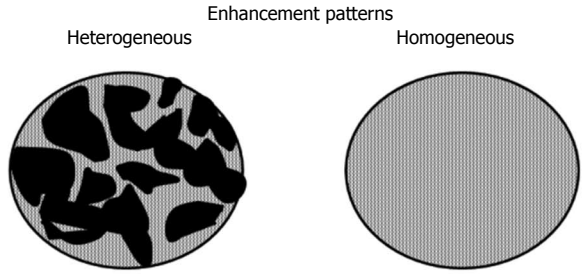

Figure 3 Contrast-enhanced harmonic enhanced harmonic endoscopic ultrasonography -determined enhancement patterns of the lymph node.

homogenous[14]. Based on a previous report[14], the readers predicted that the lymph nodes were malignant if heterogeneous enhancement was observed (Figures 3 and 4, Video 1).

Both of the blinded readers initially measured the standard EUS (shape, edge characteristics, and echogenicity), color Doppler EUS (CIV presence/absence), and CH-EUS (heterogeneous/homogenous enhancement pattern) variables separately. Interobserver agreement between the two readers in terms of these measurements was assessed by calculating the κ-coefficient (Supplementary Tables 1-1 to 1-5). Thereafter, if there were discrepant findings between the two readers, they reassessed the relevant image(s) together until an agreement was reached.

EUS-guided FNA

The final diagnosis was based on histological and/or cytological analysis of samples obtained by EUS-FNA. After standard EUS, color Doppler EUS, and CH-EUS of each lymph node, EUS-FNA was performed with a 22- or 25-gauge aspiration needle (Echo Tip Ultra, Cook, Winston-Salem, NC, United States). Punctures were repeated until a sample was obtained; the maximum number of passes was five. A cytopathologist was present in the endoscopy room for on-site sample evaluation. After it was confirmed that adequate numbers of cells had been obtained, the samples were processed and evaluated in the pathology department by using Papanicolaou staining for cytology and hematoxylin-eosin staining for histology. If there were multiple apparently visible lymph nodes, EUS-FNA was performed separately on each lymph node: after each aspiration, the needles were changed.

Histology of resected lymph nodes

The lymph nodes that were surgically resected after imaging were also assessed by the pathology department for malignancy. For this, 51 patients were included (Figure 1).

Study design

The primary objective was to compare the diagnostic accuracy of standard EUS, color Doppler EUS and CH-EUS in terms of the primary end-point, which was the ability to differentiate malignant nodes from benign nodes. The secondary end-point was to compare the accuracy of CH-EUS and EUS-FNA in terms of N-stage diagnosis in patients who underwent surgical resection.

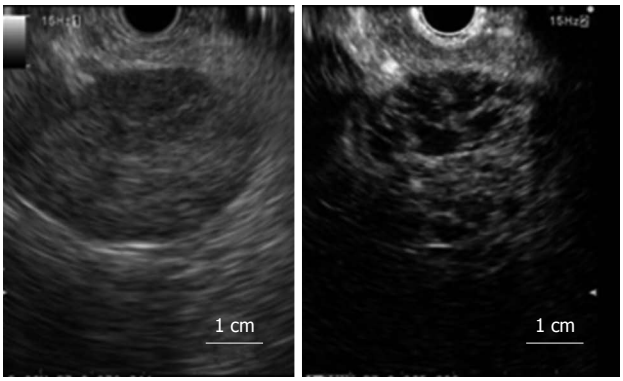

Figure 4 Typical example of a metastatic lymph node that shows heterogeneous enhancement on contrast-enhanced harmonic enhanced harmonic endoscopic ultrasonography (left, fundamental B mode; right, contrast harmonic mode).

Statistical analyses

All data were analyzed by using SAS software version 8.2 (SAS Institute, Cary, NC, United States). Differences between the EUS methods in terms of malignant lymph node detection were assessed by using McNemar's test. A difference with $P < 0.01$ was regarded as significant. This approach was also used to test differences between benign and malignant lymph nodes in terms of CH-EUS enhancement patterns. McNemar's test was also used to compare CH-EUS and EUS-FNA in terms of their N-stage diagnostic accuracy in the patients who underwent surgical resection.

Interobserver agreement in terms of the EUS variables described above was also assessed. A κ coefficient of > 0.8 was considered to indicate excellent agreement, > 0.6 was considered to indicate good agreement, and > 0.4 was considered to indicate moderate agreement. The sensitivity, specificity and accuracy with which CH-EUS differentiated malignant from benign lymph nodes were calculated and compared to the values of standard and color Doppler EUS findings (short axis, long axis, shape, edge characteristics, echogenicity and CIV). The numbers of cases of discordance are shown in Supplementary Tables 2-1 to 2-6.

RESULTS

Patient recruitment

During the study period, 183 patients suspected of pancreatobiliary disease underwent EUS and were enrolled prospectively. In 109 patients, EUS detected a pancreatobiliary carcinoma and one or more apparently visible intra-abdominal lymph nodes. The total number of detected lymph nodes was 143. The remaining 74 patients were excluded from

Table 1 Characteristics of the patients in the primary analysis

Sex (M:F)	68:35	
Median age	65 (35-82)	
Median size (long axis × short axis) (mm)	18 (8-60) × 9 (4-42)	
Final diagnosis (*n*)	Pancreatic carcinoma	67
	Bile duct carcinoma	21
	Gallbladder carcinoma	11
	Ampullary carcinoma	4

analysis because a pancreatobiliary carcinoma and/or apparently visible intra-abdominal lymph nodes were not detected. All 109 patients with apparently visible intra-abdominal lymph node(s) in standard EUS immediately underwent color Doppler EUS, CH-EUS, and EUS-FNA. In six patients (nine lymph nodes; 6.3% of the 143 apparently visible lymph nodes detected by standard EUS), the EUS-FNA samples of lymph nodes were inadequate (4 lymph nodes from 2 patients) and failed because the lymph node was in an inaccessible location (5 lymph nodes from 4 patients) (Figure 1). These patients were excluded from the primary analysis cohort. Nevertheless, among these 6 patients, 3 patients underwent surgical resection, and were included in the secondary analysis cohort (Figure 1). The remaining 103 patients (134 lymph nodes) were included in the primary analysis cohort (Figure 1).

Diagnostic accuracy of standard EUS, color Doppler EUS and CH-EUS in lymph nodes with histological diagnosis obtained by EUS-FNA (primary analysis)

Table 1 displays the characteristics of these 103 patients for the primary analysis cohort. The male: female ratio was 68:35 and the median age was 65 (range: 35-82) years. The median long and short axis lengths of the 134 lymph nodes were 18 (range: 8-60) and 9 (range: 4-42) mm, respectively. The final diagnoses were pancreatic carcinoma (n = 67), bile duct carcinoma (n = 21), gallbladder carcinoma (n = 11), and ampullary carcinoma (n = 4). Standard EUS, color Doppler EUS and CH-EUS were successfully performed in all patients and associated adverse effects were not observed. Of the 134 lymph nodes, histological and/or cytological analyses of the samples obtained by EUS-FNA revealed that 47 were malignant lymph nodes and 87 were reactive lymph nodes. Adverse effects of EUS-FNA were also not observed. All 103 patients were followed up for at least 12 mo. None of the patients who were deemed to have benign lymph nodes after EUS-FNA and the other tests, and who did not undergo surgical resection of the nodes, exhibited any signs of lymph node malignancy during follow-up, as indicated by twice yearly standard EUS.

Standard EUS

ROC analyses revealed that a short axis of 13 mm or longer and a long axis of 20 mm or longer predicted malignancy with the best sensitivity and specificity (Supplementary Figures 1 and 2). A short axis of 13 mm or longer predicted malignancy with a sensitivity, specificity and accuracy of 72% [95% confidence intervals (CI): 62%-81%)], 85% (95%CI: 79%-90%), and 81% (95%CI: 73%-86%), respectively (Table 2). A long axis of 20 mm or longer predicted malignancy with a sensitivity, specificity and accuracy of 72% (95%CI: 61%-82%), 63% (95%CI: 57%-68%), and 66% (95%CI: 59%-73%), respectively (Table 2). A round shape predicted malignancy with a sensitivity, specificity and accuracy of 62% (95%CI: 51%-72%), 75% (95%CI: 69%-80%), and 70% (95%CI: 62%-77%), respectively (Table 2). A sharp edge predicted malignancy with a sensitivity, specificity and accuracy of 81% (95%CI: 71-89%), 30% (95%CI: 24-34%), and 48% (95%CI: 41-53%), respectively (Table 2). Hypoechogenicity predicted malignancy with a sensitivity, specificity and accuracy of 66% (95%CI: 55%-76%), 61% (95%CI: 55%-66%), and 63% (95%CI: 55%-70%), respectively (Table 2). Interobserver agreement testing revealed good (κ coefficient: 0.63, $P < 0.01$), moderate (κ coefficient: 0.49, $P < 0.01$), and moderate (κ coefficient: 0.47, $P < 0.01$) agreement between the two readers in terms of the shape, edge characteristics, and echogenicity measurements, respectively (Supplementary Tables 2-1 to 2-3).

Color Doppler EUS

The absence of a CIV predicted malignancy with a sensitivity, specificity and accuracy of 70% (95%CI: 59%-80%), 72% (95%CI: 66%-78%), and 72% (95%CI: 64%-78%), respectively (Table 2). Interobserver agreement testing revealed good reproducibility between the two readers in terms of this measurement (κ coefficient: 0.69, $P < 0.01$) (Supplementary Table 2-4).

Contrast-enhanced harmonic EUS

All 134 lymph nodes yielded high-quality dynamic images on CH-EUS. Interobserver agreement testing revealed excellent reproducibility between the two readers in terms of detecting heterogeneous/homogeneous enhancement patterns (κ coefficient: 0.81, $P < 0.01$) (Supplementary Table 2-5).

Table 3 lists the number and frequency of lesions in the benign and malignant lymph node groups that had a heterogeneous or homogeneous enhancement pattern after reassessment of discrepant findings by the two blinded readers. Of the 47 malignant lymph nodes, 39 (83%) exhibited heterogeneous enhancement in which the distorted tumor vessels could be clearly visualized (Figures 3 and 4, Video 1). Of the 87 benign lymph nodes, 79 (91%) exhibited homogeneous enhancement (Figures 3 and 5, Video 2). The benign and malignant lymph node groups differed significantly in terms of the frequencies of homogeneous and heterogeneous enhancement ($P < 0.01$). When heterogeneous enhancement was deemed to indicate malignancy and homogeneous

Table 2 Sensitivity, specificity, and accuracy with which CH-enhanced harmonic endoscopic ultrasonography, color Doppler enhanced harmonic endoscopic ultrasonography, and the standard enhanced harmonic endoscopic ultrasonography variables differentiate malignant from benign lymph nodes

	Sensitivity (95%CI)	Specificity (95%CI)	Accuracy (95%CI)	*P* value[1]
Short axis 13 mm or longer	72% (34/47) (62-81)	85% (74/87) (79-90)	81% (108/134) (73-86)	0.27
Long axis 20 mm or longer	72% (34/47) (61-82)	63% (55/87) (57-68)	66% (89/134) (59-73)	0.001
Round shape	62% (29/47) (51-72)	75% (65/87) (69-80)	70% (94/134) (62-77)	0.008
Sharp edge	81% (38/47) (71-89)	30% (26/87) (24-34)	48% (64/134) (41-53)	< 0.001
Hypoechogenicity	66% (31/47) (55-76)	61% (53/87) (55-66)	63% (84/134) (55-70)	< 0.001
CIV absent	70% (33/47) (59-80)	72% (63/87) (66-78)	72% (96/134) (64-78)	0.009
Heterogeneous (CH-EUS)	83% (39/47) (77-89)	91% (79/87) (86-94)	88% (118/134) (82-93)	

[1]Compared with contrast-enhanced harmonic endoscopic ultrasonography (CH-EUS), as determined by McNemar's test. CIV: Central intranodal blood vessel.

Table 3 Number of lymph nodes in the benign and malignant groups that exhibited heterogeneous and homogeneous enhancement on contrast-enhanced harmonic endoscopic ultrasonography

	Number with each enhancement pattern		
Final diagnosis	Heterogeneous	Homogeneous	Total
Malignancy	39	8	47
Benign	8	79	87
Total	47	87	134

enhancement was deemed to indicate benignity, CH-EUS differentiated malignant from benign lymph nodes with a sensitivity, specificity and accuracy of 83% (95%CI: 77%-89%), 91% (95%CI: 86%-94%), and 88% (95%CI: 82%-93%), respectively (Table 2).

Comparison of EUS imaging methods

CH-EUS diagnosed malignant lymph nodes with a significantly higher diagnostic accuracy than most of the standard EUS variables ($P = 0.001$ *vs* the 20-mm long axis cut-off, $P = 0.008$ *vs* the round shape variable, $P < 0.001$ *vs* the sharp edge variable, and $P < 0.001$ *vs* the hypoechogenicity variable, as determined by McNemar tests) or the color Doppler EUS CIV variable ($P = 0.009$). However, CH-EUS did not differ significantly from the 13-mm short axis cut-off variable in terms of differentiating malignant from benign lymph nodes ($P = 0.27$) (Table 2).

Comparison of CH-EUS and EUS-FNA for N-staging in the surgically resected patients (secondary analysis)

Of the 109 patients in whom EUS detected a pancreato-biliary carcinoma and one or more apparently visible intra-abdominal lymph nodes, 48 patients underwent surgical resection and histological examinations of the resected lymph nodes (Figure 1). In addition, three patients whose EUS-FNA samples of lymph nodes were inadequate or failed EUS-FNA because the lymph node was in an inaccessible location underwent surgical resection, and were included in the secondary analysis cohort (Figure 1, Table 4). Thus, the secondary analysis cohort consisted of 51 patients. Comparison of the EUS-FNA and CH-EUS findings relative to surgical specimen histology revealed that six (including failed due to inadequate sampling and inaccessibility) and five of the 51 patients were misdiagnosed by EUS-FNA and CH-EUS, respectively. Thus, EUS-FNA and CH-EUS diagnosed the N-stage in the patients who underwent surgical resection with an accuracy of 88% and 90%, respectively ($P = 0.50$).

It should be noted that three patients in the secondary analysis cohort were not included in the primary analysis cohort because EUS-FNA failed due to inadequate sampling or inaccessibility of the lymph nodes. All three patients were correctly N-staged by CH-EUS. One of these three patients was shown by standard and color Doppler EUS to have a long axis of 22 mm, a sharp edge, and to lack a CIV: all of these features predicted that the lymph node was

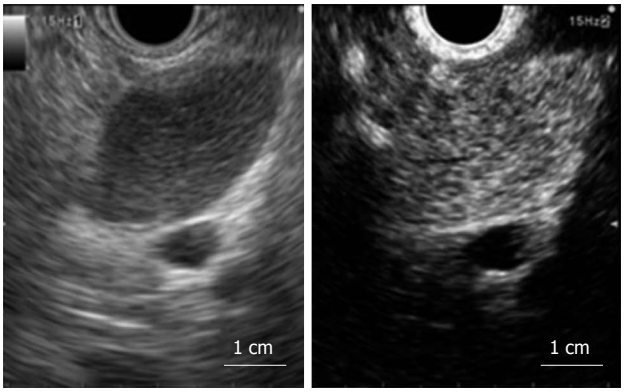

Figure 5 Typical example of a reactive lymph node that shows homogeneous enhancement on contrast-enhanced harmonic enhanced harmonic endoscopic ultrasonography (left, fundamental B mode; right, contrast harmonic mode).

Table 4 Characteristics of the patients in the secondary analysis

Sex (M:F)	32:19	
Median age	66 (37-79)	
Median size (long axis × short axis) (mm)	20 (8-60) × 10 (4-42)	
Final diagnosis (*n*)	Pancreatic carcinoma	29
	Bile duct carcinoma	12
	Gallbladder carcinoma	7
	Ampullary carcinoma	3

malignant. By contrast, CH-EUS revealed that this lymph node had homogeneous enhancement, which was deemed to indicate a benign lymph node. The patient underwent surgery and indeed, histological examination of the lymph node resected during surgery revealed that it was benign. With regard to the remaining two patients, between two and four of the six standard EUS and color Doppler EUS variables predicted that they were benign. By contrast, CH-EUS revealed that it had heterogeneous enhancement, which was deemed to indicate a malignant lymph node. Indeed, histological examination of the lymph nodes resected during surgery revealed that those lymph nodes were malignant.

DISCUSSION

A study by Gill *et al*[13] identified several morphological characteristics that can be detected by standard EUS that can help to distinguish between malignant and benign lymph nodes. Multivariable analysis revealed that in particular, a round shape, a sharp edge, and a short axis that exceeded 8.3 mm associated significantly with malignant cytology. However, the predictive accuracy of these features was limited. The present study also assessed the ability of a round shape, a sharp edge, hypoechogenicity, a $\geqslant$ 13 mm short axis length, and a $\geqslant$ 20 mm long axis length to distinguish between benign and malignant lymph nodes. However, like Gill *et al*[13], we found that the diagnostic accuracy of these features was limited.

An alternative method is color Doppler EUS. Sawhney *et al*[4] reported that the absence of CIV on color Doppler EUS is a strong and independent predictor of metastatic lymph node. In our study, however, the absence of CIV on color Doppler EUS did not predict malignancy better than the standard EUS variables. This may reflect differences between our study and theirs in terms of the way the lymph nodes were selected: Sawhney *et al*[4] only included lymph nodes that were 10 mm or longer, whereas in the present study, smaller lymph nodes were included (64 had a short axis diameter of less than 10 mm). This difference may relate to the fact that we evaluated all apparently visible lymph nodes found during the standard EUS procedure by color Doppler EUS. Therefore, the lymph nodes evaluated in the current study were relatively smaller than those examined by Sawhney *et al*[4]. Since some small benign lymph nodes may not exhibit CIV, this may have resulted in the relatively lower specificity associated with this variable in our study.

Another alternative method is contrast-enhanced color Doppler EUS with US contrast agent. Kanamori *et al*[15] reported that defective enhancement on contrast-enhanced color Doppler EUS using the first generation US contrast agent Levovist, (Nihon Schering Co., Ltd., Tokyo, Japan) predicted lymph node malignancy significantly more accurately than standard EUS variables. Hocke *et al*[8] also reported that that an irregular appearance of the vessels (or the presence of arterial vessels only) on contrast-enhanced Doppler EUS using the second generation US contrast agent SonoVue (BR1, Bracco, Italy) predicted lymph node malignancy significantly better than standard EUS variables. However, as with the study by Sawhney *et al*[4], the lymph nodes examined in these studies were relatively larger than those in our study.

Recently, the combination of the second generation US contrast agent Sonazoid and low mechanical index imaging techniques has led to CH-EUS being used for perfusion imaging, which facilitates the depiction of tumor vascularity[10,16-18]. Sonazoid resonates with a low acoustic power and thus allows us to perform CH-EUS. We showed previously that this method has an excellent ability to differentiate malignant from benign lesions without Doppler-related artifacts, even when the lesions are small[19]. Heterogeneous enhancement was observed in 39 of 47 (83%) malignant lymph nodes. This is consistent with the observation of a pathology-based study[20] that showed that the vascular architecture of malignant lymph nodes is characterized by caliber fluctuations, an irregular coarse, sinusoid formation, and arteriovenous shunts. In the current study, interobserver agreement regarding CH-EUS results revealed excellent reproducibility between the two readers (κ coefficient: 0.81). Another report also showed that CH-EUS yielded highly reproducible findings with regard to malignant lymph nodes[21]. Indeed, its reproducibility was higher than that of MDCT[22] and all of the standard EUS findings that were measured in the present study. However, it should be noted that in the current study, the readers were experts who had practiced CH-EUS for more than 8 years; each had read the data of more than 500 CH-EUS procedures. It is possible that the reproducibility of CH-EUS findings among beginners may be low, although Gincul *et al*[21] did not detect significant differences between experts and beginners. Fusaroli *et al*[23] also reported that among three parameters (uptake, pattern, and washout) of CH-EUS for solid pancreatic lesion, pancreatic cystic lesion, and submucosal lesion, the reproducibility between experienced and non-experienced endosonographers did not differ significantly. This issue must be validated in future series.

Another alternative method is EUS elastography.

EUS elastography has been presented as a novel technique to assess tissue elasticity and has been used to differentiate between malignant and benign lymph nodes. Several different variables have been used in EUS elastography as a measure of tissue elasticity, namely, color patterns[24-29], strain ratio[30,31], hue histogram analysis[32,33] and artificial neural networks[34,35]. Wei *et al*[36] report a meta-analysis that included seven articles and a large number of lymph nodes (368 patients with 431 lymph nodes). The sensitivity and specificity of EUS elastography for the differential diagnosis of benign and malignant lymph nodes were 88%, and 85%, respectively. The area under the summary receiver operating characteristic curve was 0.9456. However, the sensitivity and specificity of this method varied greatly between studies[36]. Thus, CH-EUS should be compared to EUS elastography in terms of its ability to differentiate malignant from benign lymph nodes in further studies. In addition, it may be useful to evaluate whether these imaging methods could complement each other or other methods.

EUS-FNA is also useful for differentiating malignant from benign lymph nodes. Since EUS-FNA is highly specific in terms of identifying malignant lymph nodes, most cases where EUS-FNA reveals the presence of atypical cells in the lymph nodes have a final diagnosis of malignant lymph node[37]. However, false-positive and false-negative EUS-FNA results remain possible. Jason *et al*[38] report that in their series, the EUS-FNA false-positive and false-negative rates of intra-abdominal lymph node diagnosis were 0.7% and 5.8%, respectively. The present study suffers a limitation in relation to this: we cannot be certain that the EUS-FNA findings of the lymph nodes analyzed in the primary analysis were correct. For this reason, only patients who were followed up for at least 12 mo were included in the primary analysis. None of the patients with apparently benign lymph nodes exhibited signs of lymph node malignancy during this follow-up period.

Another limitation of EUS-FNA is that it cannot be performed in all cases because of intervening vessels and/or the difficult location of the lymph node, which could, for example, lead to an excessively large scope angle or distance from the probe. These problems suggest that CH-EUS technology may complement EUS-FNA-based histological and/or cytological diagnoses. This notion is supported by the four studies that have compared CH-EUS and EUS-FNA previously. All were for pancreatic masses. Napoleon *et al*[39] report that of five adenocarcinomas that had false-negative EUS-FNA results, CH-EUS revealed hypo-enhancement in four. Gincul *et al*[21] also report that all five false-negative EUS-FNA cases were correctly classified by CH-EUS. Moreover, Kitano *et al*[19] report that when CH-EUS was combined with EUS-FNA, the sensitivity of EUS-FNA increased from 92.2% to 100%. Fusaori *et al*[40] also report that CH-EUS increased the detection of malignant pancreatic lesions in difficult cases (patients with chronic pancreatitis or biliary stents) and helped guide EUS-FNA. The present study showed that at least in the patients who underwent surgical resection, CH-EUS and EUS-FNA did not differ in terms of N-staging diagnostic accuracy. However, CH-EUS correctly N-staged three patients in which EUS-FNA sampling failed because of lymph node location or were inadequate. This is the first report to indicate that CH-EUS complements EUS-FNA in terms of N-staging in patients with pancreatobiliary neoplasms.

The present study had some limitations. Multiple lymph nodes in one patient were included in the primary analysis because it was unclear which of these lymph nodes should be sampled; thus, all apparently visible lymph nodes were sampled. This could have introduced a bias in terms of lymph node selection. In addition, EUS-FNA was the gold standard in the primary analysis, even though the accuracy of EUS-FNA may be limited, as discussed above. Histology of resected specimens yields the most accurate diagnosis. However, it is difficult to identify during surgery which lymph nodes were previously evaluated by standard EUS, color Doppler EUS, or CH-EUS. For this reason, EUS-FNA served as the gold standard in the primary analysis.

In conclusion, CH-EUS depicted the microvasculature of intra-abdominal lymph node very clearly. Thus, it may be a useful modality for differentiating malignant from benign lymph nodes in patients with pancreato-biliary carcinomas and may complement standard EUS, color Doppler EUS and EUS-FNA, all of which have limitations. In addition, it may be helpful for determining the lymph nodes that should be subjected to EUS-FNA. In view of the high accuracy described in this study, in the future, CH-EUS may help to detect the in-operable stage better and thereby helps to avoid unnecessary surgery. Hence, CH-EUS will play an important role in determining the optimal treatment of pancreatobiliary carcinomas. However, given that the sample size of this study was relatively small and all CH-EUS procedures were performed in a single medical unit, an additional study that confirms the value of CH-EUS for differentiating malignant from benign lymph nodes is warranted.

COMMENTS

Background

Accurate staging by using the tumor, node and metastasis (TNM) classification system is the most important variable for determining the optimal treatment of pancreatobiliary carcinomas. In particular, since the lymph node stage relates not only to the choice of treatment but also to the prognosis, it is essential that the techniques used for N-staging are reliable.

Research frontiers

A cyto-pathological diagnosis *via* endoscopic ultrasonography (EUS)-fine needle aspiration (FNA) is highly accurate. Noninvasive methods could facilitate EUS-FNA by identifying the target lymph node for EUS-FNA, namely, the lymph node that is most suspicious of malignancy and whose sampling will shape

treatment decisions. Standard EUS can help to distinguish between malignant and benign lymph nodes, although the predictive accuracy of these features was limited. US contrast agents that, when combined with contrast harmonic imaging, make it possible to depict the microvasculature in real time. Recently, EUS was equipped with this novel perfusion imaging technique, thus yielding contrast-enhanced harmonic EUS (CH-EUS).

Innovations and breakthroughs

This is the first study to evaluate the diagnostic accuracy of CH-EUS for differentiating malignant from benign lymph node, compared with standard and color Doppler EUS. CH-EUS was more accurate than standard and color Doppler EUS. Notably, three patients with EUS-FNA failure were correctly N-staged by CH-EUS.

Applications

The results of this study suggest that it may be a useful modality for differentiating malignant from benign lymph nodes in patients with pancreatobiliary carcinomas and may complement EUS-FNA. In addition, it may be helpful for determining the lymph nodes that should be subjected to EUS-FNA. Application of CH-EUS to staging will help patients avoid unnecessary surgery.

Terminology

Color Doppler imaging has several limitations, including blooming, overpainting and motion artifacts. It is also difficult to evaluate perfusion by using color Doppler imaging. This problem was recently overcome by a revolution in US technology, namely, contrast harmonic imaging which makes it possible to depict the microvasculature in real time. Recently, EUS was equipped with this novel perfusion imaging technique, thus yielding CH-EUS.

Peer-review

The authors demonstrated the clinical utility of CH-EUS as a diagnostic tool for detecting lymph node metastasis in patients with pancreatobiliary carcinoma. This paper is informative and interesting for the further developments of imaging approaches.

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ORIGINAL ARTICLE

Retrospective Study

Donor preoperative oxygen delivery and post-extubation hypoxia impact donation after circulatory death hypoxic cholangiopathy

Thomas J Chirichella, C Michael Dunham, Michael A Zimmerman, Elise M Phelan, M Susan Mandell, Kendra D Conzen, Stephen E Kelley, Trevor L Nydam, Thomas E Bak, Igal Kam, Michael E Wachs

Thomas J Chirichella, Elise M Phelan, Kendra D Conzen, Trevor L Nydam, Thomas E Bak, Igal Kam, Department of Surgery, Division of Transplant Surgery, University of Colorado, Aurora, CO 80045, United States

C Michael Dunham, Trauma/Critical Care Services, St. Elizabeth Health Center, Youngstown, OH 44501, United States

Michael A Zimmerman, Department of Transplant Surgery, Medical College of Wisconsin, Milwaukee, WI 53226, United States

M Susan Mandell, Department of Anesthesiology, University of Colorado, Aurora, CO 80045, United States

Stephen E Kelley, Donor Alliance Organ Procurement Organization, Denver, CO 80045, United States

Michael E Wachs, Department of Transplant Surgery, Children's Hospital Colorado, Aurora, CO 80045, United States

Author contributions: Chirichella TJ, Dunham CM, Zimmerman MA, Kam I and Wachs ME conceived and designed the study; Chirichella TJ, Dunham CM, Zimmerman MA, Phelan EM, Mandell MS, Kelley SE, Nydam TL, Bak TE, Kam I and Wachs ME performed the research; Chirichella TJ and Phelan EM acquired the data; Chirichella TJ, Dunham CM, Zimmerman MA, Phelan EM, Mandell MS, Conzen KD, Kelley SE, Nydam TL, Bak TE, Kam I and Wachs ME analysed and interpreted the data; Chirichella TJ, Dunham CM, Mandell MS and Wachs ME drafted the article or revised it critically for important intellectual content.

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Data sharing statement: Technical appendix and dataset are available from the corresponding author at tchirichella@gmail.com, after a signed data user agreement is obtained. No additional data are available.

Correspondence to: Thomas J Chirichella, MD, Transplantation Fellow, Division of Transplant Surgery, 1635 Aurora Ct., 7th Floor, University of Colorado Hospital, Aurora, CO 80045, United States. tchirichella@gmail.com
Telephone: +1-720-8480878
Fax: +1-720-8480841

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Abstract

AIM: To evaluate donation after circulatory death (DCD) orthotopic liver transplant outcomes [hypoxic cholangiopathy (HC) and patient/graft survival] and donor risk-conditions.

METHODS: From 2003-2013, 45 DCD donor transplants were performed. Predonation physiologic data from UNOS DonorNet included preoperative systolic and diastolic blood pressure, heart rate, pH, SpO_2, PaO_2, FiO_2, and hemoglobin. Mean arterial blood

pressure was computed from the systolic and diastolic blood pressures. Donor preoperative arterial O_2 content was computed as [hemoglobin (gm/dL) × 1.37 (mL O_2/gm) × SpO_2%) + (0.003 × PaO_2)]. The amount of preoperative donor red blood cell transfusions given and vasopressor use during the intensive care unit stay were documented. Donors who were transfused ⩾ 1 unit of red-cells or received ⩾ 2 vasopressors in the preoperative period were categorized as the red-cell/multi-pressor group. Following withdrawal of life support, donor ischemia time was computed as the number-of-minutes from onset of diastolic blood pressure < 60 mmHg until aortic cross clamping. Donor hypoxemia time was the number-of-minutes from onset of pulse oximetry < 80% until clamping. Donor hypoxia score was (ischemia time + hypoxemia time) ÷ donor preoperative hemoglobin.

RESULTS: The 1, 3, and 5 year graft and patient survival rates were 83%, 77%, 60%; and 92%, 84%, and 72%, respectively. HC occurred in 49% with 16% requiring retransplant. HC occurred in donors with increased age (33.0 ± 10.6 years *vs* 25.6 ± 8.4 years, P = 0.014), less preoperative multiple vasopressors or red-cell transfusion (9.5% *vs* 54.6%, P = 0.002), lower preoperative hemoglobin (10.7 ± 2.2 gm/dL *vs* 12.3 ± 2.1 gm/dL, P = 0.017), lower preoperative arterial oxygen content (14.8 ± 2.8 mL O_2/100 mL blood *vs* 16.8 ± 3.3 mL O_2/100 mL blood, P = 0.049), greater hypoxia score >2.0 (69.6% *vs* 25.0%, P = 0.006), and increased preoperative mean arterial pressure (92.7 ± 16.2 mmHg *vs* 83.8 ± 18.5 mmHg, P = 0.10). HC was independently associated with age, multi-pressor/red-cell transfusion status, arterial oxygen content, hypoxia score, and mean arterial pressure (r^2 = 0.6197). The transplantation rate was greater for the later period with more liberal donor selection [era 2 (7.1/year)], compared to our early experience [era 1 (2.5/year)]. HC occurred in 63.0% during era 2 and in 29.4% during era 1 (P = 0.03). Era 2 donors had longer times for extubation-to-asystole (14.4 ± 4.7 m *vs* 9.3 ± 4.5 m, P = 0.001), ischemia (13.9 ± 5.9 m *vs* 9.7 ± 5.6 m, P = 0.03), and hypoxemia (16.0 ± 5.1 m *vs* 11.1 ± 6.7 m, P = 0.013) and a higher hypoxia score > 2.0 rate (73.1% *vs* 28.6%, P = 0.006).

CONCLUSION: Easily measured donor indices, including a hypoxia score, provide an objective measure of DCD liver transplantation risk for recipient HC. Donor selection criteria influence HC rates.

Key words: Orthotopic liver transplantation; Ischemic cholangiopathy; Hypoxic cholangiopathy; Donation after circulatory death; Biliary complications; Reperfusion injury

Core tip: Cholangiopathy is a common and devastating clinical complication developing in recipients following donation after circulatory death liver transplantation. Numerous published investigations have attempted to link the hemodynamic instability and hypoxemia following withdrawal of life support to the development of cholangiopathy, without success. Our research indicates that cholangiopathy is linked to the magnitude of hypoxemic, ischemic, and anemic hypoxia transpiring after life support withdrawal and can be represented by a donor hypoxia score. We recommend that the historically utilized nomenclature of ischemic cholangiopathy be replaced using a more physiologic-based and expansive term, hypoxic cholangiopathy.

Chirichella TJ, Dunham CM, Zimmerman MA, Phelan EM, Mandell MS, Conzen KD, Kelley SE, Nydam TL, Bak TE, Kam I, Wachs ME. Donor preoperative oxygen delivery and post-extubation hypoxia impact donation after circulatory death hypoxic cholangiopathy. *World J Gastroenterol* 2016; 22(12): 3392-3403 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3392.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3392

INTRODUCTION

Orthotopic liver transplantation remains the gold standard for patients with end stage liver disease. The critical shortage of brain-dead organ donors has increased the utilization of donation after circulatory death (DCD) liver grafts. Many studies have compared donation after brain death to DCD liver transplants and have noted either inferior[1-3] or comparable[4,5] graft survival and biliary complications in the DCD group[5-8]. Brain-dead donors, unlike DCD donors, do not experience an agonal phase where hepatobiliary hypoxia accrues. Taner *et al*[5] stated that events during DCD procurement, such as variations in hemodynamics, a mandatory wait period, or time from incision to cross clamp, all included in the donor warm ischemic time, may impact the outcome of DCD liver transplants. The relative contribution of these factors on donor graft and recipient outcome is unknown.

The primary purpose of our study was to assess the effect of DCD donor risk conditions (age, hemodynamics prior to and after extubation, the use of vasopressors or red blood cell transfusions, pre-operative hemoglobin, and pre-operative oxygen delivery) on development of hypoxic cholangiopathy (HC). Our secondary aim was to evaluate the different eras of donors to see if these factors were predictive of recipient HC.

MATERIALS AND METHODS

Approval for the study was obtained from the Colorado Multiple Institutional Review Board. Informed consent

was waived, because this was a retrospective analysis. We reviewed information pertaining to DCD from the University of Colorado database between 2003 and 2013. Recorded data included age, date of transplant, model for end-stage liver disease (MELD) score, and whether the patient had a complication from their transplant. Using the UNOS DonorNet, the donor ID and match ID for each donor and recipient were confirmed.

Outcome conditions

Outcomes were defined as graft and patient survival, the development of recipient HC in the graft, and the need for re-transplant. Patient survival, graft survival, and the need for re-transplant were obtained from the University of Colorado Database. HC was defined as common bile duct with intrahepatic duct strictures requiring stent dependence or common bile duct and intrahepatic duct necrosis. HC was diagnosed by endoscopic retrograde cholangiopancreatogram (ERCP) or percutaneous transhepatic cholangiogram (if ERCP was performed with inability to traverse roux limb), and simple anastomotic strictures were excluded from the analysis. We believe HC is a more accurate term for DCD livers, compared to ischemic cholangiopathy which implies that the hepatobiliary insult is limited to decreased hepatic vascular perfusion. Donor and recipient variables were evaluated as potential risk factors for the development of HC. Era 1 donors were defined as those from February 2003 to November 2009 and era 2 donors were defined as those from December 2009 to September 2013. A cut-off point was created for era 1 and era 2 donors, because a more liberal donor selection criterion was used for era 2 donors. One recipient was excluded from the HC analysis due to an intraoperative death.

Donor preoperative O_2 delivery risk conditions

Predonation physiologic data from UNOS DonorNet included initial and preoperative systolic blood pressure (BP), diastolic BP, heart rate, pH, SpO_2, PaO_2, FiO_2, and positive end-expiratory pressure (PEEP) level in cm H_2O. Mean arterial BP (MAP) was computed from the systolic BP and diastolic BP. Donor preoperative arterial O_2 content was computed as (hemoglobin × 1.37 × SpO_2) + (0.003 × PaO_2). P/F O_2 was computed as PaO_2 ÷ (FiO_2% × 0.01). UNOS DonorNet provided the amount of donor red blood cell (RBC) transfusions given during hospital admission, vasopressor administration, duration of cardiac arrest prior to arriving at the hospital, pre-procurement length of stay, and pre-extubation hemoglobin. Patients who were transfused $\geqslant$ 1 unit of RBC during preoperative hospitalization were categorized as the RBC group. Donor vasopressor administration prior to the withdrawal of support was categorized as none, 1, or $\geqslant$ 2. Patients who received $\geqslant$ 2 vasopressors in the preoperative period were classified as the multi-pressor group. Patients in either the RBC or multi-pressor group were denoted as the RBC/multi-pressor group.

Donor post-extubation risk conditions

Operating room donor hemodynamic variables were taken from the local organ procurement organization (OPO) DCD operating room (OR) flowsheet. Vital signs were calculated every one-to-five minutes following extubation and then every minute at the start of the agonal phase (systolic BP < 60 mmHg or SpO_2 < 80%) per OPO protocol. Donor BP was recorded using either an arterial cannula or a blood pressure cuff in 1 min intervals. SpO_2 was monitored using either a finger or ear probe, per the OPO policy. The donor OR variables included: age, diagnosis prior to procurement, time diastolic BP < 60 mmHg, time SpO_2 < 80%, time from extubation-to-asystole, time from extubation-to-aortic cross clamping, and time from asystole-to-aortic cross clamping. Using UNOS DonorNet, the following data were also obtained for analysis: the body mass index, amount of RBC given during the hospital admission, preoperative vasopressor administration stopped at the time of extubation, duration of cardiac arrest prior to arriving at the hospital, and pre-donation length of stay. Donor hypoxia accrual time was defined as the period from the onset of extubation-to-aortic cross clamping. Donor ischemia time was defined as the elapsed number of minutes from the onset of diastolic BP < 60 mmHg until aortic cross clamping. Donor hypoxemia time was defined as the elapsed number of minutes from the onset of SpO_2 < 80% until aortic cross clamping. Donor hypoxia score was defined as (donor ischemia time + donor hypoxemia time) ÷ preoperative hemoglobin.

Recipient risk conditions

Recipient variables were: the cause of cirrhosis (hepatitis C, Laennec's, alpha-1 antitrypsin, hepatitis B, hepatocellular carcinoma, nonalcoholic steatohepatitis, autoimmune, or cholangiocarcinoma), MELD score, cold ischemic time, warm ischemic time, and type of biliary anastomosis. Duct-to-duct anastomosis was used with or without transcystic biliary tubes. When a DCD liver was used for a retransplant, a Roux-en-Y choledochojejunostomy was performed (4 DCD livers were used for re-transplant). Cold ischemic time was defined as the time of cross clamp to out of ice. Warm ischemic time was the time out of ice to reperfusion of the hepatic veins and portal vein. The University of Colorado Transplant team abandoned venovenous in 1995. Therefore, all recipient liver transplants during this time period were performed off bypass.

DCD procurement process

All DCD donors were classified as Maastricht type 3[9]. The DCD livers were procured by one organ surgical specialist provided by our local organ procurement

Table 1 Donor risk conditions for hypoxic cholangiopathy

	Result	Range
Age	29.7 ± 10.4	9-53
Body mass index (kg/m^2)	25.7 ± 4.1	18-36
Pre-op pressors = 0	29/44 (65.9%)	
Pre-op pressors = 1	9/44 (20.5%)	
Pre-op pressors ⩾ 2	6/44 (13.6%)	
Red blood cell transfusion	11/44 (25.03%)	
Pre-op hemoglobin (g/dL)	11.7 ± 2.3	4.8-16.5
Pre-op mean arterial pressure (mmHg)	88.6 ± 17.8	57-121
Pre-op SpO_2 (%)	97.3 ± 5.4	65-100
Pre-op PaO_2 (torr)	181.9 ± 114.7	41-472
Pre-op arterial O_2 content (mL/100 mL)	16.0 ± 3.3	7.5-23.2
Donor hypoxia accrual time (min)	20.8 ± 5.5	9.0-33.0
Extubation-to-asystole time (min)	12.6 ± 5.2	4.0-24.0
Asystole-to-aortic clamp time (min)	8.2 ± 2.4	5.0-14.0
Donor ischemia time (min)	12.4 ± 6.1	2-25
Donor hypoxemia time (min)	14.2 ± 6.1	1-29
Donor hypoxia score	2.3 ± 1.1	0.4-4.8
Pre-donation length of stay (d)	3.8 ± 2.3	1-13
No arrest	24 (53.3%)	
Pre-donor duration of arrest (min)	31.5 ± 20.6	3-75

organization and/or by the abdominal transplant team at the University of Colorado Hospital. The OPO obtained consent for recovery and to administer 30000 units of intravenous heparin prior to extubation. Withdrawal of life support (*i.e.*, extubation and stoppage of vasopressor agents), institution of comfort measures, and declaration of death were in compliance with donor hospital policies (*i.e.*, either in the post-anesthesia care unit with rapid transport to the OR, or in the OR). Following declaration of death, a mandatory observation of 2-5 min was performed with reconfirmation of death, depending on the donor hospital policy.

Rapid retrieval was performed using aortic cannulation through an infrarenal approach, and portal cannulation through the inferior mesenteric vein, using University of Wisconsin solution or histidine-tryptophan-ketoglutarate cold solution. Once cold perfusion was initiated, the common bile duct was transected at the duodenum and the gallbladder was opened and flushed with saline, until the effluent from the transected duct was clear. Tissue plasminogen activator was flushed through the arterial system after the liver was excised, before packaging it in cold histidine-tryptophan-ketoglutarate. Because dosing and administration was attending-dependent, these were inconsistent.

Statistical analysis

Continuous variables are expressed as means ± standard deviations. Statistical relationships were performed using the following techniques: (1) *t*-test for comparison of interval continuous data between two groups; (2) Wilcoxon rank-sum test for comparison of ordinal-rank continuous data between two groups; (3) Pearson's correlation coefficient analysis to assess the relationship between two continuous variables; (4) Fisher's exact test to assess 2 × 2 contingency tables; and (5) logistic multivariate regression analysis to assess the impact of independent variables on binary response variables. SAS System for Windows, release 9.2 (SAS Institute Inc., Cary, NC, United States) was used to perform the statistical analysis. $P < 0.05$ represented statistical significance.

RESULTS

From February 2003 to September 2013, 45 consecutive patients underwent a DCD liver transplant. HC occurred in 50.0% (n = 22) of DCD liver recipients with 15.9% (n = 7) requiring re-transplantation. The 1, 3, and 5 year graft survival rates were 83%, 77%, and 60%, respectively. The 1, 3, and 5 year patient survival rates were 92%, 84%, and 72%, respectively.

Donor risk conditions for HC

One patient was excluded from the analysis secondary to an intraoperative death. Donor risk conditions are described in Table 1. The median age of DCD liver donors was 30 years old with a body mass index < 30 kg/m^2. Most donors did not receive RBC transfusion, were not on vasopressors, and did not suffer a pre-admission cardiac arrest.

Donor preoperative O_2 delivery and hc risk

Recipient HC univariate correlations with preoperative donor oxygen delivery are presented in Table 2. Patients not developing HC were younger, more frequently received preoperative multiple vasopressor administration and RBC transfusions, had higher preoperative hemoglobin and arterial O_2 content, and had lower preoperative MAP. Multivariate analysis showed that recipient HC was independently associated with increased MAP (P = 0.07), lower arterial O_2 content (P = 0.02), decreased multiple-pressor or RBC administration (P = 0.013), and older age (P = 0.11) (r^2 = 0.4427).

Eleven of 44 (25.0%) donors underwent preoperative RBC transfusion. The transfusion group had a lower initial pH (7.23), compared to the non-transfusion group (7.33; P = 0.03). The transfusion group had a normal preoperative pH (7.39), compared to their initial value (7.23; P = 0.001). The transfusion group had a lower initial SpO_2 (91.3%), compared to the non-transfusion group (97.3%; P = 0.007). The transfusion group had a similar preoperative hemoglobin (11.0 g/dL), when compared to the non-transfusion group (12.0 g/dL; P = 0.17).

Of the 44 donors, 6 (13.6%) received ⩾ 2 pressors during the preoperative period. Initially, the multi-pressor group was acidemic (pH 7.27 ± 0.18); yet, following the administration of multiple pressor agents the acidosis improved (pH 7.37 ± 0.09; P = 0.29).

Table 2 Hypoxic cholangiopathy correlations with preoperative donor oxygen delivery

	(-) Hypoxic cholangiopathy	(+) Hypoxic cholangiopathy	*P* value
n	22	22	
Age	25.6 ± 8.4	33.0 ± 10.6	0.020
Multiple pressor administration	27.3%	0.0%	0.009
RBC transfusion	36.4%	9.5%	0.040
RBC or multiple pressor administration	54.6%	9.5%	0.001
Hemoglobin	12.3 ± 2.1	10.7 ± 2.2	0.020
Arterial O_2 content (mL/100 mL)	16.8 ± 3.3	14.8 ± 2.8	0.049
Mean arterial pressure (mmHg)	83.8 ± 18.5	92.7 ± 16.2	0.100

RBC: Red blood cell.

Table 3 Comparison of patients with preoperative multiple pressors or red blood cell transfusion

	(-) Multiple-pressor/RBC	(+) Multiple-pressor/RBC	*P* value
n	29 (67.4%)	14 (32.6%)	
Hypoxic cholangiopathy	19 (65.5%)	2 (14.3%)	0.001
Initial pH	7.33 ± 0.14	7.25 ± 0.12	0.08
Pre-op pH	7.43 ± 0.06	7.39 ± 0.08	0.07
Initial PaO_2	156.4 ± 84.0	133.6 ± 117.6	0.48
Pre-op PaO_2	171.5 ± 106.1	191.2 ± 129.8	0.60
Initial SpO_2 (%)	97.5 ± 2.7	92.2 ± 9.9	0.012
Pre-op SpO_2 (%)	97.9 ± 2.0	95.9 ± 8.8	0.41
Initial P/F O_2	228.5 ± 116	191.4 ± 93	0.40
Pre-op P/F O_2	212.5 ± 101	243.8 ± 126	0.41
Initial PEEP (cmH_2O)	6.0 ± 2.7	5.6 ± 3.9	0.69
Pre-op PEEP (cmH_2O)	7.1 ± 4.6	7.0 ± 4.5	0.96
Pre-op hemoglobin (g/dL)	11.8 ± 1.9	11.8 ± 2.5	0.99
Arterial O_2 content (mL/100 mL)	16.2 ± 2.5	16.1 ± 4.0	0.93
Pre-op systolic pressure (mmHg)	133.6 ± 21.9	123.5 ± 26.8	0.19
Pre-op diastolic pressure (mmHg)	74.3 ± 15.7	64.3 ± 18.1	0.06
Pre-op mean pressure (mmHg)	92.1 ± 16.6	82.1 ± 19.2	0.08
Pre-op heart rate (bpm)	92.3 ± 20.2	96.9 ± 22.3	0.49

Complete data available for 43/45 (95.6%) patients. RBC: Red blood cell; PEEP: Positive end-expiratory pressure.

Initial SpO_2 was insignificantly higher in the non-multi-pressor group (96.6%), compared to the multi-pressor cohort (89.4%; P = 0.37), despite a mean PaO_2 of 120.4 ± 65.0 in the latter group. Following multi-pressor administration, the SpO_2 increased to 99.3% ± 0.8% (P = 0.24). The preoperative SpO_2 was higher in the multi-pressor group (99.3%), compared to the non-multi-pressor group (96.8%; P = 0.02). The preoperative P/F O_2 was higher in the multi-pressor group (305.3) compared to the non-multi-pressor group (210.8; P = 0.07); however, the PEEP levels were comparable (8.2 and 6.9 cmH_2O; P = 0.55). Multi-pressor use did not create preoperative hypertension (systolic BP 113.2 ± 22.1; diastolic BP 67.5 ± 16.3) or tachycardia (heart rate 105.0 ± 18.6).

A comparison of the preoperative RBC/multi-pressor group with the non-intervention group is presented in Table 3. The initial pH in the RBC/multi-pressor group was more acidemic, compared to the non-RBC/multi-pressor group. The RBC/multi-pressor group's preoperative pH was significantly higher, compared to their initial pH (P = 0.001). The RBC/multi-pressor group had a 51.3 torr increase in the preoperative PaO_2 relative to the initial PaO_2 (P = 0.22). The RBC/multi-pressor group's preoperative SpO_2 increased, compared to their initial value (P = 0.29). The RBC/multi-pressor group's preoperative P/F O_2 was insignificantly higher, compared to their initial P/F O_2 with an almost identical preoperative PEEP and preoperative hemoglobin, compared with the non-RBC/multi-pressor group. The non-RBC/multi-pressor group's preoperative P/F O_2 had a minimal decrease, compared to their initial P/F O_2. The increment from the initial P/F O_2 to the preoperative P/F O_2 in the two groups trended towards significance (P = 0.09).

Donor post-extubation hypoxia and HC risk

Recipient HC correlations with donor post-extubation hypoxia are presented in Table 4. Neither cold nor warm ischemia had a statistical association with HC. HC was not associated with the duration of donor hypoxia accrual, extubation-to-asystole, asystole-to-aortic clamping, donor ischemia, or donor hypoxemia times. HC was associated with donor preoperative hemoglobin and the donor hypoxia score. HC was associated with a donor hypoxia score > 2.0 (relative

Table 4 Hypoxic cholangiopathy correlations with donor post-extubation hypoxia

	(-) Hypoxic cholangiopathy	(+) Hypoxic cholangiopathy	*P* value
n	22	22	
Donor hypoxia accrual time (min)	20.3 ± 5.8	21.6 ± 5.2	0.470
Extubation-to-asystole time (min)	12.0 ± 5.1	13.4 ± 5.4	0.380
Asystole-to-aortic clamp time (min)	8.3 ± 2.6	8.1 ± 2.3	0.800
Donor ischemia time (min)	11.7 ± 7.4	13.5 ± 4.2	0.340
Donor hypoxemia time (min)	13.2 ± 6.5	15.5 ± 5.6	0.220
Preoperative hemoglobin (g/dL)	12.3 ± 2.1	10.7 ± 2.2	0.017
Donor hypoxia score	2.0 ± 1.2	2.7 ± 0.9	0.030
Donor hypoxia score > 2.0	25.0%	69.6%	0.006

Table 5 Recipient risk variables - *n* = 45 *n* (%)

	Result	Range
Hepatitis-C	13 (28.9)	
Ethanol	6 (13.3)	
Alpha-1 antitrypsin	9 (20.0)	
Hepatitis B	3 (6.7)	
Hepatocellular carcinoma	18 (40.0)	
⩾ 2 diagnoses	21 (46.7)	
MELD	28.7 ± 5.6	17-40
Duct to duct anastamosis	38 (84.4)	
Cold-ischemia time (min)	429.9 ± 127.3	60-660
Warm-ischemia time (min)	35.4 ± 13.6	16-76
Total-ischemia time (min)	465.6 ± 131.0	95-688

MELD: Model for end-stage liver disease.

Table 6 Era 1 and era 2 risk comparisons

	Era 1	Era 2	*P* value
DCD liver transplants	17	27	
Hypoxic cholangiopathy	29.4%	63.0%	0.030
Transplants per year	2.5	7.1	0.100
Hepatocellular carcinoma	23.5%	51.9%	0.060
Donor body mass index (kg/m^2)	24.1 ± 3.1	26.7 ± 4.3	0.030
Donor hypoxia accrual time (min)	17.3 ± 5.0	22.7 ± 4.8	0.001
Donor extubation-to-asystole time (min)	9.3 ± 4.5	14.4 ± 4.7	0.001
Donor aystole-to-crossclamp time (min)	8.1 ± 2.7	8.3 ± 2.3	0.779
Donor ischemia time (min)	9.7 ± 5.6	13.9 ± 5.9	0.030
Donor hypoxemia time (min)	11.1 ± 6.7	16.0 ± 5.1	0.013
Donor hypoxia score	1.6 ± 0.9	2.7 ± 1.1	0.003
Donor hypoxia score > 2.0	28.6%	73.1%	0.006
Recipient cold ischemia time (min)	381 ± 139	461 ± 111	0.040
Recipient warm ischemia time (min)	31 ± 10	39 ± 15	0.030
Recipient total ischemia time (min)	411.8 ± 135	499.4 ± 119	0.030

risk = 2.8). Multivariate analysis showed that the recipient HC was independently associated with increased donor age (P = 0.10), increased donor preoperative MAP (P = 0.06), decreased donor preoperative arterial O_2 content (P = 0.08), less donor preoperative RBC or multiple-pressor administration (P = 0.03), and a greater rate of donor hypoxia score > 2 (P = 0.06, r^2 = 0.6197).

Recipient risk conditions

Select recipient risk conditions are presented in Table 5. HC had no association with any of the conditions listed in the Table (P > 0.05).

Era 1 and era 2 donor preoperative O_2 delivery/post-extubation hypoxia and HC

Era 2 donors had a higher HC rate and more transplants per year, compared to era 1 (Table 6). A comparison of era 1 and era 2 showed that era 2 times were longer for donor hypoxia accrual, donor extubation-to-asystole, donor ischemia, donor hypoxemia, and recipient cold ischemia. Era 2 donors also had a higher donor hypoxia score and a higher rate for donor hypoxia scores > 2.0. The correlations for era 2 donors *vs* era 1 donors were greater for donor hypoxia score (r^2 = 0.2443) and extubation-to-asystole time (r^2 = 0.1839), when compared to donor cold ischemia time (r^2 = 0.1045). Multivariate analysis identified era 2 *vs* era 1 as independently associated with a greater donor hypoxia score > 2.0 rate (P = 0.04), a longer recipient cold ischemia time (P = 0.02), and longer extubation-to-asystole time (P = 0.07, r^2 = 0.3905).

Multivariate analysis showed that HC was independently associated with increasing age (P = 0.02), less preoperative RBC or multiple-pressor administration (P = 0.04), greater donor hypoxia score > 2.0 (P = 0.03), and era 2 (P = 0.06, r^2 = 0.6502). Cold ischemia times were the same for recipients not developing HC (432 m) and those acquiring HC (431 m, P = 1.0). Recipient warm ischemia times were also similar (33 m *vs* 39 m, P = 0.18).

DISCUSSION

Donor post-extubation hypoxia and recipient HC

We found, following withdrawal of life support, an association between recipient HC and lower donor hemoglobin and longer durations of donor diastolic BP < 60 mmHg (ischemic time) and donor SpO_2 < 80% (hypoxemic time) until aortic cold-perfusion and cross clamping. These findings are summarized by the significant association between recipient HC and the donor hypoxia score. The time until aortic cold-perfusion and cross clamping (donor hypoxia accrual time) following the withdrawal of life support was not associated with recipient HC. It is clear from the

data in our study that ischemic hypoxia or hypoxemic hypoxia did not occur for several minutes following life support withdrawal. That is, the average time from the withdrawal of life support until cross clamping exceeded the times for SpO_2 < 80% and diastolic BP < 60 mmHg.

The DCD liver transplant literature has historically and commonly referred to the donor hypoxia accrual time as the donor warm ischemic time (DWIT)[10]. Abt *et al*[11] have shown that this time period was not associated with graft survival in DCD liver transplantation. However, de Vera *et al*[12] has shown that DWIT > 20 min is associated with poor graft survival. More specifically, DeOliveira *et al*[7] demonstrated that DWIT does not correlate with biliary complications. Their group defined DWIT as the time from life support withdrawal until aortic cannulation, where the SpO_2 fell < 70% or systolic BP decreased < 50 mmHg. Their lack of correlation may be related to the failure to separately consider each as a hypoxic risk condition or that their criteria are too conservative. A meta-analysis by O'Neill *et al*[10] demonstrated that DWIT correlated with DCD HC; however, the definitions of DWIT in the published literature were variably defined. Taner *et al*[5] comprehensively examined DWIT and failed to show significant associations with overall biliary complications or intrahepatic bile duct strictures, despite using multiple criteria for critical hypotension and hypoxemia.

A review of the DCD liver transplant literature showed that interval definitions for DWIT have been variable and included the time from extubation to cold perfusion, cardiac arrest, or aortic cross-clamping, or was the time from arrest to hepatic perfusion[10]. We were unable to identify any study where the magnitude of ischemic and hypoxemic hypoxia were combined to assess correlation with adverse outcomes; relevant examples include the studies by Elaffandi *et al*[13], Skaro *et al*[14], and Ho *et al*[15]. Although a hemoglobin of > 10 mg/dL is recommended as a critical care donor management goal[16], we have not found literature that explored the potential effect of donor hemoglobin on DCD liver transplant outcomes.

Relevant to our study observations are multiple etiologic factors for hepatic hypoxia, including ischemia, arterial hypoxemia, and severe anemia[17-19]. Two publications emphasize that decreased hepatic blood flow is not the sole mechanism of hypoxic liver injury, and therefore hypoxic hepatitis or hypoxic liver injury is more appropriate terminology[19,20]. These literature findings support study observations that the ischemic, hypoxemic, and anemic elements of the donor hypoxia score are clinically reasonable.

Also relevant to the study finding that the donor hypoxia score correlated with HC is the recognized physiologic concept of systemic oxygen delivery, with constitutive elements of ischemia, hypoxemia, and anemia. Although the investigation by Kostopanagiotou *et al*[21] emphasized the importance of oxygen delivery during orthotopic liver transplantation, the concepts are applicable to the issue of donor hypoxia. Systemic tissue oxygen delivery depends on an adequate cardiac output and arterial oxygen content, where the latter is affected by SaO_2 and hemoglobin. Of importance, MAP is directly related to cardiac output and systemic vascular resistance[22,23]. Thus, critical decreases in cardiac output or BP (ischemic hypoxia), SaO_2 (hypoxemic hypoxia), or hemoglobin (anemic hypoxia) cause cellular hypoxia and lactic acidosis[24-26].

Based on the aforementioned line of reasoning and our study findings, we suggest the historic term DWIT is ambiguous relative to time intervals, and does not fully encompass the three physiologic elements that can interactively cause cellular hypoxia. Since elements of hypoxia other than ischemia are present, the historic use of DWIT is not an adequately descriptive term. Because there is no donor cold ischemic or hypoxic period, it is unnecessary to include the word warm in the definitive terms. We propose that the historic DWIT be changed to donor hypoxia accrual time, which is the time from the withdrawal of life support until aortic cold-perfusion and cross clamping. We recommend that donor ischemia time represent the number of minutes from the onset of diastolic BP < 60 mmHg, following life support withdrawal, until aortic cold-perfusion and cross clamping. Finally, we suggest donor hypoxemia time be used to signify the number of minutes elapsing from the onset of SpO_2 < 80%, following life support withdrawal, until aortic cold-perfusion and cross clamping. The study findings indicate that the donor hypoxia score, an interactive variable that includes donor ischemia time, donor hypoxemia time, and preoperative hemoglobin, can be used to assess the DCD post-transplantation risk for recipient HC.

Donor preoperative O_2 delivery and recipient HC

Donor characteristics of younger age, higher preoperative hemoglobin, greater preoperative arterial O_2 content, lower preoperative MAP, and greater preoperative RBC/multi-pressor use were associated with decreased recipient HC. We reason that donor age was a significant finding because older patients have increased celiac axis[27] and thoracic and abdominal aortic atherosclerotic occlusive disease[28,29] and reduced cardiac output[30,31] that can affect hepatobiliary perfusion. Thus, it seems feasible that donors with a lower age and higher hemoglobin would have better preoperative hepatobiliary oxygen delivery that potentially reduces the risk for HC. Of relevance, donor ejection fraction of > 50% is a critical care donor management goal[32], that has been shown to be associated with an increase in successful organ transplantation[16]. Multiple investigators have shown that lower donor age has been associated with a reduction in DCD liver transplantation overall biliary complications[12], non-anastomotic biliary stricture[33], graft failure[2],

and HC[10,34]. This explains why pre-transplantation hemoglobin > 10 g/dL is recommended as a critical care donor management goal[16]. Our study showed that a preoperative donor hemoglobin > 12.0 gm/dL correlated with less HC.

The current investigation showed that the pre-operative RBC/multi-pressor group had a reduction in HC. The RBC group was initially more acidemic and had a lower SpO_2 compared to the non-RBC group. Following RBC transfusion, the preoperative pH improved into a normal range. The preoperative RBC group hemoglobin was comparable to those without transfusion, this suggesting that RBC transfusion was not excessive or inadequate. Of relevance, Pape *et al*[35] indicate that the decision to administer a blood transfusion should be based on clinical judgment relative to the individual's risk/benefit ratio associated with transfusion and anemia.

The donor multi-pressor group in the current study initially was severely acidemic and had a mean SpO_2 < 90%, despite a hyperoxemic PaO_2, findings suggesting the presence of shock[36] and hypotension[37]. Following the administration of multiple vasopressors, the preoperative pH and SpO_2 increased to normal ranges. Interestingly, the preoperative SpO_2 was higher in the multi-pressor group, compared to the non-multi-pressor group. It is further noteworthy that the preoperative P/F O_2 was higher in the multi-pressor group, compared to the non-multi-pressor group, despite comparable PEEP levels. Further, the multi-pressor group at the time of donor procurement did not have hypertension or tachycardia, suggesting that clinically-targeted vasopressor titration had been used.

It is important to note that a MAP of 60-100 mmHg has been established as a critical care donor management goal for successful organ transplantation[16,32]. The administration of vasopressors in organ transplant donors is controversial and Critical Care Donor Management Goal recommendations imply that the number of pressors should be limited to only one and that low-doses should be used[16,32]. However, these recommendations have been primarily based on an experience with brain-dead donors. One experience indicates that the vasopressor agent frequency is lower among DCD donors (9.4%) compared to brain-dead donors (74.7%; $P = 0.001$)[14]. Another investigation of 110 DCD donors showed that the number of vasopressors was not associated with graft survival[11]. A study by Feng *et al*[38] did not demonstrate a negative impact of donor hypotension or use of vasopressors on hepatic graft failure, by either univariate or multivariate analyses. In fact, in a review publication on DCD, Morrissey states "vasopressor use has improved outcomes that now approximate DBD liver transplantation"[39].

Relevant to a discussion regarding the use of vasopressors, it should be noted that hemodynamic instability and hypotension occur in 21%-82% of critically ill patients in an ICU[3,40,41] and the need for vasopressors has ranged from 24%-45%[3,40]. It is also germane that approximately 50% of hypotensive ICU patients will not respond to fluid administration; thus, it is imperative to determine which hypotensive patients will be responsive to fluid infusion[42,43]. Apropos, critically ill patients have hemodynamic instability typically based on one of three pathogenic mechanisms: cardiogenic (impaired contractility or altered heart rate), hypovolemic (intravascular volume deficiency), or distributive (vasogenic decrease in peripheral arterial tone)[43-45]. Appropriate patient assessment for these three states is critical in that appropriate intervention is predicated on the pathophysiology of the hemodynamic instability: fluids for hypovolemic, inotropes and echocardiography for cardiogenic, and vasopressors for distributive-vasogenic shock[42,45]. Multiple reliable devices can assist the intensivist in determining the presence of hypovolemic, cardiogenic, or distributive-vasogenic hemodynamic instability[42,46]. When hemodynamic instability fails to improve with volume-loading, inotropic-administration, or vasopressor-support, adrenal insufficiency and hypothyroidism of critical illness should be seriously considered[47].

Although the use of vasopressors is controversial for the management of hemodynamic instability, liver transplant patients commonly need inotropes and vasopressors during surgery[8,48], which reduce blood loss and lactic acidosis[8] and lessen need for tracheal re-intubation in the postoperative period[49]. An appropriate vasopressor MAP target of 60-65 mmHg has been suggested, without any clinical value for higher levels[22,44].

A couple of observations regarding arterial oxygenation are noteworthy relative to the RBC/multi-pressor group. First, the group had a clinically substantial increase in the preoperative PaO_2, relative to the initial value. Second, the group initially had a SpO_2 in the low-90s range which increased to the mid-90s following RBC transfusion or the administration of multiple pressors. Third, the P/F O_2 preoperative increment relative to the initial value in the RBC/multi-pressor group was substantially greater, compared to the non-treatment group. These findings indicate that improvements in arterial oxygenation, and thus potentially greater hepatobiliary tissue oxygen delivery, were better realized in the RBC/multi-pressor group. This potentially helps to account for the reduction in HC rates observed with these interventions.

The current study observations do not necessarily imply that donor candidates for DCD liver transplantation should carte blanche receive routine RBC transfusion or multiple vasopressors. The study findings do imply that it is reasonable in potential donor candidates with acidemia and anemia or vasogenic hemodynamic instability to provide RBC transfusion or administer vasopressors, respectively. The findings also indicate

that patients with positive physiologic responses to the interventions should be considered as acceptable DCD liver transplant donors. One can only speculate as to the reason patients receiving RBC transfusion or multiple pressors had a reduced rate of HC. It seems likely to be related, in part, to the selection of patients who are stabilized, based on objective physiologic and metabolic evidence. It also seems plausible that an astute intensivist may not only positively impact a circulatory deficiency, but also provide comprehensive care that maximally stabilizes other physiologic and metabolic systems. It is plausible that multiple pressors or RBC transfusion, in select donor patients, enhance systemic oxygen delivery and likely pre-procurement hepatobiliary oxygenation. The validity of these speculations is based on their linkage with a decreased rate of recipient HC.

Multivariate analysis showed that recipient HC was independently associated with a lower donor preoperative arterial O_2 content, older donor age, increased donor preoperative MAP and infrequent use of donor multiple pressors or RBC transfusions. The lower arterial oxygen content indicates that the interaction of hemoglobin, SaO_2, and PaO_2 likely mitigated systemic, and presumably hepatobiliary, oxygen delivery. As stated previously, it is likely that an older donor age could potentially decrease hepatobiliary blood flow due to a lower cardiac output or greater atherosclerotic occlusive disease. Although patients without HC had a lower donor preoperative MAP, compared to those developing HC, the average value remained in the normal range. Although precise, optimal MAP targets are uncertain, a MAP target recommendation for septic shock is 65 mmHg[50]. The current study's findings demonstrated an increased donor preoperative MAP in those developing recipient HC, suggesting that a higher MAP of 90 mmHg may be an undesirable target for young critically ill patients. It is plausible that an increased MAP in recipient HC patients emanates from higher preoperative peripheral arterial resistance and a reduction in cardiac output, thus impeding systemic, tissue oxygen delivery.

Era 1 and era 2 donor preoperative O_2 delivery/post-extubation hypoxia and HC

The transplants per year in era 2 represent a nearly 3-fold increase, compared to era 1. Our more liberal selection criteria was manifest by longer donor extubation-to-asystole, donor ischemia, and donor hypoxemia times and a higher donor hypoxia score and an increased donor hypoxia score > 2.0 rate in era 2. Although the recipient cold ischemia time in era 2 was increased compared to era 1, HC did not correlate with the recipient cold ischemia time. Further, the recipient cold ischemia time in era 2 is comparable to those reported by other United States liver transplantation centers[2]. The independent association of era 2 with HC, after controlling for donor age, multi-pressors/RBC transfusion status, and donor hypoxia score, suggests that donor selection criteria influence the subsequent development of recipient HC.

A direct relationship was observed with longer recipient warm ischemic times in era 2, compared to era 1, however this did not correlate with recipient HC. Although not statistically significant, more recipients in era 2 were transplanted for hepatocellular carcinoma, and whether the longer warm ischemic times are related to the quality of the recipient's hepatic artery is unknown. However, we do believe it is important to re-perfuse the hepatic veins and the portal vein prior to starting the arterial anastomosis to decrease further hypoxic injury to the biliary system, as confirmed by Farid *et al*[51]. Furthermore, moving forward ("Era 3"), our donor selection is comparable to era 1 with the use of tissue plasminogen activator (TPA) through the hepatic artery upon reperfusion, as recently demonstrated by Seal *et al*[52].

Limitations

Several methodological limitations need consideration. Although this is a retrospective study, this is an analysis of consecutive DCD liver transplants performed by identifying risk factors for the development of hypoxic cholangiopathy and we consider the UNOS DonorNet, DCD OR flow sheet, and University of Colorado's transplant database to be reliable. However, data accuracy and quality from a retrospective, database source are recognized to be lower, when compared to a prospective, dedicated database. Another limitation is the lack of consistency among the definition of donor warm ischemic time; however, we believe that this is a misnomer and should be called donor hypoxia accrual time. Also, we did not evaluate cross clamp time to liver in ice time. However, our aystole-to-cross clamp times were similar between era 1 and era 2, and we believe that explantation of the donor liver would yield similar results. Despite our limitations, to our knowledge, this is the only study to evaluate and define donor ischemia and hypoxemia time, preoperative donor hemoglobin, donor preoperative arterial O_2 content, and donor RBC transfusion requirements along with vasopressor administration so stringently.

In conclusions, HC continues to be a major morbidity of DCD liver transplantation. Our study indicated that HC was greater with an increased donor age, less donor preoperative multiple vasopressors or RBC transfusion, lower donor pre-operative hemoglobin, lower donor preoperative arterial oxygen content, and increased MAP. Higher donor pre-operative hemoglobin of 12 gm/dL is recommended prior to extubation. However, we do not recommend using vasopressors when not clinically indicated. Donor hypoxia score [a measurement of (donor ischemia time + donor hypoxemia time) ÷ donor pre-operative hemoglobin] provides an objective measure of DCD post-liver

transplantation risk for the development of recipient HC. The study also suggests that donor selection criteria have an impact on the proclivity for acquiring recipient HC.

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COMMENTS

Background

Orthotopic liver transplantation remains the gold standard for patients with end stage liver disease. The critical shortage of brain-dead organ donors has increased the utilization of donation after circulatory death (DCD) liver grafts. Many studies have compared donation after brain death to DCD liver transplants and have noted either inferior or comparable graft survival and biliary complications in the DCD group. Brain-dead donors, unlike DCD donors, do not experience an agonal phase where hepatobiliary hypoxia accrues. Taner *et al* stated that events during DCD procurement, such as variations in hemodynamics, a mandatory wait period, or time from incision to cross clamp, all included in the donor warm ischemic time, may impact the outcome of DCD liver transplants. The relative contribution of these factors on donor graft and recipient outcome is unknown.

Innovations and breakthroughs

The primary purpose of this study was to assess the effect of DCD donor risk conditions (age, hemodynamics prior to and after extubation, the use of vasopressors or red blood cell transfusions, preoperative hemoglobin, and pre-operative oxygen delivery) on development of hypoxic cholangiopathy (HC). The secondary aim was to evaluate the different eras of donors to see if these factors were predictive of recipient HC.

Applications

The present research indicates that cholangiopathy is linked to the magnitude of hypoxemic, ischemic, and anemic hypoxia transpiring after life support withdrawal and can be represented by a donor hypoxia score. The authors recommend that the historically utilized nomenclature of ischemic cholangiopathy be replaced using a more physiologic-based and expansive term, hypoxic cholangiopathy. Easily measured donor indices, including a hypoxia score, provide an objective measure of DCD liver transplantation risk for recipient HC. Donor selection criteria influence HC rates.

Peer-review

The authors face with the serious complication of hypoxic colangiopathy after liver transplantation using donors after circulatory death. In particular they analyze the different factors responsible for HC. Even if retrospective the study is well conducted with an excellent statistical analysis. In addition the study is well written, updated and give the reader useful clinical informations.

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ORIGINAL ARTICLE

Retrospective Study

Intrahepatic distribution of hepatitis B virus antigens in patients with and without hepatocellular carcinoma

Parham Safaie, Mugilan Poongkunran, Ping-Ping Kuang, Asad Javaid, Carl Jacobs, Rebecca Pohlmann, Imad Nasser, Daryl TY Lau

Parham Safaie, Mugilan Poongkunran, Ping-Ping Kuang, Asad Javaid, Daryl TY Lau, Liver Center, Division of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Carl Jacobs, Rebecca Pohlmann, Imad Nasser, Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Author contributions: Safaie P, Poongkunran M, Nasser I and Lau DT designed, implemented and supervised the analysis, manuscript preparation of the study; Kuang PP performed the immunostains of HBV antigens in liver tissues, Javaid A contributed to the interpretations of the data and manuscript writing; Pohlmann R and Jacobs C contributed to the reading and interpretation of the pathology; all authors read and approved the final manuscript; Safaie P and Poongkunran M contributed equally to this manuscript.

Institutional review board statement: The Beth Israel Deaconess Medical Center (BIDMC) Committee on Clinical Investigations (CCI); the appropriately authorized Institutional Review Board (IRB); and Privacy Board appointed to review research involving human subjects, has referenced the protocol named "factors affecting the pathogenesis and progression of chronic liver disease" (IRB Protocol No: 2008P000299).

Informed consent statement: This study used only the excessive liver biopsy samples from the IRB Protocol No: 2008P000299. Hence no informed consent from the study subjects necessary.

Conflict-of-interest statement: Parham Safaie, Mugilan Poongkunran, Ping-Ping Kuang, Asad Javaid, C. Jacobs, Rebecca Pohlmann, Imad Nasser, Daryl TY Lau declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

Correspondence to: Daryl TY Lau, MD, Associate Professor, Liver Center, Division of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 110 Francis Street, Suite 4A, Boston, MA 02215, United States. dlau@bidmc.harvard.edu
Telephone: +1-617-6321098
Fax: +1-617-6321125

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Abstract

AIM: To study the intrahepatic expression of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) in chronic hepatitis B patients with and without hepatocellular carcinoma.

METHODS: A total of 33 chronic hepatitis B patients (mean age of 40.3 ± 2.5 years), comprising of 14 HBeAg positive and 19 HBeAg negative patients; and 13 patients with hepatitis B virus related hepatocellular carcinoma (mean age of 49.6 ± 4.7 years), were included in our study. Immunohistochemical staining for HBcAg and HBsAg was done using standard streptavidin-biotin-immunoperoxidase technique on paraffin-embedded liver biopsies. The HBcAg

and HBsAg staining distributions and patterns were described according to a modified classification system.

RESULTS: Compared to the HBeAg negative patients, the HBeAg positive patients were younger, had higher mean HBV DNA and alanine transaminases levels. All the HBeAg positive patients had intrahepatic HBcAg staining; predominantly with "diffuse" distribution (79%) and "mixed cytoplasmic/nuclear" pattern (79%). In comparison, only 5% of the HBeAg-negative patients had intrahepatic HBcAg staining. However, the intrahepatic HBsAg staining has wider distribution among the HBeAg negative patients, namely; majority of the HBeAg negative cases had "patchy" HBsAg distribution compared to "rare" distribution among the HBeAg positive cases. All but one patient with HCC were HBeAg negative with either undetectable HBV DNA or very low level of viremia. Intrahepatic HBcAg and HBsAg were seen in 13 (100%) and 10 (77%) of the HCC patients respectively. Interestingly, among the 9 HCC patients on anti-viral therapy with suppressed HBV DNA, HBcAg and HBsAg were detected in tumor tissues but not the adjacent liver in 4 (44%) and 1 (11%) patient respectively.

CONCLUSION: Isolated intrahepatic HBcAg and HBsAg can be present in tumors of patients with suppressed HBV DNA on antiviral therapy; that may predispose them to cancer development.

Key words: Hepatitis B virus; Chronic hepatitis B; Hepatocellular carcinoma; Hepatitis B core antigen; Hepatitis B surface antigen

Core tip: This study described the distributions and patterns of intrahepatic hepatitis B core antigen and hepatitis B surface antigen (HBsAg) in patients with chronic hepatitis B using a novel, modified classification system. The HBeAg negative patients were found to have intense HBsAg in liver tissues despite their lower serum hepatitis B virus (HBV) DNA levels. For those with hepatocellular carcinoma (HCC), we observed high rate of HBV antigen detection in tumor tissues, but not in adjacent non-tumor livers, especially among those with optimal serum HBV DNA suppression. These data support that HCC can be derived from clonal expansion of infected hepatocytes with high carcinogenic potentials and selective resistance to antiviral agents.

Safaie P, Poongkunran M, Kuang PP, Javaid A, Jacobs C, Pohlmann R, Nasser I, Lau DT. Intrahepatic distribution of hepatitis B virus antigens in patients with and without hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(12): 3404-3411 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3404.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3404

INTRODUCTION

Hepatitis B virus (HBV) infection has a worldwide distribution affecting more than 240 million individuals[1,2]. It accounts near to 1 million deaths annually through complications such as liver failure and hepatocellular carcinoma (HCC)[3,4]. Clinical outcomes of HBV depend on the interaction between viral and host-specific factors.

Hepatitis B viral proteins include a structural nucleocapsid core protein (HBcAg), an envelope protein (HBsAg), and a soluble nucleocapsid protein (HBeAg)[5]. Serum HBeAg is a surrogate marker for active hepatitis B virus replication in hepatocytes[6]. HBcAg is an intracellular antigen that is detectable either in the nucleus or cytoplasm of HBV-infected hepatocytes[7]. There are reports that high nuclear expression of HBcAg reflects high level of circulating HBV DNA and normal alanine transaminases (ALT) level as seen in the "immune tolerance phase" of hepatitis B[8-10]. In contrast, the cytoplasmic expression reflects a lower level of circulating HBV DNA, but increased hepatocellular damage with higher ALT level during the "immune clearance phase"[11-13]. A study also suggested that the membranous expression of HBsAg relates closely to active viral replication[13]. However, immunohistochemical expression of HBcAg, HBsAg and its relationship to virological and histological activities has been inconsistent and limited, especially in HBeAg negative chronic hepatitis B and HBV related HCC[16,17]. The lack of a standardized system to describe the intrahepatic HBV antigen distribution may contribute to the inconsistent findings[18].

In this study, we developed a modified classification system that allows consistency in the description of both the distributions and patterns of intrahepatic HBcAg and HBsAg. We applied the classification system to examine the HBcAg and HBsAg distributions in tissues from patients with HBeAg positive (+ve) and HBeAg negative (-ve) chronic hepatitis B (CHB). Since patients with optimal HBV DNA suppression on antiviral therapy are still at risk for HCC, we also examined the patterns of HBcAg and HBsAg in tumor and adjacent non-tumor tissues among HCC patients with and without antiviral treatment.

MATERIALS AND METHODS

Patients

Liver biopsies from a total of 33 patients with chronic hepatitis B (CHB) and 13 patients with HBV-related hepatocellular carcinoma were included in the study. Fifty chronic hepatitis B cases were initially identified from our recent pathology database. Patients with HCV, HDV and HIV co-infection and inadequate biopsy size (< 2 cm) were subsequently excluded. Of the 33 HBsAg positive patients, 14 were HBeAg positive and 19 were HBeAg negative. None of the

Table 1 Classification system for intrahepatic hepatitis B core antigen and hepatitis B surface antigen

	HBcAg	HBsAg
Distribution		
Diffuse	Contiguous without skip zones Either in clusters or single cells	Contiguous without skip zones Either in clusters or single cells
Patchy	< 50% area with skip zones Either in clusters or single cells	< 50% area with skip zones Either in clusters or single cells
Rare	Occasional area of entire field Either in clusters or single cells	Occasional area of entire field Either in clusters or single cells
Pattern	-Nuclear -Cytoplasmic	-Membranous -Cytoplasmic

HBcAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen.

CHB patients received antiviral or immunosuppressive therapy prior to liver biopsy. The 13 HCC patients were selected from our tumor clinic database. Patients with HCV, HDV, and HIV coinfection were excluded. Nine of the 13 (69%) HCC patients were on antiviral therapy prior to the development of liver cancer. Our liver pathologists reviewed histological scoring and invasiveness of HCC on all these 46 cases consistently. All patients' demographic and clinical characteristics were obtained from our electronic medical records at Beth Israel Deaconess Medical Center (BIDMC), Boston, Massachusetts, United States.

Liver pathology and immunohistochemical assays

The histological diagnosis was established using hematoxylin and eosin (HE) staining and masson trichrome stains of formalin fixed paraffin-embedded liver tissue. Hepatic fibrosis was graded by our liver histopathologists according to the Metavir system on a 5-point scale between 0 and 4.

HBeAg, anti-HBe, anti-HCV were tested by enzyme immunoassay and HBV DNA quantification was performed using the Cobas Ampliprep/Cobas Taqman HBV at the Molecular lab. Analysis of sections of paraffin-embedded liver biopsies was performed using a previously reported procedure[7].

Using standard streptavidin-biotin-immunoperoxidase technique, immunohistochemical staining for HBcAg and HBsAg was done[19] in our laboratory at BIDMC. Briefly, it involves 4 major steps: deparaffinization of tissue, antigen retrieval, tissue permeabilization and immunostaining. The distribution and patterns of intrahepatic HBcAg and HBsAg was uniformly recorded. We developed a clinically relevant, modified classification system to analyze the HBsAg and HBeAg patterns as shown in Table 1 and Figure 1.

RESULTS

Characteristics of HBeAg-positive and HBeAg-negative chronic hepatitis B patients

HBeAg positive patients were younger than the HBeAg negative subjects. The gender distributions were not different between the groups with Asian males accounting for the majority of patients in both groups. Mean HBV DNA and ALT levels were higher in the HBeAg positive than the HBeAg negative patients. However, the histological characteristics were not different between the two groups. Over 90% of the patients in each group had mild to moderate grade 1-2 inflammation. Advanced Stage 3-4 hepatic fibrosis was present in 29% and 10% of the HBeAg positive and negative patients respectively (Table 2).

Comparison of HBcAg/HBsAg immune-staining characteristics in HBeAg-positive and HBeAg-negative CHB

Intrahepatic staining of HBcAg was seen in all 14 HBeAg positive patients with predominantly 'diffuse' distribution (79%) and "mixed cytoplasmic/nuclear" pattern (79%). Among the 19 HBeAg-negative patients, only one (5%) had positive intrahepatic HBcAg. It was 'rare' with mixed cytoplasmic/nuclear pattern. Intrahepatic HBsAg was seen in all the patients irrespective of their HBeAg status and was predominantly cytoplasmic (> 70%) in both groups. HBeAg negative patients actually had stronger intrahepatic HBsAg distributions. Majority (85%) of the HBeAg negative cases had either diffuse or patchy HBsAg distribution compared to 50% among the HBeAg positive cases (Table 3).

Clinical features of patients with hepatocellular carcinoma

All 13 patients (9 males and 4 females) were Asian patients with age range between 25 and 69 years. All but one patient were HBeAg negative. Eight of Twelve (67%) HBeAg negative HCC patients had HBcAg in their adjacent non-tumor tissues. This is in distinct contrast to the HBeAg negative CHB patients without cancer, where only 1/19 (5%) had positive HBcAg. However, HBsAg in the adjacent non-tumor tissues was less prevalent among patients with HCC (67%) compared to the HBeAg negative CHB patients without

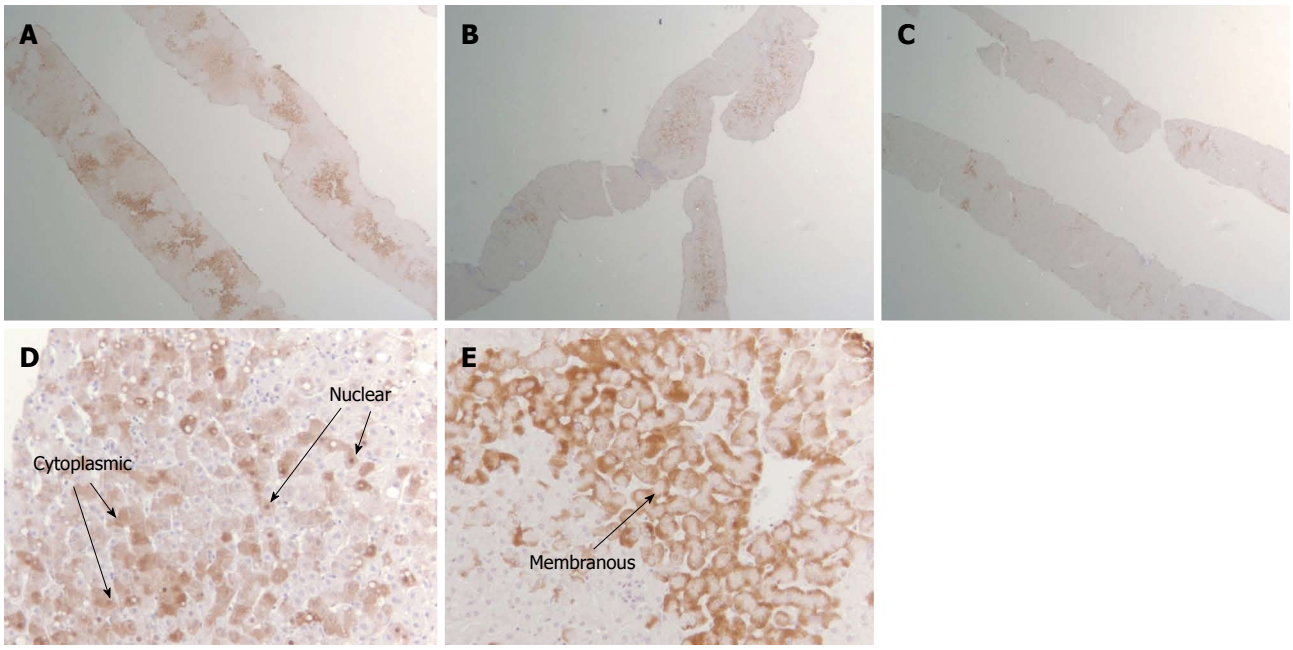

Figure 1 Images of pathology slides illustrating the classification system for intrahepatic hepatitis B surface antigen and hepatitis B core antigen. A: Diffuse distribution of HBsAg (× 2); B: Patchy distribution of HBcAg (× 2); C: Rare distribution of HBsAg (× 2); D: Cytoplasmic/nuclear pattern of HBcAg (× 20); E: Membranous pattern of HBsAg (× 20).

Table 2 Clinical, virological, and histopathological characteristics of patients with chronic hepatitis B *n* (%)

Variables	HBeAg-positive (*n* = 14)	HBeAg-negative (*n* = 19)
Mean age in years	38.5	42
Race		
Asian	13 (93)	10 (53)
White	1 (7)	1 (5)
African/African American	0 (0)	7 (37)
Hispanic	0 (0)	1 (5)
Male: female, *n*	11:3	13:6
HBV DNA (IU/mL), mean	28 million	7.3 million
ALT (U/L), mean	124	33
Inflammatory grades		
0	0 (0)	1 (5)
1-2	13 (93)	18 (95)
3-4	1 (7)	0 (0)
Fibrosis stage		
0	3 (21)	10 (53)
1-2	7 (50)	7 (37)
3-4	4 (29)	2 (10)

CHB: Chronic hepatitis B; ALT: Alanine aminotransferase.

Table 3 Immunochemical characteristics of hepatitis B core antigen/hepatitis B surface antigen in hepatitis B envelop antigen-positive and hepatitis B envelop antigen-negative patients *n* (%)

Variables	HBeAg-positive (*n* = 14)		HBeAg-negative (*n* = 19)	
Distribution,	HBcAg	HBsAg	HBcAg	HBsAg
Diffuse	11 (79)	5 (36)	0 (0)	6 (32)
Patchy	1 (7)	2 (14)	0 (0)	10 (53)
Rare	2 (14)	7 (50)	1 (5)	3 (15)
Pattern				
Cytoplasmic (+)	1 (7)	-	0 (0)	-
Nuclear (+)	2 (14)	-	0 (0)	-
Mixed: Cyto/nuclear	11 (79)	-	1 (5)	-
Cytoplasmic (+)	-	11 (79)	-	14 (74)
Membranous (+)	-	1 (7)	-	0 (0)
Mixed: Cyto/memb	-	2 (14)	-	5 (26)

HBcAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B envelop antigen.

cancer (100%) (Table 4).

Patients with HCC were further classified into two groups based on whether they were on anti-viral therapy prior to the diagnosis of HCC. All four HCC patients who were not on anti-viral therapy had invasive carcinoma and replicative serum HBV DNA. In contrast, 8 of 9 (89%) patients on anti-viral therapy had undetectable serum HBV DNA, and only 3 developed invasive carcinoma.

Detection of HBV Antigens in HCC patients on anti-viral therapy: Despite on antiviral therapy, intrahepatic HBcAg was detected in all nine patients in the treatment group. The majority had diffuse distribution. Figure 2 illustrated diffuse HBcAg in both tumor and adjacent tissue of a patient. Interestingly, 4 (44%) patients with suppressed serum HBV DNA were found to have diffuse distribution of HBcAg only in the tumor but not in the adjacent liver (representative case shown in Figure 3). Intrahepatic HBsAg was detected in seven of nine (78%) patients. The majority had patchy distribution. Only one of the nine (11%) patients had HBsAg detected in the tumor tissue alone (Table 4).

Detection of HBV Antigens in HCC patients not on anti-viral therapy: Intrahepatic staining of HBcAg

Table 4 Features of hepatocellular carcinoma patients based on the antiviral therapy status

Variables	HCC group on anti-viral therapy (n = 9)	HCC group on no therapy (n = 4)
Mean age ± SD, yr	53.2 ± 8.5	46.5 ± 20.5
Male: female	7:2	2:2
HBeAg negative	8/9	4/4
Undetectable HBV DNA	8/9	0/4
Cirrhosis	4/9	0/4
Invasive HCC	3/9	4/4
Tumor:		
Well differentiation	6/9	1/4
Moderate differentiation	3/9	3/4
Intrahepatic HBcAg staining		
(+) Only in tumor tissues	4/9	1/4
(+) Only in non-tumor tissues	3/9	1/4
(+) Both in tumor and non-tumor tissues	2/9	2/4
Intrahepatic HBsAg staining		
(+) Only in tumor tissues	1/9	0/4
(+) Only in non-tumor tissues	3/9	2/4
(+) Both in tumor and non-tumor tissues	3/9	1/4

SD: Standard deviation; HCC: Hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; HBcAg: Hepatitis B core antigen; HBeAg: Hepatitis B envelop antigen.

was seen all four patients with only one having patchy HBcAg present in the tumor tissue alone. Intrahepatic HBsAg was detected in three of four patients with none presenting only in the tumor tissue alone. Intrahepatic staining of both HBcAg and HBsAg in tumor tissues was seen only in one patient (Table 4).

DISCUSSION

In this study, we sought to examine the intrahepatic HBcAg and HBsAg distribution and patterns using a standardized, modified classification system in patients with chronic hepatitis B and in those with HBV-related hepatocellular carcinoma.

HBeAg that is considered to be a marker of HBV replication and infectivity; is usually associated with high levels of serum HBV DNA[5,20]. In our study, we found HBeAg positive patients to be younger, had higher serum levels of HBV DNA and ALT when compared to the HBeAg negative patients. These observations are in concordance with previous reports[21-23].

The presence of intrahepatic HBcAg in 100% of our HBeAg positive subjects with high level of viremia confirm the previous studies that the presence of HBcAg in hepatocytes serves as as a marker of viral replication[24,25]. The prevalence of intrahepatic HBcAg expression in HBeAg negative patients has been inconsistent and was reported between 0% and 90%[26-31]. In our current study, we detected HBcAg in only 5% of the HBeAg negative patients. Even though intrahepatic HBcAg expression was infrequent in

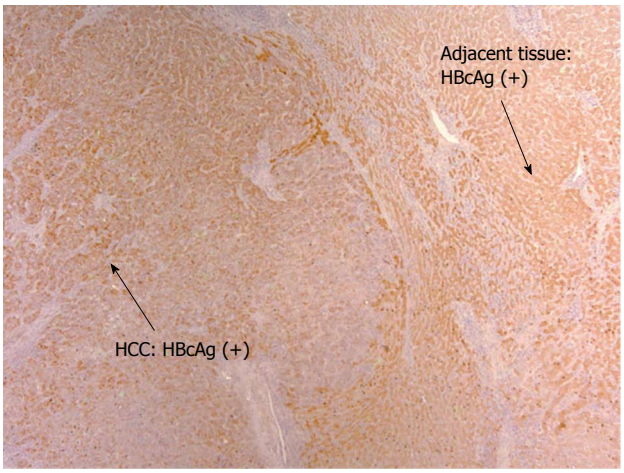

Figure 2 Diffuse hepatitis B core antigen distribution of mixed cytoplasmic, nuclear pattern in both tumor and adjacent non-tumor tissue (× 10). HCC: Hepatocellular carcinoma; HBcAg: Hepatitis B core antigen.

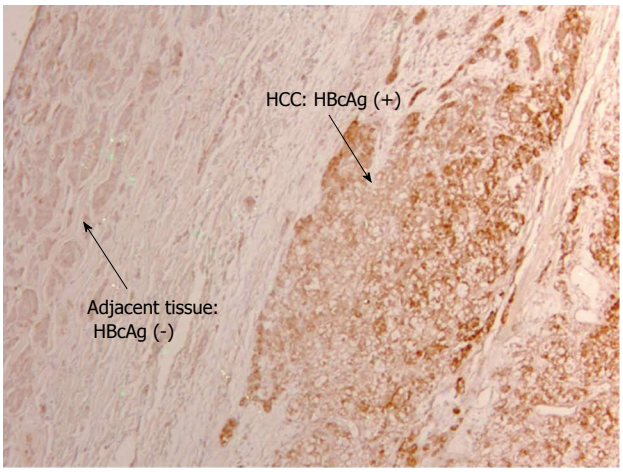

Figure 3 Diffuse hepatitis B core antigen distribution of mixed cytoplasmic, nuclear pattern in tumor tissue alone but not in adjacent non-tumor tissue (× 10). HCC: Hepatocellular carcinoma; HBcAg: Hepatitis B core antigen.

HBeAg negative CHB, its absence cannot exclude the presence of modest levels of HBV replication[18].

The mixed cytoplasmic/nuclear pattern of HBcAg staining in the majority of our HBeAg-positive patients correlates positively with the diffuse HBcAg distribution and high HBV DNA levels. These results are consistent with previous reports[7,8,13]. Among HBeAg-negative patients, only one had positive but rare distribution intrahepatic HBcAg. These results are consistent with previous reports that higher level of viral replication (or serum HBV DNA) is associated with diffuse distribution and mixed cytoplasmic/nuclear pattern of HBcAg[7,8,11,13,18,24,30].

In contrast to HBcAg, all patients had positive intrahepatic HBsAg regardless of their HBeAg status, and its distribution did not correlate with HBV DNA levels. In fact, HBeAg negative patients had more intense intrahepatic HBsAg distributions. These findings

are consistent with the hypothesis that the increased accumulation of intracellular HBsAg during inactive viral replication is secondary to the diminished synthesis of HBcAg and, therefore, reduced export of HBsAg[32,33]. Both HBeAg positive and negative patients had predominantly cytoplasmic HBsAg pattern regardless of their serum HBV DNA levels. In the literature, either cytoplasmic or membranous expression of HBsAg had been associated with active viral replication[7,28]. We could not comment on the isolated membranous pattern for the majority of our patients had either cytoplasmic or mixed cytoplasmic, membranous HBsAg pattern.

HBcAg expressions have been thought to induce liver necroinflammation through HBV specific and non-specific T cells. Previous studies indicated that patients with predominantly nuclear expression of HBcAg had lower hepatocellular damage compared to those with predominantly cytoplasmic expression[21,22,34]. Studies from European and Asian population, however, reported no significant correlation between the patterns of HBcAg, HBsAg and hepatic injury[35-40]. Similarly, our study did not find a relationship between intrahepatic HBV antigen expression and histological activity in chronic hepatitis B. These inconsistent results may signify the complex interactions between the host and the hepatitis B virus.

Chronic hepatitis B is a major cause of hepatocellular carcinoma (HCC) globally. Its risk factors include advanced age, male sex, HBV genotype, longer duration of infection, higher viral load, elevated ALT, presence of cirrhosis and co-infections[41,42]. It is known that the cumulative risk of HCC persists after the clearance of HBV DNA, HBeAg and HBsAg in CHB[43-45]. In this study, HCC patients were older (mean age, 49.6 ± 4.7 years) compared to those with chronic hepatitis B. The majority (92%) of our HCC patients were HBeAg negative, had undetectable HBV DNA on antiviral therapy and only 23% had underlying cirrhosis. These observations provided further evidence that HCC risk persists despite HBeAg seroclearance and optimal serum HBV DNA suppression. Detailed studies on the intrahepatic HBV antigens in HCC patients are limited. We detected intrahepatic HBcAg and HBsAg in 100% and 77% of our HCC patients respectively in spite of their undetectable serum HBV DNA and HBeAg negative status. These findings strongly suggest that HBV replication in HCC occurs more commonly than previously perceived by other studies[16,46-48]. Suzuki *et al*[48] reported 11.6/4.2% HBsAg/HBcAg in HCC and the highest reported was from Hsc *et al* and coworkers around 32/14.7% respectively.

To our knowledge, this is the first study to describe the distribution or patterns of HBV-associated antigens in HCC patients based on treatment status. Perhaps the most significant observation in our study is the high rate of HBV antigen detection in tumor tissue alone, but not in adjacent non-tumor liver, among those with optimal HBV DNA suppression. We identified five of 9 (56%) of HCC patients with either HBcAg or HBsAg present only in tumor tissues despite prolonged nucleoside analogue treatment. These data may imply that these tumors were derived from clonal expansion of infected hepatocytes with high rate of proliferation, carcinogenic potentials and selective resistance to antiviral agents[49,50]. Our findings also support the possible mechanisms of HBV-induced carcinogenesis secondary to HBV DNA integration into host genome leading to potential activation of oncogenes and induction of genetic instability. For instance, protein HBx is reported to be involved in activation of numerous signaling pathways and cellular promoters and to host DNA mutations[51,52].

This study, while providing new observations on the potential mechanisms of HCC development, has a number of limitations. It is a retrospective study with especially patients with HCC referred from other health centers. Hence, details about other potential confounding factors of tumor development such as alcoholism and other environmental exposure were unavailable for the analyses. Lastly, the enrolled subjects are mostly Asians, and hence our findings should be confirmed with other populations.

In conclusion, the presence of intrahepatic HBcAg is associated with HBeAg positive status. Hepatocyte expression of diffuse, mixed (cytoplasmic/nuclear) expression of HBcAg correlates with serum HBV DNA levels, but not with the histological activity. Intrahepatic HBsAg was detected in all chronic hepatitis B patients regardless of their HBeAg status or levels of viremia. While intrahepatic HBcAg is rarely detectable in HBeAg negative chronic HBV patients, majority of the HBeAg negative patients with hepatocellular carcinoma had positive HBcAg even with optimal viral suppression. The presence of either HBcAg or HBsAg within tumor tissues of HCC patients on treatment may indicate that these tumors were derived from clonal expansion of infected hepatocytes with high carcinogenic potentials and drug resistance.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a globally prevalent liver disease that can progress to cirrhosis and hepatocellular carcinoma (HCC). The outcome of the chronic HBV infection depends on both viral and host-specific factors. The disease outcomes have been correlated to serum HBV DNA levels and HBeAg status. Intra-hepatic distributions and localization patterns of viral antigens detected by immunohistochemistry could potentially provide additional prognostic values. In this study, the authors developed a modified classification system to characterize the expressions of HBV core antigen (HBcAg) and HBV surface antigen (HBsAg) in liver tissues of patients with chronic hepatitis B with and without HCC.

Research frontiers

The authors observed a high rate of HBV antigen detection in tumor tissue alone, but not in adjacent non-tumor liver in HCC patients with optimal HBV DNA suppression. This raised the question of HBV DNA integration into host genome leading to potential activation of oncogenes, induction of genetic instability and antiviral drug resistance.

Innovations and breakthroughs

The authors developed a modified classification system to standardize the reporting of the intrahepatic distributions and patterns of HBcAg and HBsAg. The authors discovered distinct differences in the distributions of these antigens in HBeAg positive and HBeAg negative chronic hepatitis B patients. Interestingly, authors observed variable HBV antigen expressions in the tumor tissues. These results provided insights into the potential mechanisms of disease persistence and carcinogenesis.

Applications

These findings open up new research study to confirm that these liver cancers were derived from clonal expansion of infected hepatocytes with high rate of proliferation, carcinogenic potentials and selective resistance to antiviral agents.

Terminology

Immunohistochemical staining of liver tissues: it applies standard streptavidin-biotin-immunoperoxidase technique to identify the various proteins in the liver.

Peer-review

The author of this paper evaluated the distributions and patterns of intrahepatic HBcAg and HBsAg in patients with chronic hepatitis B using a novel, modified classification system. The HBcAg expression is associated with high levels of serum HBV DNA and HBeAg status. The extensive HBsAg distribution even in HBeAg negative patents with low level of viremia explains the persistence of hepatitis B. The distinct expressions of viral antigens in tumor tissues deserve further evaluations to understand the carcinogenesis of HBV.

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ORIGINAL ARTICLE

Retrospective Study

Two strategies for prevention of cytomegalovirus infections after liver transplantation

Philipp Simon, Max Sasse, Sven Laudi, David Petroff, Michael Bartels, Udo X Kaisers, Sven Bercker

Philipp Simon, Max Sasse, Sven Laudi, Udo X Kaisers, Sven Bercker, Department of Anesthesia and Intensive Care Medicine, Medical Faculty, University of Leipzig, 04103 Leipzig, Germany

David Petroff, Clinical Trial Centre Leipzig, University of Leipzig, 04103 Leipzig, Germany

Michael Bartels, Department of Visceral, Transplantation, Vascular and Thoracic Surgery, Medical Faculty, University of Leipzig, 04103 Leipzig, Germany

Author contributions: Simon P and Bercker S designed research; Simon P and Sasse M performed research; Simon P and Bercker S contributed new reagents or analytic tools; Simon P, Sasse M and Petroff D analyzed data; Simon P, Petroff D, Bercker S, Laudi S, Bartels M and Kaisers UX wrote the paper.

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Correspondence to: Philipp Simon, MD, Department of Anesthesia and Intensive Care Medicine, Medical Faculty, University of Leipzig, Liebigstraße 20, 04103 Leipzig, Germany. philipp.simon@medizin.uni-leipzig.de
Telephone: +49-341-971700
Fax: +49-341-9717709

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Abstract

AIM: To analyze differences in patients' clinical course, we compared two regimes of either preemptive therapy or prophylaxis after liver transplantation.

METHODS: This retrospective study was reviewed and approved by the institutional review board of the University of Leipzig. Cytomegalovirus (CMV) prophylaxis with valganciclovir hydrochloride for liver transplant recipients was replaced by a preemptive strategy in October 2009. We retrospectively compared liver transplant recipients 2 years before and after October 2009. During the first period, all patients received valganciclovir daily. During the second period all patients included in the analysis were treated following a preemptive strategy. Outcomes included one year survival and therapeutic intervention due to CMV viremia or infection.

RESULTS: Between 2007 and 2010 n = 226 patients underwent liver transplantation in our center. n = 55 patients were D^+/R^- high risk recipients and were excluded from further analysis. A further 43 patients had to be excluded since CMV prophylaxis/preemptive strategy was not followed although there was no clinical reason for the deviation. Of the remaining 128 patients whose data were analyzed, 60 received

prophylaxis and 68 were treated following a preemptive strategy. The difference in overall mortality was not significant, nor was it significant for one-year mortality where it was 10% (95%CI: 8%-28%, P = 0.31) higher for the preemptive group. No significant differences in blood count abnormalities or the incidence of sepsis and infections were observed other than CMV. In total, 19 patients (14.7%) received ganciclovir due to CMV viremia and/or infections. Patients who were treated according to the preemptive algorithm had a significantly higher rate risk of therapeutic intervention with ganciclovir [n = 16 (23.5%) *vs* n = 3 (4.9%), P = 0.003)].

CONCLUSION: These data suggest that CMV prophylaxis is superior to a preemptive strategy in patients undergoing liver transplantation.

Key words: Transplantation; Liver; Cytomegalovirus; Preemptive; Prophylaxis; Valganciclovir; Therapy

Core tip: This retrospective study compares a preemptive therapy to prophylaxis for cytomegalovirus (CMV) infection in 128 patients after liver transplantation (LTx). CMV infections are frequent and increase morbidity and mortality so that preventive strategies are routine procedures. The one-year mortality did not differ significantly between the preemptive (n = 68) and prophylaxis (n = 60) groups, though it was 10% (95%CI: 8%-28%, P = 0.31) higher for the former. Preemptive patients had a significantly higher rate of intervention with ganciclovir (23.5% *vs* 4.9%, P = 0.003). Our data suggest that CMV prophylaxis is superior to a preemptive strategy after LTx.

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INTRODUCTION

Patients who undergo immunosuppressive therapy after solid organ transplantation are at higher risk for opportunistic bacterial, fungal, and viral infections. Infections with cytomegalovirus (CMV) are frequent and have been shown to increase morbidity and mortality in particular shortly after transplantation[1]. Estimates for the incidence of CMV infections after liver transplantation (LTx) range from 22% to 29%[1-3]. Therefore, strategies for preventing CMV infections are a routine procedure after solid organ transplantation[4]. Prophylaxis with antiviral substances leads to a reduction in the incidence and the severity of CMV infections. However, appropriate substances for prophylaxis have major side effects including myelodepression. Therefore, preemptive therapy has been proposed as an alternative regimen[1,5-7]. Preemptive therapy aims at suppressing viral replication after detection of CMV viremia, but prior to the onset of clinical symptoms. Antiviral therapy is then initiated in order to prevent clinically relevant infections[8-13]. However, to date, there is no strong clinical evidence indicating superiority of one regimen in liver transplant recipients over the other. To accumulate more evidence on the clinical course in patients after liver transplantation, we used retrospective data to compare preemptive therapy to prophylaxis during the liver transplantation program of the university hospital of Leipzig.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of the university of Leipzig (No. 122-12-16042012).

Prophylaxis with valganciclovir hydrochloride had been the standard treatment for liver transplant recipients in our center irrespective of their CMV serological status. Since patients presented with serious side effects including pancytopenia, prophylaxis was replaced by a preemptive strategy in October 2009. To assess differences in safety and efficacy of the two regimens, we retrospectively compared all liver transplant recipients two years before and after October 2009 during hospital stay after liver transplantation. Data on mortality was collected up to one year after transplantation. All patients treated according to the prophylaxis regimen received 450mg valganciclovir twice daily. If necessary doses were adapted to renal function. For patients treated after October 2009, only high-risk seronegative recipients, who received an organ from a seropositive donor (D^+/R^-), received prophylaxis and therefore all D^+/R^- patients in both groups were excluded from analysis. For both regimens, CMV polymerase chain reaction (PCR) was performed twice weekly. When PCR was positive, all patients in both groups were treated with ganciclovir for at least 14 d, whether or not there were clinical symptoms.

The data collected included baseline data, antibody patterns against CMV for donor and recipients (D/R), viremia during the intensive care unit (ICU) stay and occurrence of CMV infections. Furthermore, occurrence of sepsis, thrombocytopenia [platelet count < 50 giga particles (GPT)/L], leukocytopenia (white blood cells < 4 GPT/L) and anemia (hematocrit < 30%) starting 72 h after ICU admission was documented. LabMELD (Model of end stage liver disease) score was calculated for each patient on the day of transplantation. All MELD points were calculated retrospectively using validated laboratory data. Mortality data were collected during initial hospital stay and at day 28, day 90 and 1 year after transplantation. All patients received the same

Table 1 Patient demographics *n* (%)

	All (*n* = 128)	Prophylaxis (*n* = 60)	Preemptive therapy (*n* = 68)	*P* value
Age (yr)	54 ± 10	52 ± 12	56 ± 8.5	0.04
Sex				
Male	90 (70)	42 (70)	48 (71)	1.00
Female	38 (30)	18 (30)	20 (29)	
Weight (kg)	80 ± 16	81 ± 13	79 ± 19	0.52
SOFA	9.1 ± 4.0	8.8 ± 3.8	9.4 ± 4.3	0.34
APACHE II	14.2 ± 6.7	13.3 ± 5.5	15.0 ± 7.5	0.13
Lab. MELD at Transplantation	18.5 ± 9.4	18.8 ± 8.8	18.2 ± 9.9	0.73
CMV-status D/R				0.24
-/-	19 (17)	5 (10)	14 (22)	
-/+	33 (29)	16 (33)	17 (27)	
+/+	60 (54)	28 (57)	32 (51)	
+/-	Excluded			

Data are expressed as mean ± SD or *n* (%). Complete CMV-status was unavailable for 16 patients, but was known not to be +/-. D/R: Donor/receptor; CMV: Cytomegalovirus; Lab.MELD: Model of end stage liver disease.

standard immunosuppression with mycophenolate mofetil, steroids (tapered within 8 wk) and tacrolimus (days 1-14 FK506 level 10 ng/mL, days 15-28 8 ng/mL and continuing with 5 ng/mL). In the event of severe infection or side effects, immunosuppressive therapy was chosen by the physicians on an individual basis.

Statistical analysis

Data were collected in an Excel 2010 spreadsheet (Microsoft Corp., Redmond, United States). Statistical analysis was performed using SPSS Statistics 20.0 (SPSS GmbH Software, Munich, Germany) and R version 3.1.0. (R Foundation for Statistical Computing, Vienna, Austria). Survival analyses were performed using a log-rank test with the R package "survival". Categorical data are expressed as absolute or relative frequencies and the χ^2 or Fisher's exact test was used for inferential statistics depending on the number of expected counts. The confidence interval for differences in proportions makes use of a Wilson confidence interval. Continuous data or categorical ones with fewer than six levels are expressed as mean and standard deviation and a *t*-test was used for comparing groups. If categorical data has five or fewer levels, then median and interquartile range are presented and differences between groups are compared using the Wilcoxon-Mann-Whitney *U* test. A *P* value of less 0.05 was considered to be statistically significant.

RESULTS

Between 2007 and 2010 *n* = 226 patients underwent liver transplantation in our center. *n* = 55 patients were D^+/R^- high risk recipients and were excluded from further analysis. A further 43 patients had to be excluded since CMV prophylaxis/preemptive strategy was not followed although there was no clinical reason for the deviation. These 43 patients do not differ markedly from the remainder with respect to sex, age or labMELD. Of the remaining 128 patients whose data were analyzed, 60 received prophylaxis and 68 were treated following a preemptive strategy.

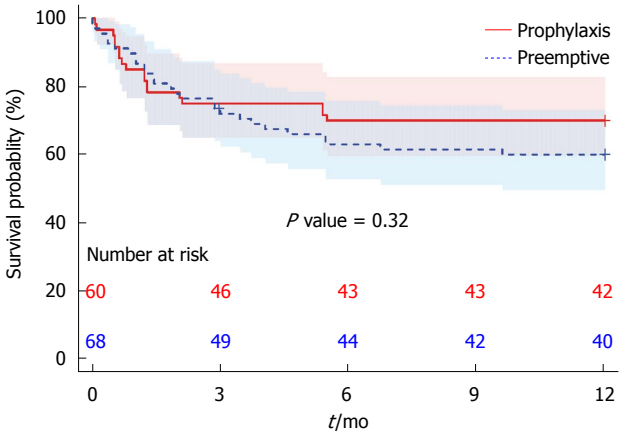

Figure 1 Survival curve. Mortality did not differ significantly between the groups.

Mean age of all analyzed patients was 54 ± 10 years, 90 patients were male and 38 were female (Table 1). The mean labMELD score before transplantation was 18.5 ± 9.4. At day 1 after transplantation patients had a mean APACHE Ⅱ (Acute Physiology And Chronic Health Evaluation Ⅱ) score of 14.2 ± 6.7 and a SOFA (simplified organ failure assessment) of 9.1 ± 4.0.

Mortality did not differ significantly between the groups (Figure 1). At one year, the mortality was 18/60 (30%, 95%CI: 20%-43%) for the prophylaxis strategy and 27/67 (40%, 95%CI: 29%-52%) for the preemptive strategy, where one censored case accounts for the denominator of 67 instead of 68 patients. However, this difference did not reach significance (*P* = 0.31).

There was a nonhomogeneous distribution of donors and recipients serologic CMV patterns in our series, which was not significant. We found 22% D^-/R^- in the preemptive group compared with 10% in the prophylaxis group (Table 1).

There were no significant differences in blood count abnormalities or the incidence of sepsis and infections other than CMV (Table 2). In total 19 patients (15%) received ganciclovir due to CMV viremia and/or infections. The therapy started 29 ± 20 d after transplantation on average and lasted for a mean of 18 ± 12 d. Patients who were treated according to the preemptive algorithm had a significantly higher rate risk of therapeutic intervention with ganciclovir [*n* = 16 (24%) *vs n* = 3 (5%), *P* = 0.005]. From these 19 patients 16 patients (84.0%) had clinical symptoms allegeable by CMV in context of the viremia [prophylaxis *n* = 2 (12.5%), preemptive therapy *n* = 14 (87.5%)].

Table 2 Infection/blood count abnormalities *n* (%)

	All (*n* = 128)	Prophylaxis (*n* = 60)	Preemptive therapy (*n* = 68)	*P* value
Re - LTx	17 (13)	10 (17)	7 (10)	0.31
Infection	86 (71)	40 (69)	46 (72)	0.84
Sepsis	63 (52)	30 (52)	33 (52)	1.00
Thrombocytopenia (PLT < 50 Gpt/L; > 72 h post-LTx	69 (57)	32 (55)	37 (58)	0.86
Leukocytopenia (WBC < 4 Gpt/L; > 72 h post-LTx)	38 (31)	15 (26)	23 (36)	0.25
Anaemia (Hct < 30%; > 72 h post-LTx)	114 (93)	54 (93)	60 (94)	1.00

GPT: Giga particles; LTx: Liver transplantation; PLT: Platelet count; WBC: White blood cells; Hct: Haematocrit.

DISCUSSION

We retrospectively compared the effects of a preemptive and a prophylactic strategy to prevent CMV infections during the early phase after liver transplantation. We could demonstrate that a preemptive strategy was associated with significantly more episodes of CMV viremia/infections in patients with mid/low risk without evidence for more side effects of the antiviral substances.

Recommendations for prevention of CMV infections in solid organ transplantation are heterogeneous and rather difficult to interpret. There are a few prospective randomized studies comparing both regimens after kidney transplantation. Kliem *et al*[15] demonstrated that prophylaxis reduced the incidence of CMV infections by 65% and the authors suggest that prophylaxis might improve long-term graft survival and recommend limiting preemptive strategies to low-risk patients. In a series of 296 kidney transplant patients, there was a reduction of CMV infections by 28 percentage points in the group who received prophylactic therapy (38.7% *vs* 11%, $P < 0.0001$)[16]. On the other hand, Reischig *et al*[17] and Khoury *et al*[18] found a similar incidence of CMV infections in both groups (6% *vs* 9%, $P = 0.567$) and therefore suggested a comparable efficacy of both strategies. Gerna *et al*[19] performed a controlled, randomized, open-label study in 21 children after liver transplantation and did not report any CMV disease, irrespective of the procedure. A recent Cochrane analysis concluded that data is not yet sufficient to recommend one strategy over the other prophylaxis or preemptive strategies[14].

For adult liver transplant recipients, data from randomized studies are not available. In clinical practice there is a broad variety of strategies concerning the selection of substances, the timing and the duration of either prophylaxis or preemptive strategies. However, most centers tend to use prophylaxis at least in high-risk patients[16].

All told, there is no strong evidence from randomized studies indicating which of the two strategies is superior after liver transplantation. Accordingly, several authors consider both treatment options to be similarly effective[8,11,12,20-22].

Positive effects of a preemptive strategy can be the reduction of side effects and costs. When compared to no preventive strategy at all, preemptive therapy leads to less graft rejection and may improve CMV specific cell-mediated immunity and lead to a decreased risk of late CMV infections[11,13,23]. On the other hand, the use of prophylactic strategies has been shown to reduce mortality, the incidence of graft loss and opportunistic viral[24], bacterial or fungal infections[25]. Otherwise, prophylaxis increases the risk of drug-resistance and the incidence of late-onset CMV disease[26].

The incidence of CMV infection varies depending upon donor and/or recipient serological status[19,27,28]. Hodson *et al*[25] suggested in a systematic review of randomized trials, that CMV infections are more frequently in sero-negative patients and current guidelines suggest antiviral prophylaxis at least in D^+/R^- patients[29,30]. In the presented study, these patients have been excluded from analysis. There is an imbalance of sero-negative patients between the two groups in our study (Table 1) and following the above argument, one might have supposed that the prophylaxis group was at higher risk of infection. It turns out that none of the 19 sero-negative patients in the study had CMV viremia/infection, however, so that this could not have contributed to our findings.

The presented retrospective study of consecutive treatment groups has limitations. Due to the retrospective character and lack of randomization, a variety of factors such as slight changes in therapeutic regimens over-time may have influenced results. On the other hand, we demonstrated that groups did not differ concerning demographic baseline data, severity of liver failure or severity of disease at admission, and therefore suggest that our results strongly support the hypothesis that the higher incidence of CMV viremia was mainly influenced by the introduction of a preemptive strategy. Attributing clinical symptoms such as diarrhea or elevation of liver enzymes in the early course after LTX to CMV remains uncertain as there are a broad variety of possible causes. However, we suggest that our results support the hypothesis that prophylaxis is more effective in preventing CMV viremia and infection even in patients with low or mid risk for CMV infection. In conclusion, we demonstrated a significantly lower rate of CMV viremia/infection with prophylaxis when compared to a preemptive strategy. Our data indicate that prophylaxis might be superior to a preemptive strategy. To confirm this hypothesis, randomized prospective trials in liver transplant recipients are needed.

COMMENTS

Background

Patients after solid organ transplantation who undergo immunosuppressive therapy are at higher risk for infections with cytomegalovirus. They are frequent and have been shown to increase morbidity and mortality, particularly during the early stages after liver transplantation. Therefore, strategies for preventing cytomegalovirus (CMV) infections are a routine procedure after liver transplantation. Prophylaxis with antiviral substances leads to a reduction in the incidence and the severity of CMV infections. Preemptive therapy aims at suppression of viral replication after detection of CMV viremia and prior to the onset of clinical symptoms. The authors compared preemptive therapy to prophylaxis during the liver transplantation program of the university hospital of Leipzig to analyze differences in the clinical course in patients after liver transplantation.

Research frontiers

There is no strong evidence from randomized studies demonstrating which of the strategies is superior after liver transplantation. Accordingly, several authors consider both treatment options to be similarly effective. The authors demonstrated a significantly lower rate of CMV viremia/infection using prophylaxis when compared to a preemptive strategy. The data indicate that prophylaxis might be superior to a preemptive strategy.

Innovations and breakthroughs

Current study found a lower incidence of CMV viremia/infection with prophylaxis compared to a preemptive strategy without significant differences in blood count abnormalities or the incidence of infections and differences in mortality at any time. These data indicate that prophylaxis might be superior to a preemptive strategy.

Applications

The data indicate that prophylaxis might be superior to a preemptive strategy. To confirm this hypothesis randomized prospective trials in liver transplant recipients are needed.

Peer-review

The topic has a great interest given the lack of studies in this field in liver transplant recipients. Nevertheless the higher rate of seronegative patients in the pre-emptive group could explain the higher rate of infections in this group. It would be interesting to include some data regarding duration of treatment in both groups and regarding the specific period of time after transplant in which the CMV infection occurred (early infection *vs* late infection).

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ORIGINAL ARTICLE

Clinical Trials Study

Daclatasvir *vs* telaprevir plus peginterferon alfa/ribavirin for hepatitis C virus genotype 1

Ira Jacobson, Stefan Zeuzem, Robert Flisiak, Brygida Knysz, Stefan Lueth, Dorota Zarebska-Michaluk, Ewa Janczewska, Peter Ferenci, Moises Diago, Anna Linda Zignego, Rifaat Safadi, Yaacov Baruch, Dzhamal Abdurakhmanov, Stephen Shafran, Dominique Thabut, Rafael Bruck, Adrian Gadano, Alexander James Thompson, Justin Kopit, Fiona McPhee, Tracy Michener, Eric A Hughes, Philip D Yin, Stephanie Noviello

Ira Jacobson, Weill Cornell Medical College, New York, NY 10029, United States

Ira Jacobson, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

Stefan Zeuzem, JW Goethe University Hospital, 60323 Frankfurt, Germany

Robert Flisiak, Uniwersytet Medyczny w Białymstoku, 15-089 Białystok, Poland

Brygida Knysz, EMC and Medical University, 50-220 Wrocław, Poland

Stefan Lueth, Universitatsklinikum Hamburg-Eppendorf, 20246 Hamburg, Germany

Dorota Zarebska-Michaluk, Wojewodzki Szpital Zespolony w Kielcach, 25-736 Kielce, Poland

Ewa Janczewska, ID Clinic, 41-400 Mysłowice, Poland

Peter Ferenci, Medizinische Universität Wien, 1090 Vienna, Austria

Moises Diago, Clinica Quiron, 46010 Valencia, Spain

Anna Linda Zignego, Università degli Studi di Firenze, 50121 Florence, Italy

Rifaat Safadi, Holy Family Hospital, Nazareth 6004, Israel

Yaacov Baruch, Rambam Medical Center, Haifa 31096, Israel

Dzhamal Abdurakhmanov, 1st Moscow State Medical University N.A.I.M. Sechenov, 119991 Moscow, Russia

Stephen Shafran, University of Alberta Hospital, Edmonton, Alberta T6G 2B7, Canada

Dominique Thabut, Groupe Hospitalier Pitié-Salpêtrière, 75013 Paris, France

Rafael Bruck, Tel Aviv Sourasky Medical Center and Tel Aviv University, Tel Aviv 64239, Israel

Adrian Gadano, Hospital Italiano de Buenos Aires, Buenos Aires C1181ACH, Argentina

Alexander James Thompson, St. Vincents Hospital and the University of Melbourne, Melbourne 3065, Australia

Justin Kopit, Fiona McPhee, Philip D Yin, Bristol-Myers Squibb Research and Development, Wallingford, CT 06492, United States

Tracy Michener, Eric A Hughes, Stephanie Noviello, Bristol-Myers Squibb Research and Development, Princeton, NJ 08450, United States

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Correspondence to: Dr. Ira Jacobson, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, United States. ijacobson@chpnet.org
Telephone: +1-917-7978812

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Abstract

AIM: To evaluate daclatasvir *vs* telaprevir, each combined with peginterferon alfa-2a/ribavirin (pegIFN/RBV), in treatment-naive hepatitis C virus (HCV) genotype (GT) 1-infected patients.

METHODS: In this phase 3, randomized, open-label, noninferiority study, 602 patients were randomly assigned (2:1) to daclatasvir *vs* telaprevir, stratified by *IL28B* rs12979860 host genotype (CC *vs* non-CC), cirrhosis status (compensated cirrhosis *vs* no cirrhosis), and HCV GT1 subtype (GT1a *vs* GT1b). Patients were selected by study inclusion criteria from a total of 793 enrolled patients. Patients received daclatasvir 60 mg once daily or telaprevir 750 mg 3 times daily plus pegIFN/RBV. Daclatasvir recipients received 24 wk of daclatasvir plus pegIFN/RBV; those without an extended rapid virologic response (eRVR; undetectable HCV-RNA at weeks 4 and 12) received an additional 24 wk of pegIFN/RBV. Telaprevir-treated patients received 12 wk of telaprevir plus pegIFN/RBV followed by 12 (with eRVR) or 36 (no eRVR) wk of pegIFN/RBV. The primary objective was to compare for noninferiority of sustained virologic response rates at posttreatment week 12 (SVR12) in GT1b-infected patients. Key secondary objectives were to demonstrate that the rates of anemia (hemoglobin < 10 g/dL) and rash-related events, through week 12, were lower with daclatasvir + pegIFN/RBV than with telaprevir + pegIFN/RBV among GT1b-infected patients. Resistance testing was performed using population-based sequencing of the NS5A region for all patients at baseline, and for patients with virologic failure or relapse and HCV-RNA $\geqslant$ 1000 IU/mL, to investigate any link between NS5A polymorphisms associated with daclatasvir resistance and virologic outcome.

RESULTS: Patient demographics and disease characteristics were generally balanced across treatment arms; however, there was a higher proportion of black/African Americans in the daclatasvir groups (6.0% and 8.2% in the GT1b and GT1a groups, respectively) than in the telaprevir groups (2.2% and 3.0%). Among GT1b-infected patients, daclatasvir plus pegIFN/RBV was noninferior to telaprevir plus pegIFN/RBV for SVR12 [85% (228/268) *vs* 81% (109/134); difference, 4.3% (95%CI: -3.3% to 11.9%)]. Anemia (hemoglobin < 10 g/dL) was significantly less frequent with daclatasvir than with telaprevir [difference, -29.1% (95%CI: -38.8% to -19.4%)]. Rash-related events were also less common with daclatasvir than with telaprevir, but the difference was not statistically significant. In GT1a-infected patients, SVR12 was 64.9% with daclatasvir and 69.7% with telaprevir. Among both daclatasvir and telaprevir treatment groups, across GT1b- or GT1a-infected patients, lower response rates were observed in patients with *IL28B* non-CC and cirrhosis - factors known to affect response to pegIFN/RBV. Consistent with these observations, a multivariate logistic regression analysis in GT1b-infected patients demonstrated that SVR12 was associated with *IL28B* host genotype (CC *vs* non-CC, P = 0.011) and cirrhosis status (absent *vs* present, P = 0.031). NS5A polymorphisms associated with daclatasvir resistance

(at L28, R30, L31, or Y93) were observed in 17.3% of GT1b-infected patients at baseline; such variants did not appear to be absolute predictors of failure since 72.1% of these patients achieved SVR12 compared with 86.9% without these polymorphisms. Among GT1b-infected patients, treatment was completed by 85.4% (229/268) in the daclatasvir group, and by 85.1% (114/134) in the telaprevir group, and among GT1a-infected patients, by 67.2% (90/134) and 69.7% (46/66), respectively. Discontinuations (of all 3 agents) due to an AE were more frequent with telaprevir than with daclatasvir, whereas discontinuations due to lack of efficacy were more frequent with daclatasvir, due, in part, to differences in futility criteria.

CONCLUSION: Daclatasvir plus pegIFN/RBV demonstrated noninferiority to telaprevir plus pegIFN/RBV for SVR12 and was well-tolerated in treatment-naive GT1b-infected patients, supporting the use of daclatasvir with other direct-acting antivirals.

Key words: Direct-acting antiviral; Chronic hepatitis C; Daclatasvir; Genotype 1b; NS5A inhibitor; Liver disease

Core tip: This phase 3 study describes the first prospective comparison of an NS5A inhibitor and an NS3/4A protease inhibitor in peginterferon-based regimens. Combinations of peginterferon alfa-2a/ribavirin (pegIFN/RBV) with boceprevir or telaprevir were the standard-of-care for genotype (GT) 1-infected patients at the time of study design. In treatment-naive GT1b-infected patients, daclatasvir (NS5A inhibitor) plus pegIFN/RBV achieved a sustained virologic response at posttreatment week 12 (SVR12) of 85% and demonstrated noninferiority to telaprevir plus pegIFN/RBV showing 81% SVR12. Daclatasvir plus pegIFN/RBV was well-tolerated, with a superior safety profile for anemia compared with telaprevir plus pegIFN/RBV. These results support the ongoing investigation of daclatasvir in all-oral combinations in multiple patient populations.

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) affects 130-150 million people globally and is a major cause of cirrhosis and hepatocellular carcinoma[1]. Of the 7 HCV genotypes (GTs) identified[2], GT1 is the most prevalent worldwide, and accounts for 75% of all infections in the United States (US)[3]. GT1 can be classified into the two main subtypes GT1a and GT1b, of which GT1b is the most common worldwide, predominating in Europe, Japan, and China; in the US 36% of all GT1 infections are subtype 1b[4].

Peginterferon alfa plus ribavirin (pegIFN/RBV) has traditionally been used to treat HCV. However, this regimen achieves only limited sustained virologic response (SVR) rates of 40%-50%[5,6] and is associated with a high frequency of adverse events (AEs)[7]. Although pegIFN/RBV-based therapies continue to be the standard of care in some countries[8], HCV treatment has evolved toward direct-acting antiviral agents (DAAs) that target specific viral proteins[5,6], with the first all-oral combinations for GT1 recently approved in Japan, Europe, the US, and Canada[9-15].

Combinations of pegIFN/RBV with one of the NS3/4A protease inhibitors boceprevir or telaprevir were the first DAA-based regimens approved and at the time of study design, the standard-of-care for GT1-infected patients. With telaprevir plus pegIFN/RBV, SVR rates increased from < 50% with pegIFN/RBV alone to 72%-75% in GT1-infected, treatment-naive patients[16-18]. However, skin rash and anemia are frequent, and sometimes severe, adverse events (AEs) observed with telaprevir[16-19]. In patients treated with telaprevir plus pegIFN/RBV, rash has been reported in 35%-37% of patients (compared with 24% with pegIFN/RBV), necessitating premature discontinuation of telaprevir in 7% of patients, and anemia in 37%-42% (compared with 19% with pegIFN/RBV)[16-18]. Combinations of pegIFN/RBV with more recent DAAs, such as the NS5B inhibitor sofosbuvir or the NS3/4A protease inhibitor simeprevir, achieved SVR rates of 80%-90% and demonstrated a more favorable safety profile than telaprevir plus pegIFN/RBV[20-22]. Telaprevir plus pegIFN/RBV has been compared with simeprevir plus pegIFN/RBV in treatment-experienced GT1-infected patients[23]; however, for treatment-naive patients, no direct comparison of telaprevir *vs* a non-protease inhibitor DAA has been performed to date.

Daclatasvir is a potent, once-daily, pangenotypic NS5A inhibitor[24,25] that has been studied and shown to be well-tolerated in > 13000 patients. In phase 2 trials in treatment-naive patients infected with GT1-4, daclatasvir + pegIFN/RBV demonstrated greater efficacy than pegIFN/RBV alone[26,27]. In GT1-infected patients, daclatasvir plus pegIFN/RBV achieved SVR at posttreatment week 24 (SVR24) rates of 60% compared with 38% with pegIFN/RBV; response rates were consistently higher in patients with GT1b (77%) than in those with GT1a (55%)[27], a finding that has also been observed with other DAA + pegIFN/RBV

combinations[16,21,28]. Daclatasvir-containing pegIFN-free regimens are approved for treatment of chronic HCV infection in a number of countries: daclatasvir plus asunaprevir (ASV, NS3 inhibitor) was approved as the first all-oral treatment for GT1 in Japan[9], and daclatasvir plus sofosbuvir (with or without ribavirin) is approved in Europe for GT1, 3, and 4[10], and in Canada for GT1, 2, and 3[29]. Daclatasvir is also approved in the US, indicated in combination with sofosbuvir for the treatment of chronic HCV GT3 infection[30].

This phase 3 COMMAND-3 study compared the safety and efficacy of daclatasvir, an NS5A inhibitor, with that of telaprevir, a protease inhibitor, each in combination with pegIFN/RBV, in treatment-naive patients with GT1 infection, with a focus on GT1b-infected patients.

MATERIALS AND METHODS

Study design

This was a phase 3, randomized, open-label, non-inferiority study in treatment-naive patients with GT1 infection (Study AI444-052; ClinicalTrials.gov number NCT01492426). Overall, 602 patients were randomly assigned (2:1) to daclatasvir *vs* telaprevir, stratified by *IL28B* rs12979860 host genotype (CC *vs* non-CC), cirrhosis status (compensated cirrhosis *vs* no cirrhosis), and HCV GT1 subtype (GT1a *vs* GT1b). Patients with compensated cirrhosis were capped at 25%. GT1a-infected patients were capped at 200 (33%); this cap was introduced during enrollment (May 2012) based on data from a previous daclatasvir phase 2 study indicating that daclatasvir was more effective in GT1b-infected than in GT1a-infected patients[27]. Patients were treated with daclatasvir 60 mg/d (n = 402) or telaprevir 750 mg 3 times/d (n = 200) in combination with pegIFN alfa-2a 180 μg once weekly and RBV [weight-based dosing of 1000 mg/d (< 75 kg) or 1200 mg/d (≥ 75 kg)]. Daclatasvir-treated patients with undetectable HCV-RNA at weeks 4 and 12 [extended rapid virologic response (eRVR)] had a planned treatment duration of 24 wk of daclatasvir plus pegIFN/RBV; those without eRVR received an additional 24 wk of pegIFN/RBV (total of 48 wk of therapy), provided they did not experience treatment futility. Telaprevir-treated patients received 12 wk of telaprevir plus pegIFN/RBV, followed by 12 (with eRVR) or 36 (without eRVR) weeks of pegIFN/RBV alone, provided they did not experience treatment futility. Patients in both groups were followed for 24 wk (without eRVR) or 48 wk (with eRVR) posttreatment.

In the daclatasvir group, treatment futility, which mandated discontinuation of all study drugs, was defined as: (1) virologic breakthrough [> 1-$\log_{10}$ increase in HCV-RNA over nadir or confirmed HCV-RNA ≥ lower limit of quantification (LLOQ) after confirmed undetectable HCV-RNA while on treatment beginning at week 2 of therapy]; (2) week 12 HCV-RNA > 1000 IU/mL; or (3) week 24 HCV-RNA ≥ LLOQ. In the telaprevir group, treatment futility was defined per prescribing information as week 4 or 12 HCV-RNA > 1000 IU/mL or week 24 HCV-RNA detectable confirmed; virologic breakthrough was not included in the telaprevir futility criteria, per the prescribing information[19]. Relapse was defined as undetectable HCV-RNA at the end of treatment (EOT) followed by confirmed HCV-RNA ≥ LLOQ at any follow-up visit.

Patients

The study included treatment-naive patients aged 18 years or older, with GT1a or GT1b infection, and HCV-RNA ≥ 10000 IU/mL at screening. Patients with no cirrhosis or compensated cirrhosis [by liver biopsy at any time, or by FibroScan™ (≥ 14.6 kPa) within 1 year of screening] were eligible for inclusion. No previous treatment of HCV with interferon-based regimens or DAAs was allowed. Other exclusion criteria included evidence of decompensated liver disease (including a history or presence of ascites, bleeding varices, or hepatic encephalopathy), evidence of a medical condition contributing to chronic liver disease other than HCV, documented or suspected hepatocellular carcinoma or other malignancies, co-infection with HIV or hepatitis B virus, alanine aminotransferase ≥ 5 × the upper limit of normal, hemoglobin < 12 g/dL (120 g/L) for women and < 13 g/dL (130 g/L) for men, platelet count < 90 × 10^9 cells/L, international normalized ratio ≥ 1.7, albumin < 3.5 g/dL (35 g/L), or any criterion that would exclude the patient from receiving pegIFN/RBV or telaprevir. Patients were randomized to a regimen within stratum *via* block randomization (block size of 6) and using an interactive voice response system prepared by the sponsor.

Objectives and assessments

Primary and secondary objectives were amended during enrollment to focus on GT1b-infected patients based on the results of previous phase 2 data[27]. The primary objective was to demonstrate that daclatasvir plus pegIFN/RBV was noninferior to telaprevir plus pegIFN/RBV for SVR at posttreatment week 12 (HCV-RNA < LLOQ at posttreatment week 12) in GT1b-infected patients. The first two secondary objectives were to demonstrate that the rates of anemia (hemoglobin < 10 g/dL) and of rash-related events, through week 12, were lower with daclatasvir plus pegIFN/RBV than with telaprevir plus pegIFN/RBV, among GT1b-infected patients (see Supplementary Material and Methods for the definition of rash-related events). Additional secondary objectives included noninferiority comparisons between arms of undetectable HCV-RNA at week 4 [rapid virologic response (RVR)], week 12 [complete early virologic response (cEVR)], and weeks 4 and 12 (eRVR), and HCV-RNA < LLOQ at post-treatment week 24 (SVR24),

in GT1b-infected patients. The final secondary objective was a noninferiority comparison between arms of SVR12 in GT1a-infected patients.

HCV-RNA was assayed using the Roche HCV COBAS® TaqMan® test v2.0 (LLOQ = 25 IU/mL; limit of detection approximately 10 IU/mL). HCV GT and subtype were determined by Versant HCV GT 2.0 assay (LIPA) and were analyzed by ICON Central Laboratories, Inc. *IL28B* genotype was determined by polymerase chain reaction amplification coupled with allelic discrimination. Resistance testing was performed using population-based sequencing of the NS5A region for all patients at baseline, and for patients with virologic failure or relapse and with amplifiable (HCV-RNA $\geqslant$ 1000 IU/mL) plasma samples. Safety monitoring was based on the incidences of AEs, serious AEs (SAEs), discontinuations due to AEs, laboratory abnormalities, vital signs, and physical examinations.

Statistical analysis

The statistical methods of this study were reviewed by the biometrics group at Bristol-Myers Squibb. A noninferiority margin of -12% was employed in this study. A 2-sided 95% confidence interval (CI) for the difference in rates, daclatasvir plus pegIFN/RBV minus telaprevir plus pegIFN/RBV, was used to test for noninferiority. To demonstrate noninferiority, the lower bound of the CI had to be > -0.12. A sample size of 400 GT1b-infected patients, randomized 2:1 to daclatasvir plus pegIFN/RBV *vs* telaprevir plus pegIFN/RBV, provided 91% power to show that the SVR12 rate of daclatasvir plus pegIFN/RBV was noninferior to that of telaprevir plus pegIFN/RBV at the 5% significance level, assuming SVR12 rates of 85% for both regimens[16].

Each secondary comparison was conducted at the 5% level and proceeded hierarchically according to the order of the objectives (see Objectives and assessments). Testing of an endpoint was performed only if the null hypothesis of the preceding endpoint was rejected. Safety comparisons, for anemia and rash-related events, were for superiority, *i.e.*, to show that daclatasvir plus pegIFN/RBV was less toxic than telaprevir plus pegIFN/RBV. Secondary efficacy comparisons were for noninferiority (noninferiority margin -12%).

Efficacy analyses were restricted to all treated patients and were performed using a modified intent-to-treat (mITT) analysis (patients with missing HCV-RNA measurements were considered failures). For the primary endpoint, an analysis based on SVR documented on or after (if follow-up week 12 HCV-RNA was missing) posttreatment week 12 was also conducted. A stratum-adjusted, 2-sided, asymptotic 95%CI was used to compute the difference in SVR12 rates between arms[31]. The strata were those used in the randomization. Stratum-adjusted CIs were also used for testing differences between rates for secondary efficacy endpoints.

All authors had access to the study data and have reviewed and approved the final manuscript.

RESULTS

Patient disposition

Overall, 793 patients were screened; of these, 402 GT1b-infected patients were randomized and treated with daclatasvir plus pegIFN/RBV (*n* = 268) or telaprevir plus pegIFN/RBV (*n* = 134), and 200 GT1a-infected patients were randomized and treated with daclatasvir plus pegIFN/RBV (*n* = 134) or telaprevir plus pegIFN/RBV (*n* = 66) (Figure 1). Among GT1b-infected patients, treatment was completed by 85.4% (229/268) in the daclatasvir group, and by 85.1% (114/134) in the telaprevir group, and among GT1a-infected patients, by 67.2% (90/134) and 69.7% (46/66), respectively. Discontinuations (of all three agents) due to an AE were more frequent with telaprevir than with daclatasvir, whereas discontinuations due to lack of efficacy were more frequent with daclatasvir. This was due, in part, to the different futility criteria in the two treatment groups. Posttreatment follow-up was initiated by 384 patients in the daclatasvir group and by 191 patients in the telaprevir group and was completed by 359 (93.5%) and 181 (94.8%), respectively.

Baseline characteristics

Patient demographics and disease characteristics were generally balanced across treatment arms (Table 1); however, there was a higher proportion of black/African Americans in the daclatasvir groups (6.0%-8.2%) than in the telaprevir groups (2.2%-3.0%). Mean HCV-RNA levels ranged from 6.23-6.31 $\log_{10}$ IU/mL, 9.7%-13.6% of patients had cirrhosis, and there was a high proportion of patients with a non-CC *IL28B* genotype (68.7%-79.9%) across all groups.

Efficacy in GT1b-infected patients

In GT1b-infected patients, SVR12 rates were 85.1% (228/268) with daclatasvir plus pegIFN/RBV *vs* 81.3% (109/134) with telaprevir plus pegIFN/RBV (mITT; primary endpoint) (Table 2); the difference between treatment arms was 4.3% (95%CI: -3.3% to 11.9%), demonstrating noninferiority (lower bound of the 95%CI greater than -12%). Similar SVR12 rates in GT1b-infected patients were obtained when using the next available HCV-RNA value for patients with missing posttreatment week 12 measurements [daclatasvir plus pegIFN/RBV: 85.8% (230/268); telaprevir plus pegIFN/RBV: 82.1% (110/134)]. Response rates observed for the secondary efficacy endpoints (RVR, eRVR, cEVR, SVR24) in GT1b-infected patients appeared similar between treatment arms (Table 2); however, no formal comparisons could be made because the test for the difference in rash-

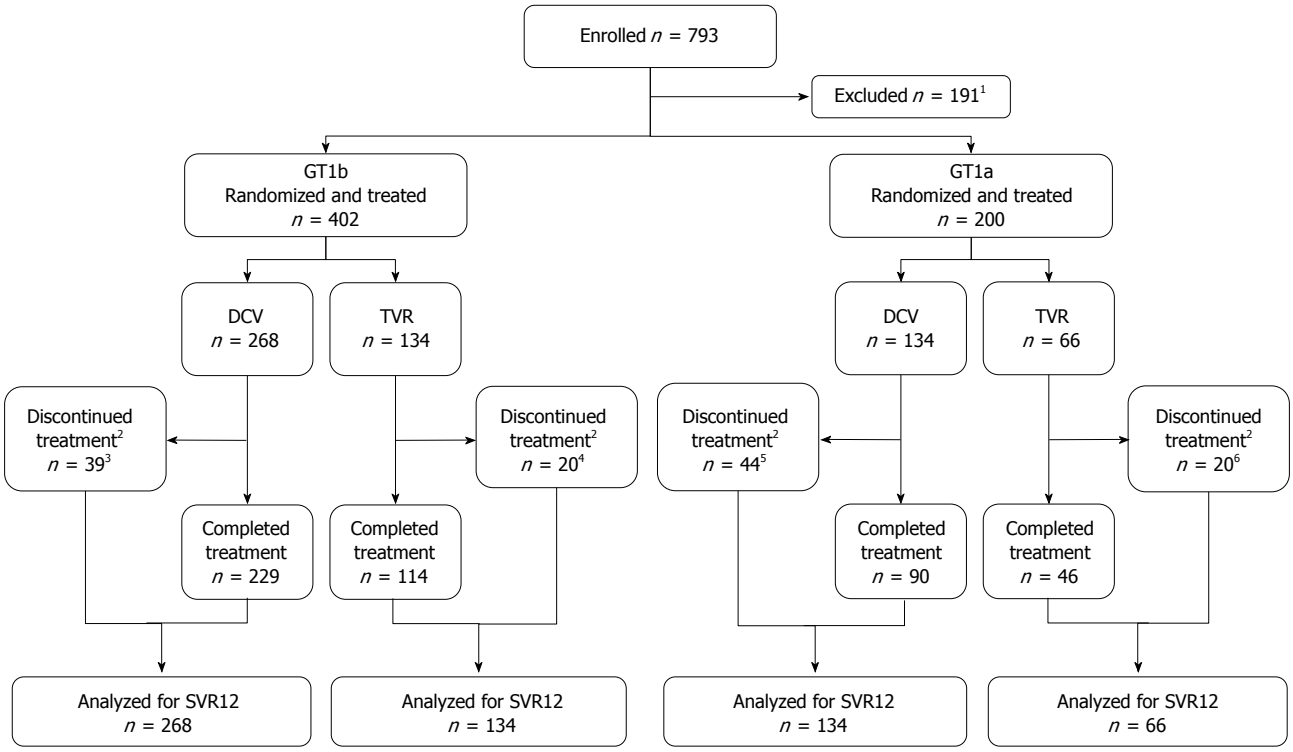

Figure 1 Patient disposition. Efficacy analyses were based on a modified intent-to-treat analysis (included all randomized and treated patients who had received ⩾ 1 dose of study medication; patients with missing HCV RNA measurements were considered failures). [1]No longer met study entry criteria during the screening period (*n* = 139), withdrew consent (*n* = 28), administrative reason by sponsor (*n* = 5), lost to follow-up (*n* = 3), pretreatment AE (*n* = 2), or other reasons (*n* = 11); [2]All 3 study drugs discontinued; [3]Lack of efficacy (*n* = 15; 11 virologic breakthroughs, 3 futility, and 1 other), AE (*n* = 14), patient request (*n* = 6), lost to follow-up (*n* = 3), and withdrew consent (*n* = 1); [4]AE (*n* = 17), withdrew consent (*n* = 2), and lost to follow-up (*n* = 1); [5]Lack of efficacy (*n* = 23; 11 virologic breakthroughs, 4 futility, 8 other), adverse event (*n* = 11), lost to follow-up (*n* = 6), patient request (*n* = 1), and other (*n* = 3); [6]Lack of efficacy (*n* = 5; 3 futility, 2 other), AE (*n* = 8), patient request (*n* = 3), lost to follow-up (*n* = 3), and death (*n* = 1; the death occurred in a patient who had discontinued treatment at week 16 due to bacteremia and died at posttreatment week 4 due to sepsis secondary to HCV-related cirrhosis). DCV: Daclatasvir; GT: Genotype; SVR12: Sustained virologic response (HCV-RNA < LLOQ) at posttreatment week 12; TVR: Telaprevir.

Table 1 Patient demographics and baseline disease characteristics

n (%)	GT1b		GT1a	
	DCV + pegIFN/RBV (*n* = 268)	TVR + pegIFN/RBV (*n* = 134)	DCV + pegIFN/RBV (*n* = 134)	TVR + pegIFN/RBV (*n* = 66)
Age (yr), median (range)	46.0 (18-71)	48.0 (19-69)	49.0 (19-67)	51.5 (28-69)
Male	159 (59.3)	72 (53.7)	98 (73.1)	47 (71.2)
Race				
White	243 (90.7)	129 (96.3)	120 (89.6)	63 (95.5)
Black/African American	16 (6.0)	3 (2.2)	11 (8.2)	2 (3.0)
Asian	6 (2.2)	2 (1.5)	1 (0.7)	0
Other	3 (1.1)	0	2 (1.5)	1 (1.5)
HCV-RNA $\log_{10}$ (IU/mL), mean (SD)	6.23 (0.701)	6.23 (0.577)	6.30 (0.637)	6.31 (0.636)
HCV-RNA ⩾ 800000 IU/mL	196 (73.1)	97 (72.4)	104 (77.6)	51 (77.3)
IL28B genotype				
CC	53 (19.8)	27 (20.1)	42 (31.3)	20 (30.3)
CT	161 (60.1)	86 (64.2)	73 (54.5)	37 (56.1)
TT	53 (19.8)	21 (15.7)	19 (14.2)	9 (13.6)
Not reported	1 (0.4)	0	0	0
Cirrhosis				
Present	26 (9.7)	15 (11.2)	16 (11.9)	9 (13.6)

DCV: Daclatasvir; GT: Genotype; HCV: Hepatitis C virus; pegIFN: Peginterferon alfa-2a; RBV: Ribavirin; TVR: Telaprevir.

related events between arms, which preceded the comparisons of secondary efficacy endpoints in the testing hierarchy, was not statistically significant (see Safety section). Among GT1b-infected patients with SVR12, three patients did not achieve SVR24 (one patient in each arm relapsed between posttreatment

Table 2 Efficacy endpoints and failures in GT1b-infected patients

Outcome, *n*/*n* (%)	DCV + pegIFN/RBV	TVR + pegIFN/RBV
Efficacy		
SVR12 (mITT)[1,2]	228/268 (85.1)	109/134 (81.3)
SVR12 on or after PT week 12[3]	230/268 (85.8)	110/134 (82.1)
RVR (HCV-RNA undetectable at week 4)[1,4]	207/268 (77.2)	106/134 (79.1)
cEVR (HCV-RNA undetectable at week 12)[1,4]	243/268 (90.7)	121/134 (90.3)
eRVR (HCV-RNA undetectable at weeks 4 and 12)[1,4]	201/268 (75.0)	98/134 (73.1)
EOTR (HCV-RNA undetectable at EOT)	244/268 (91.0)	131/134 (97.8)
SVR24[1,4]	226/268 (84.3)	108/134 (80.6)
Failures		
Non-SVR12	40/268 (14.9)	25/134 (18.7)
On-treatment failures	21/268 (7.8)	3/134 (2.2)
Virologic breakthrough	11/268 (4.1)	NA[5]
Treatment futility other than virologic breakthrough	3/268 (1.1)	0
HCV-RNA detectable at EOT	7/268 (2.6)	3/134 (2.2)
Posttreatment relapse[6]	12/244 (4.9)	20/131 (15.3)
HCV-RNA undetectable at EOT but missing PT week 12 data	7/244 (2.9)	2/131 (1.5)

[1]Patients with missing data at posttreatment week 12 were considered failures; [2]Difference, 4.3%; 95%CI: -3.3% to 11.9%; noninferior at the 5% significance level (lower bound of the 95%CI was > -12%); [3]Patients with missing data at posttreatment week 12 were considered responders if the next available HCV-RNA value was < LLOQ; [4]Differences and 95%CIs were as follows: RVR, -1.5% (-9.8% to 6.8%); cEVR, 0.6 (-5.5% to 6.6%); eRVR, 2.2% (-6.8% to 11.2%); SVR24, 4.4% (-3.5% to 12.2%); [5]Not included in TVR futility criteria per prescribing information; [6]Assessed in patients with undetectable HCV-RNA at EOT. cEVR: Complete early virologic response; DCV: Daclatasvir; EOT: End of treatment; EOTR: End of treatment response; eRVR: Early rapid virologic response; GT: Genotype; HCV: Hepatitis C virus; LLOQ: Lower limit of quantitation; mITT: Modified intent-to-treat; NA: Not applicable; pegIFN: Peginterferon alfa-2a; PT: Posttreatment; RBV: Ribavirin; SVR12: Sustained virologic response (HCV-RNA < LLOQ) at posttreatment week 12; RVR: Rapid virologic response; SVR24: Sustained virologic response at posttreatment week 24; TVR: Telaprevir.

Table 3 Proportion of GT1b-infected patients with SVR12 by subgroups

SVR12[1], *n*/*n* (%)	DCV + pegIFN/RBV (*n* = 268)	TVR + pegIFN/RBV (*n* = 134)
Age (yr)		
< 65	218/256 (85.2)	103/126 (81.7)
⩾ 65	10/12 (83.3)	6/8 (75.0)
Sex		
Male	134/159 (84.3)	61/72 (84.7)
Female	94/109 (86.2)	48/62 (77.4)
Race		
White	208/243 (85.6)	105/129 (81.4)
Black/African American	11/16 (68.8)	2/3 (66.7)
Asian	6/6 (100.0)	2/2 (100.0)
Other	3/3 (100.0)	0
Baseline HCV-RNA		
< 800000 IU/mL	66/72 (91.7)	33/37 (89.2)
⩾ 800000 IU/mL	162/196 (82.7)	76/97 (78.4)
Cirrhosis		
Absent	208/242 (86.0)	99/119 (83.2)
Present	20/26 (76.9)	10/15 (66.7)
IL28B genotype		
CC	51/53 (96.2)	23/27 (85.2)
CT	132/161 (82.0)	69/86 (80.2)
TT	44/53 (83.0)	17/21 (81.0)

[1]mITT analysis (patients with missing data at posttreatment week 12 were considered failures). DCV: Daclatasvir; GT: Genotype; HCV: Hepatitis C virus; pegIFN: Peginterferon alfa-2a; RBV: Ribavirin; SVR12: Sustained virologic response (HCV-RNA < LLOQ) at posttreatment week 12; TVR: Telaprevir.

weeks 12 and 24, and one patient in the daclatasvir group had a missing posttreatment week 24 HCV-RNA measurement).

In GT1b-infected patients, observed SVR12 rates tended to be higher in the daclatasvir arm than in the telaprevir arm in subgroups based on demographic and disease status, such as age, sex, *IL28B*, cirrhosis, and baseline HCV-RNA level (Table 3). Of note, among cirrhotics, a higher proportion of patients receiving daclatasvir *vs* telaprevir achieved SVR12 (76.9% *vs* 66.7%). In the daclatasvir group, SVR12 appeared to be independent of age and sex. In both treatment groups, lower response rates were observed in patients with *IL28B* non-CC, baseline HCV-RNA ⩾ 800000 IU/mL, or cirrhosis, baseline factors known to affect response to pegIFN/RBV[32]. Consistent with these observations, a multivariate logistic regression analysis in GT1b-infected patients demonstrated that SVR12 was associated with *IL28B* host genotype (CC *vs* non-CC, $P = 0.011$), baseline HCV-RNA (< 800000 IU/mL *vs* ⩾ 800000 IU/mL, $P = 0.016$), and cirrhosis status (absent *vs* present, $P = 0.031$). Virologic response was not associated with age, sex, race, or type of treatment (Supplementary Table 3).

Virologic failure in GT1b-infected patients

Forty (14.9%) patients in the daclatasvir group and 25 (18.7%) patients in the telaprevir group did not achieve SVR12 (Table 2). Virologic breakthrough occurred in 11 (4.1%) patients in the daclatasvir group. Virologic breakthrough was not assessed as a futility criteria for the telaprevir group, per the telaprevir prescribing information[19]. Relapse (among patients with undetectable HCV-RNA at EOT) was reported in 12/244 (4.9%) patients in the daclatasvir group and 20/131 (15.3%) patients in the telaprevir group. Other posttreatment failures, occurring in

Table 4 Overall on-treatment safety in GT1-infected patients

Event, *n* (%)	DCV + pegIFN/RBV (*n* = 402)	TVR + pegIFN/RBV (*n* = 200)[1]
Death	1 (0.2)[2]	1 (0.5)[2]
SAEs	26 (6.5)[2]	20 (10.0)[2]
AEs leading to discontinuation of any study drug	28 (7.0)[2]	37 (18.5)[2]
AEs leading to discontinuation of all 3 study drugs	25 (6.2)	25 (12.5)
AEs (grade 1-4) ⩾ 20%		
Fatigue	140 (34.8)	81 (40.5)
Headache	137 (34.1)	57 (28.5)
Asthenia	109 (27.1)	53 (26.5)
Pruritus	107 (26.6)	75 (37.5)
Anemia	96 (23.9)	99 (49.5)
Rash	93 (23.1)	69 (34.5)
Nausea	88 (21.9)	74 (37.0)
Neutropenia	87 (21.6)	27 (13.5)
Alopecia	86 (21.4)	32 (16.0)
Influenza-like illness	85 (21.1)	38 (19.0)
Dry skin	84 (20.9)	34 (17.0)
Pyrexia	80 (19.9)	42 (21.0)
Grade 3 or 4 emergent laboratory abnormalities		
Hemoglobin	26 (6.5)	41 (20.5)
Absolute neutrophil count	104 (25.9)	41 (20.5)
Lymphocytes	67 (16.7)	44 (22.0)
Platelet count	15 (3.7)	8 (4.0)
ALT	3 (0.7)	4 (2.0)
AST	7 (1.7)	1 (0.5)
Total bilirubin	4 (1.0)	6 (3.0)
Serum creatinine increased	1 (0.2)	0

[1]*n* = 198 for grade 3/4 laboratory abnormalities; [2]Further information provided in supplementary material. AE: Adverse event; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; DCV: Daclatasvir; GT: Genotype; pegIFN: Peginterferon alfa-2a; RBV: Ribavirin; SAE: Serious adverse event; TVR: Telaprevir.

7/244 (2.9%) patients in the daclatasvir group and 2/131 (1.5%) patients in the telaprevir group, were due to undetectable HCV-RNA at EOT but missing posttreatment week 12 HCV-RNA.

At baseline, 249/268 GT1b-infected patients treated with daclatasvir plus pegIFN/RBV had available NS5A population-based sequencing data. In 43/249 (17.3%) of these patients, one or more of the NS5A polymorphisms L28M/V, R30H/Q, L31M, or Y93H were detected at baseline; of these, 72.1% (31 patients) achieved SVR12, of which 61.3% (19 patients) had a non-CC *IL28B* genotype. Of the remaining 12 patients who did not achieve SVR12, 11 had a non-CC *IL28B* genotype. Among patients without NS5A polymorphisms at baseline, 87% (179/206) achieved SVR12.

Among the 40 GT-1b patients in the daclatasvir group who did not achieve SVR12, 32 had evaluable samples at baseline and at the time of failure. In two patients, the same NS5A resistance-associated variants (RAVs) were detected at both baseline and failure: L31M-Y93H in one patient, and L28V-R30Q-L31M-Q62D in a second patient. Among the remaining 30 patients, the most common treatment-emergent NS5A RAVs were L31F/I/M/V (22 patients) and Y93H (21 patients); RAVs at L31 and Y93 emerged together in 18 patients. NS5A L31 and Y93 RAVs also emerged together in one daclatasvir-treated patient who achieved SVR12 but relapsed at posttreatment week 24. Of the eight non-SVR12 patients who were not tested at failure [due to undetectable HCV-RNA at last available visit (*n* = 5), missing HCV-RNA measurement (*n* = 2), or lost to follow-up while HCV-RNA was < 1000 IU/mL (*n* = 1)], seven had no NS5A polymorphisms at baseline, and one had no baseline NS5A sequence available.

Efficacy outcomes in GT1a-infected patients

In GT1a-infected patients, SVR12 rates were 64.9% in the daclatasvir group and 69.7% in the telaprevir group. Other outcomes and treatment failures in GT1a-infected patients are shown in Supplementary Table 1. Baseline NS5A sequences were available for 123 GT1a-infected patients treated with daclatasvir; the polymorphisms Q30R, L31M, and/or Y93N were detected in 6 (5%) patients, of whom 5 achieved SVR12. Among 34 GT1a-infected virologic failures with evaluable samples, the most common emergent NS5A RAVs at failure were Q30E/H/R (32 patients). In patients with emergent Q30 RAVs, L31 variants were also frequently detected, either as on-treatment emergent RAVs (19 patients) or pre-existing at baseline (one patient). In a daclatasvir-treated patient who relapsed after achieving SVR12, the NS5A RAV Q30E was detected at posttreatment week 24.

Safety

Safety, pooled across GT1 subtypes, is shown in

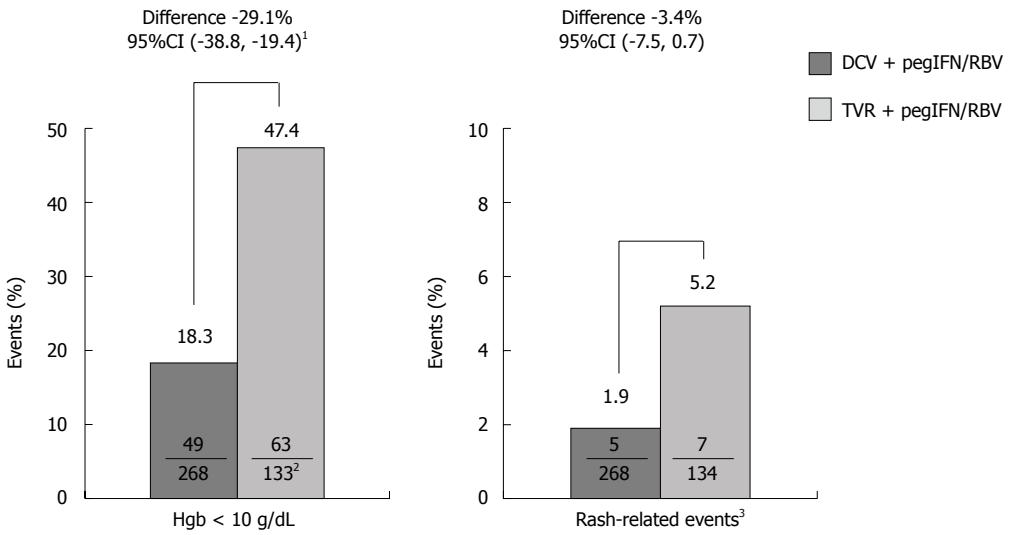

Figure 2 Safety secondary endpoints in GT1b-infected patients (first 12 wk). mITT analysis (patients with missing data at posttreatment week 12 were considered failures). [1]Superior at the 5% significance level; upper bound of the 95%CI was 0%; [2]One patient in the TVR arm had no measurement due to discontinuation prior to on-treatment laboratory assessment; [3]Includes rash-related SAEs, AEs leading to discontinuation, and grade 3 or 4 AEs. AE: Adverse event; CI: Confidence interval; DCV: Daclatasvir; GT: Genotype; Hgb: Hemoglobin; pegIFN: Peginterferon alfa-2a; RBV: Ribavirin; SAE: Serious adverse event; TVR: Telaprevir.

Table 4. AEs leading to discontinuation of any study drug were more frequent with telaprevir (18.5%) *vs* daclatasvir (7.0%). In the daclatasvir group, the most common (≥ 1%) AEs leading to discontinuation of any study drug were psychiatric (1.5%), skin (1.5%), or hemolytic events (1.2%); in the telaprevir group the most common AEs were skin events (9.0%), hemolytic events (4.5%), general disorders (3.0%), gastrointestinal events (2.0%), psychiatric events (1.5%), infections and infestations (1.5%), nervous system disorders (1.5%), or renal events (1.0%). Serious AEs (SAEs) were reported in 6.5% of daclatasvir-treated patients and in 10.0% of telaprevir-treated patients. Drug-related SAEs were reported in 3.5% (n = 14) and 8.0% (n = 16) of treated patients in the daclatasvir and telaprevir arms, respectively, including one case of drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome in each treatment group and five cases of anemia in the telaprevir group. The daclatasvir-treated patient with DRESS syndrome, a 62-year-old male with GT1b-infection, developed grade 3 rash after approximately 2.5 mo of therapy, followed by hyperthermia and hypereosinophilia (eosinophils 1.12 × 10^9 cells/L) leading to hospitalization. Topical corticosteroid (betamethasone) and trimeprazine resulted in rapid improvement and normalization of the eosinophil count (0.41 × 10^9 cells/L) within 10 d of discontinuing study therapy. The patient achieved SVR12. The five telaprevir-treated patients with a related SAE of anemia all had grade 4 anemia with hemoglobin levels of 54-72 g/L; all 5 patients received blood transfusions and 2 discontinued study therapy. The most common (> 25%) AEs were fatigue, headache, asthenia, and pruritus in the daclatasvir group, and anemia, fatigue, nausea, rash, pruritus, headache, and asthenia in the telaprevir group. Grade 3 or 4 laboratory abnormalities were comparable between treatment groups, except for grade 3 or 4 hemoglobin levels, which were more frequent with telaprevir (20.5%) than with daclatasvir (6.5%). Grade 3 or 4 bilirubin elevations occurred in 3% of patients in the telaprevir group compared with 1% in the daclatasvir group.

One death was reported in each treatment group, both of which occurred during off-treatment follow-up and were considered unrelated to therapy. In the daclatasvir group, the patient died during posttreatment week 4 from multiple fractures and a subdural hematoma related to a fall. In the telaprevir group, the patient, who had discontinued treatment at week 16 (due to bacteremia), died during posttreatment week 4 from sepsis secondary to cirrhosis due to hepatitis C.

Grade 1-4 anemia was experienced by 23.9% of GT1-infected patients in the daclatasvir group, and 49.5% in the telaprevir group, including 3.2% (n = 13) and 13.5% (n = 27), respectively, with grade 3 or 4 anemia. Incidences for the secondary endpoint of hemoglobin < 10 g/dL in GT1b-infected patients through week 12 were significantly lower in the daclatasvir group than in the telaprevir group (daclatasvir: 18.3% *vs* telaprevir: 47.4%; difference, -29.1; 95%CI: -38.8% to -19.4%; Figure 2). Grade 1-4 rash [single Medical Dictionary for Regulatory Activities (MedDRA) term] was reported in 23.1% of GT1-infected patients in the daclatasvir group and in 34.5% in the telaprevir group, including 1.0% (n = 4) and 3.5% (n = 7), respectively, with grade 3 or 4 rash. Incidences for the secondary endpoint of rash-related events (composite MedDRA term) in GT1b-infected patients through week 12 were also lower with daclatasvir than with telaprevir, however the difference was not statistically significant given the

low event rates in both arms (daclatasvir: 1.9%; telaprevir: 5.2%; difference, -3.4; 95%CI: -7.5% to 0.7%; Figure 2).

Safety was also assessed in subgroups of patients with and without cirrhosis at baseline (Supplementary Table 2). In both treatment arms, anemia, reduced platelet count, and elevated total bilirubin were more frequent in patients with than in those without cirrhosis. Among cirrhotic patients, those treated with telaprevir experienced a higher frequency of the AEs anemia (58.3% *vs* 33.3%), rash (41.7% *vs* 21.4%), fatigue (50.0% *vs* 38.1%), and nausea (37.5% *vs* 11.9%), and of SAEs (16.7% *vs* 7.1%); in contrast, neutropenia was more common with daclatasvir (26.2%) than with telaprevir (16.7%) but was not associated with an increase in infections (daclatasvir: 21.4%; telaprevir: 41.7%).

DISCUSSION

This study evaluated the efficacy and safety of daclatasvir plus pegIFN/RBV compared with telaprevir plus pegIFN/RBV in HCV GT1-infected patients and is the first head-to-head comparison of two different DAA classes - an NS5A inhibitor *vs* an NS3/4A protease inhibitor - in combination with pegIFN/RBV in treatment-naive patients. In treatment-experienced GT1-infected patients simeprevir (NS3/4A protease inhibitor) plus pegIFN/RBV has been compared with telaprevir plus pegIFN/RBV, with simeprevir plus pegIFN/RBV demonstrating noninferiority to telaprevir plus pegIFN/RBV for SVR12 (54% *vs* 55%) and a more favourable safety profile[23].

In the present study in treatment-naive, GT1b-infected patients, SVR12 rates achieved with daclatasvir plus pegIFN/RBV were noninferior to those observed with telaprevir plus pegIFN/RBV, and anemia was significantly less frequent among daclatasvir-treated patients than in telaprevir-treated patients. Rash-related events were also less common among patients receiving daclatasvir *vs* telaprevir. Daclatasvir plus pegIFN/RBV demonstrated high SVR12 rates in GT1b-infected patients across all subgroups of baseline factors known to affect response rates to pegIFN/RBV (cirrhosis, *IL28B* genotype, age, sex, baseline viral load). Importantly, in difficult-to-cure patients with cirrhosis, SVR12 rates were higher with daclatasvir plus pegIFN/RBV than with telaprevir plus pegIFN/RBV (76.9% *vs* 66.7%). On-treatment treatment futility was more common with daclatasvir than with telaprevir; however this may have been related to the different futility criteria between the two treatment groups. However, posttreatment relapse was more frequent with telaprevir than with daclatasvir (15% *vs* 5%).

Using population-based sequencing of the NS5A region, NS5A polymorphisms associated with daclatasvir resistance (L28, R30, L31, and/or Y93) were observed in 17% of GT1b-infected patients at baseline. Although such variants may be associated with virologic outcome to daclatasvir plus pegIFN/RBV, they do not appear to be absolute predictors of failure because 72% of GT1b-infected patients with these polymorphisms at baseline achieved SVR12 in this study. Furthermore, since most (11/12) patients with baseline NS5A polymorphisms who did not achieve SVR12 also had a non-CC *IL28B* genotype, it may be that *IL28B* genotype has a stronger association with virologic failure. A similar observation was reported in a previous daclatasvir plus pegIFN/RBV phase 2 study, in which only 2/10 GT1b-infected patients with L31 or Y93 variants failed to achieve a response, but 30/32 GT1b virologic failures were non-CC *IL28B*[27].

A relevant consideration is that the persistence of RAVs may influence further treatment options in an IFN-free context. In this study, patients were not monitored beyond post-treatment week 24, thus analysis of the persistence of NS5A RAVs was not undertaken. However, persistence beyond one year has been described previously in GT1b- and GT1a-infected patients[33,34]. Persistence of emergent NS5A RAVs has also been described from an interim analysis of a three-year follow-up study[35]. Detection of viral variants by direct sequencing limits the ability to detect low-frequency variants within a viral population; however, despite this limitation, the results of these analyses have been useful for describing relationships between RAVs and clinical outcomes.

In this study, response rates with daclatasvir plus pegIFN/RBV were lower and virologic failure was more frequent in patients infected with HCV GT1a than in patients infected with GT1b. Most likely, this is due to a lower resistance barrier of daclatasvir in GT1a-infected patients rather than to a higher potency in GT1b-infected patients, since the EC_{50} of daclatasvir was low in both GT1a-infected patients (20 pM) and GT1b-infected patients (4 pM)[24]. These lower response rates observed among GT1a-infected patients are consistent with previous studies of daclatasvir[27] and have also been observed with other DAAs, including telaprevir[16], boceprevir[28], and simeprevir[21].

Daclatasvir plus pegIFN/RBV was generally well-tolerated, with an overall safety profile similar to that of pegIFN/RBV alone, with no new safety or tolerability concerns attributable to daclatasvir. In the overall GT1-infected population, anemia events (grade 1-4 and grade 3 or 4) were less common in the daclatasvir group (24% and 3%, respectively) than in the telaprevir group (50% and 14%, respectively), and were also less frequently a reason for treatment discontinuation (daclatasvir: 0.5%; telaprevir: 4.0%). Among GT1b-infected patients, daclatasvir plus pegIFN/RBV demonstrated superiority over telaprevir plus pegIFN/RBV for the secondary endpoint of hemoglobin < 10 g/dL (18% *vs* 47%).

Grade 1-4 and grade 3 or 4 rash (single MedDRA

term) were also less frequent in the overall GT1-infected population in patients receiving daclatasvir (23% and 1%, respectively) than in those receiving telaprevir (35% and 4%, respectively), as were discontinuations due to rash (daclatasvir: 0.2%; telaprevir: 4.5%). The grade 1-4 events of rash observed in the daclatasvir group are most likely related to pegIFN/RBV, because the observed rate (23%) is comparable to that historically reported with pegIFN/RBV alone[16,27], and no cases of rash have been reported with daclatasvir all-oral regimens, including daclatasvir plus sofosbuvir[36]. Rash-related events (composite MedDRA term) were evaluated as a secondary endpoint in this study in GT1b-infected patients, and a lower incidence was observed in those receiving daclatasvir than in those receiving telaprevir, but this difference was not statistically significant due to low event rates in both arms. The single case of DRESS syndrome observed with daclatasvir is the only case reported to date in the daclatasvir development program and real-word experience.

At the time this study was designed, telaprevir plus pegIFN/RBV was the standard of care for the treatment of HCV GT1 infection; however, other DAAs have now been approved, both in combination with pegIFN/RBV and as part of IFN-free regimens. Currently approved all-oral treatment options for GT1-infected patients include daclatasvir plus sofosbuvir ± RBV (approved for GT1, 3, and 4 in the EU[10]), which has been shown to achieve SVR12 rates of up to 98% in treatment-naive GT1-infected patients[36], and the dual combination daclatasvir plus asunaprevir (approved for GT1b in Japan[9]), which has been shown to achieve SVR12 rates of 90% in GT1b-infected treatment-naive patients[37]. Other approved all-oral regimens include the combinations of sofosbuvir + ledipasvir ± RBV[11,12] and simeprevir plus sofosbuvir ± RBV[13,14,38], which achieved SVR rates of up to 98% and 94%, respectively, in this patient population. Ombitasvir/paritaprevir/ritonavir plus dasabuvir, which was recently approved, provided SVR rates of 90% (without RBV) and 95%-97% (with RBV) in treatment-naive noncirrhotic patients infected with GT1a, and of 99% (without RBV) and 98%-100% (with RBV) in those infected with GT1b[36,39,40]; in GT1-infected patients with cirrhosis SVR12 was 96%[41]. Daclatasvir is also being evaluated as part of an all-oral, fixed-dose combination with asunaprevir and beclabuvir (formerly BMS-791325). In phase 3 studies, this regimen without RBV has provided SVR12 rates of 92% in GT1a- and 1b-infected treatment-naive patients without cirrhosis[42], and SVR12 rates of 93%-98% in GT1a- and 1b-infected patients with compensated cirrhosis, with or without RBV[43]; in these studies, SVR12 rates were lower in patients infected with GT1a than in those infected with GT1b when RBV was excluded from the regimen.

Although all-oral regimens have become the new standard of care in chronic hepatitis C management, pegIFN/RBV-based therapies will remain a potentially important treatment option in such settings as low-income countries or possibly for patients who have failed DAA-only therapies.

In conclusion, this first and only head-to-head comparison of two classes of DAAs in treatment-naive patients demonstrates that daclatasvir plus pegIFN/RBV is noninferior to telaprevir plus pegIFN/RBV for SVR12 for treatment of HCV GT1b infection. Daclatasvir plus pegIFN/RBV was generally well-tolerated, with a significantly lower rate of anemia, and an observed lower rate of rash-related events compared with telaprevir plus pegIFN/RBV. The results of this study and other studies support the role of daclatasvir as an effective and well-tolerated component of interferon-free, all-oral HCV regimens across multiple genotypes.

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COMMENTS

Background

Chronic infection with hepatitis C virus (HCV) affects 130-150 million people globally and is a major cause of cirrhosis and hepatocellular carcinoma. Peginterferon alfa plus ribavirin (pegIFN/RBV) has historically been used to treat HCV, although this regimen achieves only limited sustained virologic response (SVR) rates of 40%-50% in genotype 1 (GT1) infection. Whilst pegIFN/RBV-based therapies remain the standard of care in some countries, HCV treatment has evolved toward direct-acting antiviral agents (DAAs) that target specific viral proteins.

Research frontiers

The combination of pegIFN/RBV with the NS3/4A protease inhibitor telaprevir was one of the first approved DAA-based regimens, and replaced pegIFN/RBV as the standard-of-care for GT1-infected patients. Skin rash and anemia are frequent, and at times severe, adverse events (AEs) observed with telaprevir. No direct comparison of telaprevir *vs* a non-protease inhibitor DAA has been performed to date. Daclatasvir is a potent, once-daily, pangenotypic NS5A inhibitor; this phase 3 study compared the safety and efficacy of daclatasvir with that of telaprevir, each in combination with pegIFN/RBV, in treatment-naive patients with GT1 infection, with a focus on GT1b-infected patients.

Innovations and breakthroughs

Among GT1b-infected patients, daclatasvir plus pegIFN/RBV was noninferior to telaprevir plus pegIFN/RBV for SVR12 [85% (228/268) *vs* 81% (109/134); difference, 4.3% (95%CI: -3.3% to 11.9%)]. Daclatasvir was generally well-tolerated: anemia (hemoglobin < 10 g/dL) was significantly less frequent with daclatasvir than with telaprevir [difference, -29.1% (95%CI: -38.8% to -19.4%)], and rash-related events were less frequent with daclatasvir plus pegIFN/RBV compared with telaprevir plus pegIFN/RBV. Discontinuations of all 3 agents due to an adverse event were more frequent with telaprevir than with daclatasvir, whereas discontinuations due to lack of efficacy were more frequent with daclatasvir, due, in part, to differences in futility criteria.

Applications

Although all-oral regimens have become the new standard of care in chronic HCV management - for which daclatasvir is currently approved for the treatment of HCV GT1, GT3, and GT4 infections (approvals vary by country) - pegIFN/

RBV-based therapies will remain a potentially important treatment option in such settings as low-income countries or possible for patients who have failed DAA-only therapies. The results of this study and other studies support the role of daclatasvir as an effective and well-tolerated component of interferon-free, all-oral HCV regimens across multiple genotypes.

Terminology

SVR12/24, sustained virologic response [HCV-RNA < lower limit of quantitation (LLOQ)] at posttreatment week 12/24; Relapse, undetectable HCV-RNA at the end of treatment followed by confirmed HCV-RNA ⩾ LLOQ at any follow-up visit. In the daclatasvir group, treatment futility, which mandated discontinuation of all study drugs, was defined as (1) virologic breakthrough (> 1-$\log_{10}$ increase in HCV-RNA over nadir or confirmed HCV-RNA ⩾ LLOQ after confirmed undetectable HCV-RNA while on treatment beginning at week 2 of therapy); (2) week 12 HCV-RNA >1000 IU/mL; or (3) week 24 HCV-RNA ⩾ LLOQ. In the telaprevir group, treatment futility was defined per prescribing information as HCV-RNA > 1000 IU/mL (at weeks 4 or 12) or HCV-RNA detectable confirmed (at week 24).

Peer-review

The arena within which HCV is treated has moved at a very rapid pace over the past 5 years. The range of DAA options has expanded since telaprevir and boceprevir were the first protease inhibitors to become available. While globally, the world is focused on DAAs as the treatments of choice for HCV, these drugs are expensive and not all countries will be in a position to treat infection *vs* disease. In fact, some first-world countries are currently only treating patients with advanced disease due to the cost of these drugs. The authors make this point nicely and rightly state that there may be a place for IFN based anti-viral strategies for the management and treatment of HCV.

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ORIGINAL ARTICLE

Observational Study

Intracorporeal esophagojejunostomy after totally laparoscopic total gastrectomy: A single-center 7-year experience

Ke Chen, Yu Pan, Jia-Qin Cai, Xiao-Wu Xu, Di Wu, Jia-Fei Yan, Rong-Gao Chen, Yang He, Yi-Ping Mou

Ke Chen, Yu Pan, Jia-Qin Cai, Xiao-Wu Xu, Di Wu, Jia-Fei Yan, Rong-Gao Chen, Yang He, Yi-Ping Mou, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Institute of Micro-Invasive Surgery, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Author contributions: Chen K, Pan Y and Cai JQ wrote the manuscript; Mou YP, Xu XW, Chen K, and Yan JF performed the operations; Chen RG, He Y, and Wu D reviewed the medical records and collected data; Mou YP proofread and revised the manuscript; all authors read and approved the final manuscript.

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Correspondence to: Yi-Ping Mou, MD, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Institute of Micro-Invasive Surgery, Zhejiang University, 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. mouyiping2002@163.com
Telephone: +86-571-86006952
Fax: +86-571-86044817

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Abstract

AIM: To assess the efficacy and safety of intracorporeal esophagojejunostomy in patients undergoing laparoscopic total gastrectomy (LTG) for gastric cancer.

METHODS: A retrospective review of 81 consecutive patients who underwent LTG with the same surgical team between November 2007 and July 2014 was performed. Four types of intracorporeal esophagojejunostomy using staplers or hand-sewn suturing were performed after LTG. Data on clinicopatholgoical characteristics, occurrence of complications, postoperative recovery, anastomotic time, and operation time among the surgical groups were obtained through medical records.

RESULTS: The average operation time was 288.7 min, the average anastomotic time was 54.3 min, and the average estimated blood loss was 82.7 mL. There were no cases of conversion to open surgery. The first flatus was observed around 3.7 d, while the liquid diet was started, on average, from 4.9 d. The average postoperative hospital stay was 10.1 d. Postoperative complications occurred in 14 patients, nearly 17.3%.

However, there were no cases of postoperative death.

CONCLUSION: LTG performed with intracorporeal esophagojejunostomy using laparoscopic staplers or hand-sewn suturing is feasible and safe. The surgical results were acceptable from the perspective of minimal invasiveness.

Key words: Gastric cancer; Total gastrectomy; Esophagojejunostomy; Laparoscopy; Hand-sewn

Core tip: Totally laparoscopic distal gastrectomy using intracorporeal anastomosis has gradually increased with advances in laparoscopic surgical instrumentation. However, intracorporeal esophagojejunostomy is still uncommon after totally laparoscopic total gastrectomy due to technical difficulties. Herein, we evaluate various types of intracorporeal esophagojejunostomy using laparoscopic staplers and a hand-sewn technique.

Chen K, Pan Y, Cai JQ, Xu XW, Wu D, Yan JF, Chen RG, He Y, Mou YP. Intracorporeal esophagojejunostomy after totally laparoscopic total gastrectomy: A single-center 7-year experience. *World J Gastroenterol* 2016; 22(12): 3432-3440 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3432.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3432

INTRODUCTION

Since the first reported laparoscopy assisted distal gastrectomy (LADG) was performed for gastric disease in 1994[1], for tumors relatively low in the stomach, interest for this surgical approach has continued to grow. For middle or upper gastric adenocarcinoma, laparoscopy assisted total gastrectomy (LATG) has become more popular than laparoscopy assisted proximal gastrectomy (LAPG) due to the relatively lower rate of reflux esophagitis. In addition, LATG is the treatment of choice for middle or upper gastric adenocarcinoma with deeper invasion[2,3].

Because of the narrow operating window, however, technical problems during extracorporeal esophagojejunostomy may necessitate performing an exit *via* a mini-laparotomy. This relatively small incision has a number of disadvantages, including removal of the possibly challenging specimen, contamination through the incision, and extra pulling over the residual stomach[4].

Based on our extensive laparoscopic experience obtained from performing laparoscopic pancreatic, gastric, and other operations[5-11], we developed totally laparoscopic total gastrectomy (TLTG) for middle or upper gastric cancer. Here, we describe our 7-year experience using this technique and the short-term clinical results obtained using different kinds of intracorporeal esphagojejunostomy using laparoscopic staplers or a hand-sewn suture technique.

MATERIALS AND METHODS

Patients

From November 2007 to July 2014, a total of 81 consecutive patients with gastric cancer underwent TLTG that was performed by a single surgical team at Sir Run Run Shaw Hospital. Preoperative clinical evaluation, which included clinical grading, was assessed with computed tomography, abdominal ultrasonography, endoscopic ultrasonography, esophagogastroduodenoscopy, and gastrointestinal radiography.

Clinical statistics were collected from medical records of the patients: sex, age, body mass index (BMI), method of anastomosis, time required for anastomosis, total operation time, estimated blood loss, the day of first flatus and liquid diet, postoperative complications, and length of the postoperative hospital stay. Pathological and clinical staging was determined based on the American Joint Committee on Cancer (the 7th edition) and the tumor-node-metastasis (TNM) classification scheme. This research was approved by the Zhejiang University's Ethics Committee. Written consent was obtained from every patient prior to enrollment in the study.

General surgical procedures

A former depicted approach was used to position the patient and the trocar location[6]. Five major trocars were inserted in the V-shape arrangement. The guidelines of gastric cancer in Japan served as the principle, including the No. 8a, 9, 10, 11p, 11d, and 12a apart from the dissection of D_1. The operation was started by retracting the greater omentum and then bluntly dissembling along with the transverse colon, which is the border that accesses the lesser sac. In addition, the gastroepiploic vessels were confirmed, clipped, and then excised. Mobilization was begun at the comparative superior edge of the pancreas, thereby unveiling the celiac trunk. The common hepatic artery, the right gastric artery, and the left gastric artery together with the splenic artery and the neighboring lymph nodes were excised and identified. Meanwhile, those veins were excised and clipped. The hepatogastric and hepatoduodenal ligaments were dissected. The duodenum was later transected with 3 cm from the pylorus using the endoscopic linear stapler. The jejunum was then stapled using an endoscopic linear stapler, which was 20 cm from Treitz's ligament. The detailed lymphadenectomy was described in our previously published study[7]. In general, there were two approaches for intracorporeal gastrointestinal reconstruction, mechanical circular or

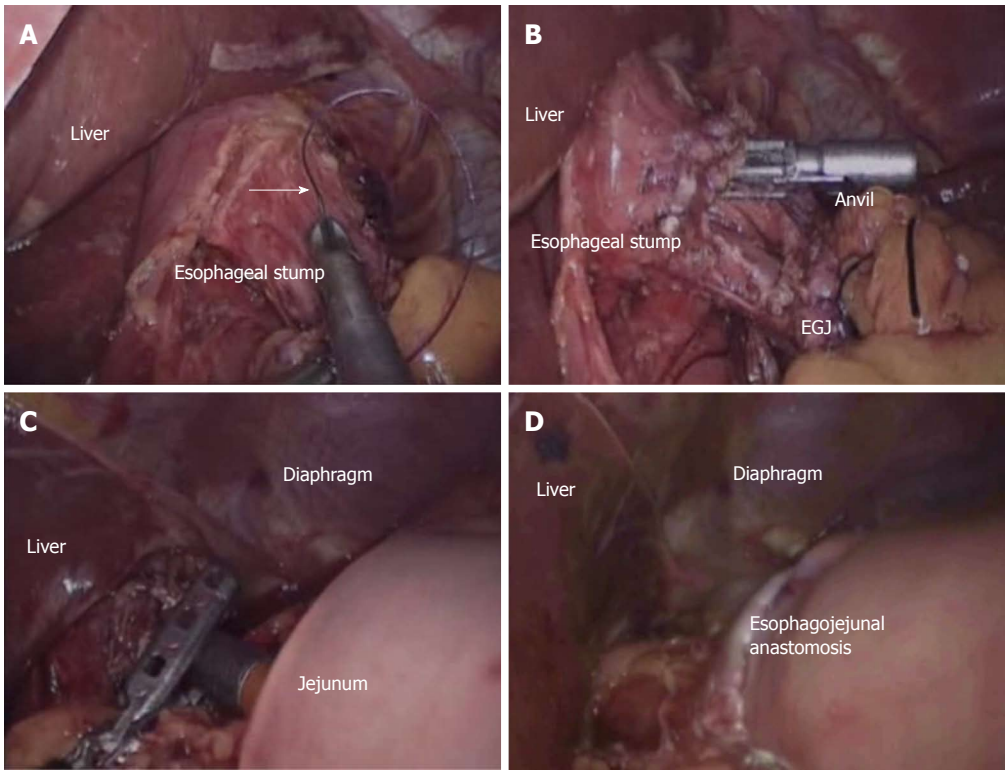

Figure 1 Conventional circular stapler-anvil method. A: The purse-string suture (white arrow) was placed in the esophagus; B: The anvil was introduced into the esophageal stump through the hole; C: The circular stapler was introduced into the jejunum through the jejunal stump and attached with the anvil; D: The circular stapler was fired and the esophagojejunostomy was completed.

linear staplers and the hand-sewn suture technique. However, in our experience, we found some limitations using these mechanical approaches. Therefore, we have used intracorporeal hand-sewn gastrointestinal anastomosis since September 2012.

Methods of intracorporeal esophagojejunostomy

Conventional circular stapler-anvil method (Type A): This technique is an anvil method. The stomach was lifted up and then a purse-string construction was placed 1 cm above the line that was predetermined transected (Figure 1A). A hole was made with the esophagogastric junction using a harmonic scalpel. The anvil was introduced to the esophageal stump *via* the hole of purse-string construction, and the hole was tied (Figure 1B). The esophagogastric junction was later divided, and the stomach was also extracted. The circular stapler was utilized in the jejunum *via* the jejuna stump and was later attached with the anvil (Figure 1C). The esophagojejunostomy was finished by firing the circular stapler (Figure 1D). The jejunum stump was closed using the endoscopic linear stapler.

Linear stapler side-to-side method (Type B): A relative small opening was made into the 10 cm ranking from the stump over the distal jejunum, and the latter was then pulled up to the esophagus, in which a small side opening was also made. A antiperistalic side-to-side esophagojejunostomy was later presented with a linear stapler (Figure 2A). The esophagus and the residual hole were closed using the stapler (Figure 2B).

Linear stapler delta-shaped method (Type C): The escophageogastric junction was transected through the endoscopic linear stapler. Small holes were then created alongside the edge of the jejunum and the esophageal stump. The posterior walls of both the esophageal stump and the jejunum were approximated and joined by the endoscopic linear stapler (Figure 3A). The staple line was inspected later for hemostasis and defects. Stay sutures were placed to lift the common opening (Figure 3B); which was then closed with two applications of the linear stapler (Figure 3C), leading to the reconstruction over the intracorporeal tract (Figure 3D).

Hand-sewn end-to-side method (Type D): The jejunal loop was brought up to reach the esophageal stump. The jejunum was anchored to the esophageal stump through several serosal muscularis interrupted sutures that were placed in the posterior layer of the esophageal stump (Figure 4A). Two small holes were created: one on the esophageal stump and the other over the anti-mesenteric aspect of the jejunum. The posterior wall was latterly closed through a number of full-thickness sutures (Figure 4B). In addition, the closure over the anterior wall was carried out *via* the full-thickness sustainable suture (Figure 4C). Lastly, the seromuscular layer was finally strengthened through

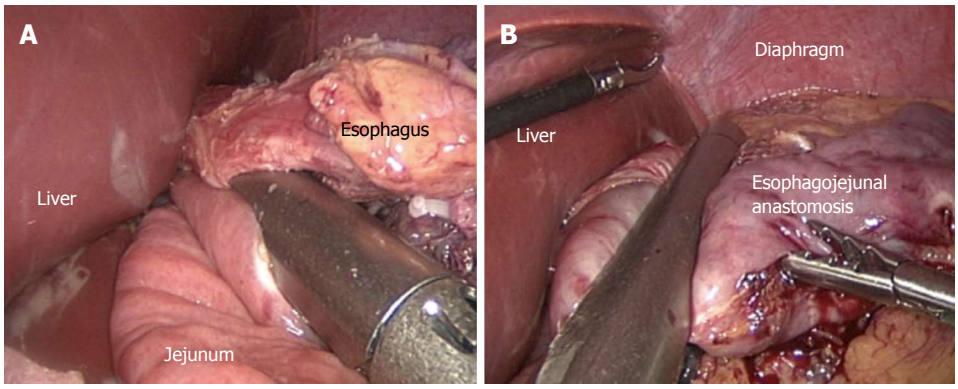

Figure 2 Linear stapler side-to-side method. A: Each jaw of the linear stapler was inserted into the holes on the esophageal stump and the jejunum and then the linear stapler was fired; B: The entry hole and esophagus were closed using the stapler.

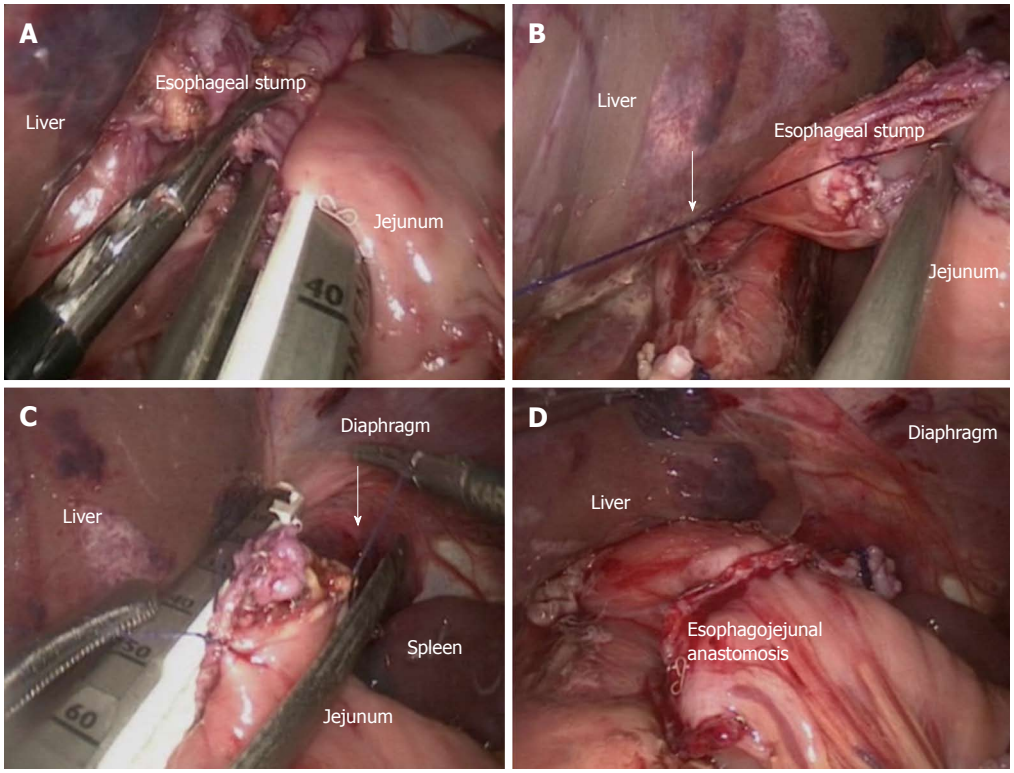

Figure 3 Linear stapler delta-shaped method. A: Small holes were created along the edge of the esophageal stump and the jejunum that were approximated and joined with the endoscopic linear stapler; B: Stay sutures (white arrow) were placed to lift the common opening; C: The common opening was then closed with two applications of the linear stapler; D: Reconstruction of the intracorporeal alimentary tract was completed.

interrupted sutures to decrease the tension (Figure 4D).

Procedure after esophagojejunostomy

After the strengthening over the seromuscular layer, which was facilitated with interrupted sutures to decrease tension, the esophageal wall was sutured to those of the diaphragm to minimize tension. The specimen was placed over the retrieval bag and latterly removed *via* an enlarged umbilical incision. The pneumoperitoneum was latterly established again. The routine of the Roux-en-Y anatomosis was presented laparoscopically among the distal jejunum (40 cm from the esophagojejunostomy) and then the proximal jejunum. Any specific defect over the mesentery was closed.

Postoperative management

After surgery, all patients were cared for in the general ward. The nasogastric tube was removed at the end of surgery in the operating room. Patients were supported through the TPN before they finally took a liquid diet. If the patients could tolerate the liquid diet, they were offered a semiliquid diet. The anastomosis was checked on postoperative days 3-7 *via* conducting an upper gastrointestinal radiography facilitated with

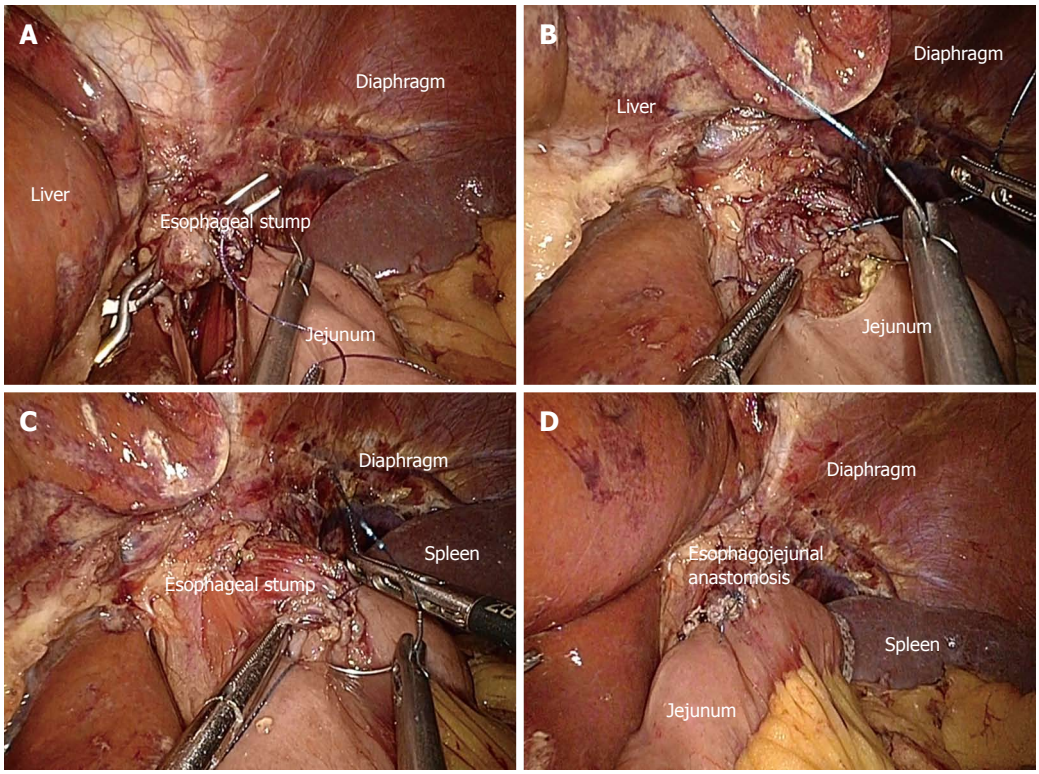

Figure 4 Hand-sewn end-to-side method. A: The jejunum was anchored to the esophageal stump by several serosal muscularis interrupted sutures placed in the posterior layer of the esophageal stump; B: The posterior wall was closed by several full-thickness interrupted sutures; C: Closure of the anterior wall was carried out by a full-thickness continuous suture; D: The seromuscular layer was strengthened with interrupted sutures to reduce tension.

Gastrografin as the opposite medium. Patients were discharged from the hospital if they resumed a semi-liquid diet and their average blood temperature and work panel was without obvious abnormalities. The anastomoses were assessed again within 3 mo by gastroscopy.

Patients were followed up every 3 mo during the next 2 years after surgery and every 6 mo during the 3 years after that. Routine follow-up covered the physical test, laboratory examination, endoscopy, computed tomography or ultrasonography, and chest radiography. Every patient was observed until death or the last follow-up date of May 2015.

RESULTS

Clinical features and pathological characteristics

Table 1 illustrates the clinical features and pathological characteristics held by the patients. The average age of the patients was 59.0 years, and the ratio of females to male was 2:1. The average BMI was 22.2 kg/m^2. Well over one-third of the patients suffered from comorbidities, with the most common being hypertension. The average neoplasm size was 4.0 cm. Approximately half of the patients had lesions that were staged as T1 (39.5%), N0 (49.4%), and stage I (43.2%) neoplasms. Sixty percent of patients suffered from advanced gastric cancer, which was defined as tumor invasion into the layer of the proper muscular.

Operative outcomes and postoperative clinical course

The operative results together with the following postoperative clinical process statistics are presented in Table 2. The anastomotic approaches adopted served as type A among 18 patients, type B among 22 patients, type C among 10 patients, and type D among 31 patients. The operation was completed successfully in every case without conversion to open surgery or laparoscopy assisted surgery. The average operation time was 288.7 min. The average time to intracorporeal esophagojejunostomy was 54.3 min, with an average blood loss of 82.7 mL. The average number of retrieved lymph nodes per patient was 34.3. The distal and proximal margins were checked in frozen sectors, with R0 resection realized in each case. The average time until the first flatus was 3.7 d. The average time to start soft and liquid diets were 4.9 d and 6.6 d, respectively. The average postoperative hospital stay was 10.1 d.

Postoperative complications are listed in Table 3. The rate of postoperative morbidity was 17.3%, and there were no cases of postoperative death. Morbidity included anastomotic leakage (n = 1), anastomosis stricture (n = 3), intraluminal bleeding (n = 3), intestinal stasis (n = 2), abdominal abscess (n = 1), ileus (n = 1), lymphorrhea (n = 1), pulmonary infection (n = 1), and pulmonary embolism (n = 1). All complications were controlled by conservative treatment.

Table 1 Clinical characteristics and pathologic features of 81 patients who underwent totally laparoscopic total gastrectomy

Variable	Value (%)
Gender (male/female)	54 (66.6)/27 (33.3)
Age (yr)	59.0 ± 10.6
BMI (kg/m^2)	22.2 ± 3.1
ASA classification (Ⅰ/Ⅱ/Ⅲ)	43 (53.1)/36 (44.4)/2 (2.5)
Comorbidities[1]	31 (38.3)
Hypertension	20 (24.7)
Diabetes mellitus	6 (7.4)
Cardiovascular	7 (8.6)
Pulmonary	6 (7.4)
Liver	2 (2.5)
Others	2 (2.5)
Tumor size (cm)	4.0 ± 2.4
Histology (differentiated/ undifferentiated)	42 (51.9)/39 (48.1)
T stage (T1/T2/T3/T4)	32 (39.5)/10 (12.3)/11 (13.6)/28 (34.6)
N stage (N0/N1/N2/N3)	40 (49.4)/11 (13.6)/13 (16.0)/17 (21.0)
TNM stage (Ⅰ/Ⅱ/Ⅲ/Ⅳ)	35 (43.2)/17 (21.0)/29 (35.8)/0 (0.0)

[1]Nine (11.1%) of 81 patients had more than two comorbidities. Data are mean ± SD or number (%). BMI: Body mass index; ASA: American Society of Anesthesiologists.

DISCUSSION

The use of laparoscopic gastrectomy (LG) for gastric cancer has been gradually accepted because of its short-term benefit and the comparable oncological results as open gastrectomy. Most available studies on LG have focused on LDG for tumors located in the lower third of the stomach, which is the most common type in East Asia[12,13]. The incidence of tumors located in the upper and middle stomach has been evaluated recently and has resulted in an increased demand for LTG.

The major indications and technical aspects for LTG are similar to those for LDG. Several technical points relevant to the safety and feasibility of this technique should be addressed. The additional requirement set for the lymph nodes over the splenic hilum or any other short gastric artery represents a difficulty that has been encountered in fundamental gastrectomy. This requirement exists because the splenic vessels flow circuitously, with the branches substantially varying from one to another in deep locations. It is well known that hemorrhage or ischemia could occur because of splenic vascular injury during dissembling of lymph nodes neighboring splenic artery branches. Laparoscopy, compared with the laparotomy, permits the operator to finish dissembling the lymph nodes, which are quite clear, and assists in developing surgical safety. Therefore, the most obvious obstacle to LTG is still the difficulty involved in esophagojejunostomy, which can be performed extracorporeally or intracorporeally. Indeed, it is quite difficult in many cases to conduct the anastomosis *via* a mini-laparotomy, because space is quite restricted and narrow, specifically in obese victims with relatively thick abdominal walls or the specific tumors that exist in an upper location. It is for this reason that we started to conduct extracorporeal anatomosis, hoping to deal with the barricades of cumbersome reconstruction. We first introduced the use of intracorporeal anastomosis after LTG in 2007.

Two approaches for mechanical esophagojejunostomy have been used in LTG, linear stapling and circular stapling. Circular stapling enjoys similar merits as open total gastrectomy (OTG). The esophagojejunostomy can be performed successfully by adopting a circular stapler *via* a structure technique, which was hand-sewn in the first 18 patients of our series to mend the anvil that was applied in the esophageal stump. Based on our experience, the esophagus was not cut off initially, and the cardia was tightly tied with a band and then stretched down to expose the esophagus. Purse-string sutures were undertaken, and the anterior wall of the esophagus was then cut with a harmonic scalpel to form a half-circle. After placement of the anvil, the suture line was tightened and the esophagus was finally cut off with the harmonic scalpel. However, the circular stapler was inappropriate for placement during laparoscopic surgery due to its larger size and the absence of a matching tube. The pneumoperitoneum was vulnerable to its placement, and vision was unclear. Therefore, it was not an ideal method and may have resulted in a higher rate of postoperative complications[14,15], especially in our early experience. Thus, a novel transorally inserted anvil (OrVil; Covidien, Mansfield, MA, United States) was introduced to address the challenge of anvil placement[16]. OrVil is not only good in terms of operation safety and treatment effects but also in the reduction in operation time. However, due to its high cost, possibility of bacterial contamination in the abdominal cavity, and injury to the esophageal mucosa, this method is adopted only in some specialized medical centers.

A linear stapler was used for side-to-side esophagojejunostomy. This method was first reported by Uyama *et al*[17] and was modified by Wang *et al*[18]. Circular stapling was performed to isolate separately the esophagus and jejunum prior to anastomosis. The linear stapler was inserted at the two ends to complete the anastomosis and then closed the collective opening. Benefits of the linear stapling method involved no initial dissection of the jejunum and esophagus to pull down the esophagus. Thus the esophagus did not need to be completely retracted to the chest; and instead, the specimen was removed, and the common opening was closed together after completion of the anastomosis, economizing the staplers. This method was simple to perform, and the anastomotic stoma was bigger, which may have helped avoid postoperative complications, such as anastomotic stenosis. With regard to specific surgical methods, some surgeons advise that the position taken by the esophageal and jejunum stump should be fixed, adopting at least one stitch prior to the endoscopic

Table 2 Surgical outcomes of 81 patients who underwent totally laparoscopic total gastrectomy

	Type A (*n* = 18)	Type B (*n* = 22)	Type C (*n* = 10)	Type D (*n* = 31)	Total (*n* = 81)
Operation time (min)	305.6 ± 45.9 (250-380)	266.8 ± 38.7 (230-360)	278.0 ± 16.2 (250-300)	297.9 ± 33.2 (240-420)	288.7 ± 39.1 (230-420)
Anastomotic time (min)	57.5 ± 18.5 (35-90)	40.0 ± 11.2 (25-60)	39.0 ± 3.9 (35-45)	67.2 ± 18.8 (45-105)	54.3 ± 19.9 (25-105)
Blood loss (mL)	80.6 ± 29.4 (50-160)	86.4 ± 39.7 (50-200)	87.0 ± 24.5 (50-120)	80.0 ± 32.5 (50-180)	82.7 ± 32.7 (50-200)
Retrieved lymph nodes	30.9 ± 5.8 (25-45)	34.6 ± 4.1 (25-42)	34.8 ± 6.1 (28-47)	35.8 ± 11.4 (24-69)	34.3 ± 8.2 (24-69)
First flatus after operation (d)	4.2 ± 0.8 (3-5)	3.6 ± 1.3 (2-7)	3.4 ± 0.8 (2-5)	3.6 ± 0.8 (2-5)	3.7 ± 1.0 (2-7)
Liquid diet (d)	5.2 ± 0.8 (4-6)	4.9 ± 1.1 (3-7)	4.6 ± 0.7 (4-6)	4.7 ± 1.0 (3-7)	4.9 ± 0.9 (3-7)
Soft diet (d)	6.7 ± 1.3 (5-11)	6.3 ± 1.1 (5-8)	6.6 ± 0.8 (5-8)	6.7 ± 2.1 (5-15)	6.6 ± 1.6 (5-15)
Postoperative hospital stay (d)	10.9 ± 2.9 (9-20)	10.2 ± 2.4 (8-17)	10.1 ± 2.9 (8-18)	9.6 ± 1.9 (7-17)	10.1 ± 2.4 (7-20)

Data are mean ± SD (range).

Table 3 Postoperative complications in 81 patients who underwent totally laparoscopic total gastrectomy

	Type A (*n* = 18)	Type B (*n* = 22)	Type C (*n* = 10)	Type D (*n* = 31)	Total (*n* = 81)
Postoperative complications	5	5	2	2	14
Anastomotic leakage	1				1
Anastomosis stricture	1	2			3
Intraluminal bleeding		1	1	1	3
Intestinal stasis	1		1		2
Abdominal abscess		1			1
Ileus				1	1
Lymphorrhea	1				1
Pulmonary infection		1			1
Pulmonary embolism	1				1

linear staple that has been applied[19]. However, in our practice, it has been found that the suture angulates and suspends the jejunum, posing great challenges to the endoscopic linear stapler, sacrificing the mobility of the jejunum. According to our experience, it is more favorable to place one arm of the endoscopic linear stapler, which has been applied into the opening of the jejunum, and to clamp the two arms with no stapling. Later, with the assistance of the stapler, attracting the jejunum close to the esophageal stump's rear and then unveiling the two arms to replace the second one finishes the anastomosis following the apposition, which has been proven to be quite satisfactory. However, even using this approach, possible issues remain, including taking the distortion over the mesenterium and Roux limb as well as the slipping over the esophagojejunal anastonomic site applied in the comparative lower mediastinum. The surgical margin is limited as a longer esophageal stump should be reserved.

The delta-shaped esophagojejunostomy provides a considerable lumen, regardless of the size of the esophageal stump. More importantly, as a long esophageal stump and anastomosed intestine are not required, this technique achieves less flexion in order to provide enough blood supply for the anastomotic site, thus decreasing the risk of anastomotic leakage. However, surgeons of the technique should have proficient laparoscopic suturing skills, or the outcome of the anastomosis will be impacted because of the unfavorable apposition of the average opening. The assistant should maneuver the stay suture efficiently to ensure that the stapler is applied adequately on all layers of the esophagus[20]. Hand-sewn end-to-side esophagojejunostomy overcomes the limitations caused by the mechanical method. The suturing process can be clearly observed under high definition laparoscopy, and the anastomosis is reliable. The operating space is large, and there is no tension in the whole anastomosis procedure, thus avoiding injury. More importantly, this method completes the anastomosis after removal of the specimen, as the anastomosis can be performed after negative margins are confirmed using intraoperative frozen sections. This method does not require a long esophageal stump. In patients with a positive resection margin, the removal length can be expanded appropriately to confirm a negative resection margin. Therefore, the R0 resection can be improved, and the conversion rate to laparotomy is reduced. However, the hand-sewn method requires operators with extensive experience in laparoscopic suturing, which may increase surgery time. In our experience, progressive practice (from practice on the simulator to practice on animal models and simple suture under laparoscopy, and finally to laparoscopic gastrointestinal anastomosis) can effectively shorten the learning curve. At the same time, the application of new laparoscopic instruments can simplify intracorporeal hand-sewn suturing. Knotless barbed sutures (V-Loc™; Covidien, Mansfield, MA, United States) can reduce the time of anastomosis and can ensure the safety of anastomosis, with no need for permanent traction during the whole anastomosis procedure.

We recommend that the reconstruction method using a stapler should be selected on the basis of tumor location. In our experience, side-to-side or delta-shaped esophagojejunostomy using a linear stapler can be adopted for patients with lesions in the body and fundus of the stomach as well as the lower cardia. For patients with lesions in the upper and middle cardia, end-to-side esophagojejunostomy using a circular stapler is chosen to ensure a negative surgical margin. In addition, if the surgeon is well experienced with the laparoscopic hand-sewn technique, it can be used after total gastrectomy, regardless of tumor location.

In conclusion, we demonstrated that LTG facilitated with intracorporeal anastomosis is feasible and safe, leading to quite favorable contemporary results. However, the clinical efficacy should be demonstrated further in a perfectly designed randomized controlled clinical trial. The choice of approach for intracorporeal anastomosis depends mainly on the surgeon's preference and experience.

COMMENTS

Background

Laparoscopic gastrectomy for gastric cancer has gained wide popularity in the past few decades. However, laparoscopic total gastrectomy (LTG) is rarely performed due to some technical difficulties.

Research frontiers

It is important to study the feasibility and safety of LTG with intracorporeal anastomosis. New approaches are needed in order to improve the surgical experience for patients and provide easier and simpler surgical procedures. Based on our study, LTG with intracorporeal anastomosis is likely to be the best option.

Innovations and breakthroughs

This study demonstrated that LTG with intracorporeal anastomosis can be a safe and favorable alternative to open gastrectomy and laparoscopy-assisted gastrectomy. Furthermore, several esophagojejunostomy methods are available, depending on patients' condition and surgeon preference.

Applications

LTG with intracorporeal anastomosis can be safely performed with the proper esophagojejunostomy method.

Terminology

LTG with intracorporeal anastomosis can be safely performed with quite favorable results. However, the clinical efficacy should be demonstrated with more well designed studies.

Peer-review

The authors concluded that LTG with intracorporeal esophagojejunostomy using laparoscopic staplers was safe and feasible for patients with gastric cancer. This paper is well studied and well written.

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ORIGINAL ARTICLE

Prospective Study

Osteopontin: A non-invasive parameter of portal hypertension and prognostic marker of cirrhosis

Radan Bruha, Marie Jachymova, Jaromir Petrtyl, Karel Dvorak, Martin Lenicek, Petr Urbanek, Tomislav Svestka, Libor Vitek

Radan Bruha, Jaromir Petrtyl, Karel Dvorak, Tomislav Svestka, 4th Department of Internal Medicine, 1st Faculty of Medicine and General University Hospital, Charles University in Prague, 12808 Prague, Czech Republic

Marie Jachymova, Martin Lenicek, Libor Vitek, Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, 12808 Prague, Czech Republic

Petr Urbanek, Department of Internal Medicine, Central Military Hospital and 1st Faculty of Medicine, Charles University in Prague, 12808 Prague, Czech Republic

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Clinical trial registration statement: This study is registered at SUKL - State Institute for Drug Control of the Czech Republic (http://www.sukl.eu/modules/evaluation/detail.php?id=31445&lang=2). The registration identification number is EudraCT number: 2011-001132-30.

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Correspondence to: Radan Bruha, MD, PhD, 4th Department of Internal Medicine, General University Hospital, U Nemocnice 2, 12808 Prague, Czech Republic. bruha@cesnet.cz
Telephone: +420-22-4962506
Fax: +420-22-4923524

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Abstract

AIM: To investigate the relationship between osteopontin plasma concentrations and the severity of portal hypertension and to assess osteopontin prognostic value.

METHODS: A cohort of 154 patients with confirmed liver cirrhosis (112 ethylic, 108 men, age 34-72 years)

were enrolled in the study. Hepatic venous pressure gradient (HVPG) measurement and laboratory and ultrasound examinations were carried out for all patients. HVPG was measured using a standard catheterization method with the balloon wedge technique. Osteopontin was measured using the enzyme-linked immunosorbent assay (ELISA) method in plasma. Patients were followed up with a specific focus on mortality. The control group consisted of 137 healthy age- and sex- matched individuals.

RESULTS: The mean value of HVPG was 16.18 ± 5.6 mmHg. Compared to controls, the plasma levels of osteopontin in cirrhotic patients were significantly higher ($P < 0.001$). The plasma levels of osteopontin were positively related to HVPG ($P = 0.0022$, $r = 0.25$) and differed among the individual Child-Pugh groups of patients. The cut-off value of 80 ng/mL osteopontin distinguished patients with significant portal hypertension (HVPG above 10 mmHg) at 75% sensitivity and 63% specificity. The mean follow-up of patients was 3.7 ± 2.6 years. The probability of cumulative survival was 39% for patients with HVPG > 10 mmHg and 65% for those with HVPG ⩽ 10 mmHg ($P = 0.0086$, odds ratio (OR), 2.92, 95% confidence interval (CI): 1.09-7.76). Osteopontin showed a similar prognostic value to HVPG. Patients with osteopontin values above 80 ng/mL had significantly lower cumulative survival compared to those with osteopontin ⩽ 80 ng/mL (37% *vs* 56%, $P = 0.00035$; OR = 2.23, 95%CI: 1.06-4.68).

CONCLUSION: Osteopontin is a non-invasive parameter of portal hypertension that distinguishes patients with clinically significant portal hypertension. It is a strong prognostic factor for survival.

Key words: Cirrhosis; Complications of cirrhosis; Hepatic venous pressure gradient; Osteopontin; Portal hypertension; Prognosis; Survival prediction

Core tip: Data presented in our study are based on a 7-year follow-up interval with systematic hemodynamic evaluations of more than 150 cirrhotic patients. We report for the first time a close relationship between osteopontin (OPN) and portal hypertension. Our findings suggest that OPN in plasma could be a marker of clinically significant portal hypertension. Importantly, we found that OPN is a strong prognostic indicator in patients with liver cirrhosis; and, similar to hepatic venous pressure gradient (HVPG) value, it significantly determined survival probability. Moreover, the combination of HVPG and OPN increased the validity of prognosis.

Bruha R, Jachymova M, Petrtyl J, Dvorak K, Lenicek M, Urbanek P, Svestka T, Vitek L. Osteopontin: A non-invasive parameter of portal hypertension and prognostic marker of cirrhosis. *World J Gastroenterol* 2016; 22(12): 3441-3450 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3441.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3441

INTRODUCTION

Osteopontin (OPN), first described in 1979[1], is a multifunctional protein that is physiologically expressed in the kidney and bone[2]. Under pathological conditions, OPN expression has been found in various organs and has been attributed to many pathological conditions, including inflammation, angiogenesis, fibrosis, and carcinogenesis[3]. Hepatic expression of OPN was first described in rats after carbon tetrachloride intoxication[4]. OPN was shown to contribute to the migration of macrophages into the necrotic areas in liver tissue[5] and to serve as a key cytokine within the extracellular matrix; thus, contributing to fibrogenesis[6,7]. OPN is involved in the evolution and progression of various cancers, including hepatocellular carcinoma (HCC)[8] and cholangiocarcinoma[9]. In fact, plasma OPN levels have been found to be significantly elevated in patients with liver cirrhosis and HCC compared to those without HCC[10].

Recently, plasma OPN levels were shown to predict liver fibrosis in various chronic liver diseases, such as non-alcoholic steatohepatitis[11], alcoholic liver disease[12], and chronic viral hepatitis B[13] and C[14]. As OPN levels correlate significantly with the fibrosis stage in alcohol-induced liver disease[12], it follows that OPN levels could be related to the degree of portal hypertension and, hence, serve as a surrogate non-invasive marker of portal hypertension. This relationship has not been studied until now. Portal hypertension, which is pathogenically related to liver injury and fibrosis, leads to major complications of cirrhosis. In the clinical setting, portal hypertension is evaluated by invasive measurement of the hepatic venous pressure gradient (HVPG)[15]. Recently, non-invasive biomarkers of cirrhosis[16] have been suggested as substitutes for invasive measurement of portal pressure in some indications.

The aim of our study was to evaluate the relationship between OPN plasma concentration and the degree of portal hypertension, as measured by HVPG, and to assess the impact of OPN values on prognosis in patients with liver cirrhosis.

MATERIALS AND METHODS

Patients

A group of 154 patients with confirmed liver cirrhosis

and portal hypertension were included in the study. They were recruited from a list of consecutive patients referred to the 4th Department of Internal Medicine of the General University Hospital in Prague between 2007 and 2014 for hemodynamic evaluation of portal hypertension. A total of 286 patients were examined, out of which 154 were included in the study. The main criteria for exclusion from the study were: active uncontrolled alcohol abuse, known HCC, concomitant antiviral treatment, the use of any drug affecting splanchnic hemodynamics or portal pressure within 2 wk before HVPG measurement, portal vein thrombosis, lack of initial clinical data in the database, lack of appropriate blood sample volume in storage, and refusal of patients to store blood samples for future evaluation or to collect clinical data. Blood samples were collected during hepatic vein catheterization and immediately separated and stored at -70 ℃ until OPN evaluation.

Liver cirrhosis was diagnosed on the basis of biochemical tests, clinical and ultrasound findings, and/or liver biopsies; the presence of portal hypertension was diagnosed by hepatic vein catheterization.

Hepatic vein catheterization and HVPG measurement were performed in all patients. Eighty-three patients included in the study were referred for HVPG measurement in cases of primary or secondary prophylaxis of bleeding from esophageal varices, according to the Baveno criteria[17]. Indications for HVPG measurement in the remaining 71 patients were staging of liver cirrhosis and portal hypertension. The clinical and laboratory data of all patients were collected at the time of hepatic vein catheterization. The follow-up data were collected from the hospital charts at the time of OPN evaluation. The patients were carefully followed up with a special focus on mortality. The data of six transplanted patients were censored at the time of transplantation.

The control group, used for the purposes of comparing OPN levels, consisted of 137 healthy individuals. The control population was recruited from the staff of the university hospital in Prague. These individuals consisted of healthy subjects without a history of liver disease, coronary artery disease, or other chronic diseases and were age- and gender-matched to the patient population.

The study was carried out in full accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Institutional Ethics Committee. Informed consent for future evaluation of blood samples for scientific purposes was obtained from all subjects at the time of hepatic vein catheterization.

Measurement of the HVPG

Measurement of the HVPG was performed using the classic wedge technique[18]. Shortly after overnight fasting, patients were transferred to the catheterization room. Under local anesthesia, a 7F catheter introducer was placed in the right jugular vein using the Seldinger technique. Under fluoroscopic control, a 7F balloon-tipped catheter (B. Braun Melsungen AG, Melsungen, Germany) was advanced into the right hepatic vein in order to measure both free hepatic venous pressure and wedged hepatic venous pressure. All measurements were performed in triplicate using a continuous recording unit. HVPG was calculated as the difference between the wedged hepatic venous pressure and the free hepatic venous pressure. Clinically significant portal hypertension was defined according to the Baveno criteria[17] with HVPG > 10 mmHg.

Laboratory analyses

Biochemical and hematology examinations were performed on automatic analyzers (Modular Analyzer; Roche Diagnostics GmbH, Mannheim, Germany) using standard laboratory assays. The severity of liver disease was evaluated by Child-Pugh scoring and the model end-stage liver disease (MELD) score and then evaluated separately according to platelet count, presence of esophageal varices, ascites, and hepatic encephalopathy.

OPN levels in plasma were measured using an enzyme-linked immunosorbent assay (ELISA) kit (DOST00, R&D Systems, Minneapolis, MI, United States), according to the manufacturer's instructions. The control group consisted of 137 healthy age- and sex-matched individuals. In each ELISA kit, both controls and patients were included to minimize the effect of inter-assay error due to group comparison.

Other non-invasive markers of portal hypertension were compared to OPN for the detection of clinically significant portal hypertension, including platelet count, platelet count/spleen diameter ratio[19], and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio[20].

Statistical analysis

The results are presented as mean values with standard deviation. Either a two-sample *t*-test or the Mann-Whitney rank test for non-Gaussian distributed variables was used to estimate intergroup differences. The correlations between different parameters were evaluated by calculation of Pearson or Spearman correlation coefficients and linear regression analyses. Logistic regression was used to assess the predictive value for mortality of risk factors. In order to prevent model over-adjustment, we merged both predictors (HVPG and OPN), as they seemed to act independently and exhibit similar ORs. All tests were two-sided, with $P < 0.05$ considered as statistically significant. Receiver operating characteristic curve analysis was used to assess the utility of OPN and other parameters when distinguishing between patients with and without clinically significant portal hypertension. Survival

Table 1 Characteristics of patients with liver cirrhosis in the study

Parameter	All patients (n = 154)	Compensated patients (without ascites) (n = 91)	Decompensated patients (with ascites) (n = 63)	P value[1]
Age (yr)	54.7 ± 11.1	54.5 ± 11.2	55.4 ± 11.3	0.276
Gender (M/F) (%)	74/26	73/27	77/23	0.340
Etiology of cirrhosis (alcohol/viral/other incl. NASH) Number of patients	112/22/20	63/15/13	47/9/7	0.490
Child-Pugh A/B/C (%)	41/34/25	69/27/4	14/39/47	< 0.001
MELD score	12.5 ± 4.9	10.9 ± 4.5	14.3 ± 4.8	< 0.001
Bleeding from varices (%)	30	28	33	0.288
Bilirubin (μmol/L)	31 (18-55)	25 (15-44)	40 (23-62)	0.011
Albumin (g/L)	33.6 ± 7.4	39.9 ± 6.7	30.1 ± 6.6	< 0.001
Creatinine (μmol/L)	83 ± 29	81.6 ± 27.2	97.8 ± 22.3	< 0.001
ALT (μkat/L)	0.65 (0.5-1.4)	0.71 (0.6-1.7)	0.58 (0.4-0.8)	0.282
AST (μkat/L)	0.96 (0.7-1.6)	1.11 (0.8-1.6)	0.75 (0.6-1)	0.193
Platelets (× 10^9/L)	107 (74-163)	98 (68-142)	130 (82-203)	0.015
Arterial mean blood pressure (mmHg)	91 ± 11.5	92 ± 13	90 ± 10	0.483
Ascites (%)	41	-	-	-
Encephalopathy (%)	12	6	19	< 0.001
HVPG (mmHg)	16.0 ± 5.4	14.2 ± 5.1	26.9 ± 6.1	< 0.001
Varices (none/small/large) (%)	18/35/47	25/30/45	10/40/50	< 0.001
Spleen length (mm)	143 ± 22	142 ± 19	146 ± 26	0.534
Diameter of the portal vein (mm)	13.3 ± 2.1	13.2 ± 1.8	13.5 ± 2.4	0.729
Portal flow velocity (cm/s)	16 ± 5.8	18.8 ± 6	13.5 ± 4	< 0.001
Diameter of the lienal vein (mm)	10.1 ± 3	10.25 ± 2.7	9.8 ± 3.5	0.348
Follow-up (yr)	3.7 ± 2.6	4.13 ± 2.4	2.88 ± 2.6	0.004
Osteopontin (ng/mL)	107 (73.7-154)	85.7 (65.7-129)	138 (106-194)	< 0.001

[1]Statistical difference between compensated and decompensated patients. Data are expressed as mean ± SD or as a median and 25-75 percentile, when non-normally distributed. HVPG: Hepatic venous pressure gradient.

probability was determined using the Kaplan-Meier method. The statistical analyses were performed using BMDP Statistical Software (Release 8.1) and Statistica 12 CZ.

RESULTS

Characteristics of patients

One hundred and fifty four patients were included in the study - 108 males and 46 females. Both gender groups exhibited no differences with regard to age, biochemical parameters, and/or the etiology and stage of cirrhosis (data not shown). Patients were divided into two groups depending on the absence or presence of ascites (compensated, n = 91; decompensated, n = 63). The clinical and laboratory parameters of the whole group as well as patients with and without ascites are given in Table 1. There were no statistical differences between patients and controls in respect to age and gender (data not shown). There were no differences in OPN values between patients with alcoholic or other etiology: 110 (73-159) ng/mL *vs* 107 (75-146) ng/mL, P = 0.95 (values as median and interquartile (IQ) range).

Relationship of OPN to HVPG

The mean value of HVPG in patients with cirrhosis was 16.18 ± 5.6 mmHg. The plasma values of OPN in cirrhotic patients were significantly higher than values in controls: 107 (74-154) ng/mL *vs* 55 (42-67) ng/mL, P < 0.001, values as median and IQ range; Figure 1A). Plasma levels of OPN were closely and positively related to HVPG values (P = 0.002, r = 0.25, Figure 2). Plasma levels of OPN above 80 ng/mL distinguished patients with HVPG > 10 mmHg with 75% sensitivity and 63% specificity (AUC 0.763, confidence interval (CI) 49.8-83.7). The positive predictive value (PPV) and negative predictive value (NPV) for the OPN cut-off of 80 of ng/mL concentration in discriminating patients with significant portal hypertension was 92% (95%CI: 85%-96%) and 31% (95%CI: 18%-47%), respectively. A cut-off value of 90 ng/mL distinguished patients with HVPG > 12 mmHg (a marker of increased risk of variceal bleeding) to 71% sensitivity and 62% specificity (area under the curve (AUC), 0.725, 95%CI: 57.3-85.1). When calculated for patients without ascites only, plasma levels of OPN above 80 ng/mL distinguished patients with clinically significant portal hypertension with similar results (sensitivity 65%, specificity 64%, AUC, 0.69; 95%CI: 42-89). The sensitivity and specificity of other non-invasive parameters for the discrimination of patients with clinically significant portal hypertension were as follows: platelet count/spleen size ratio - 32%, 50%

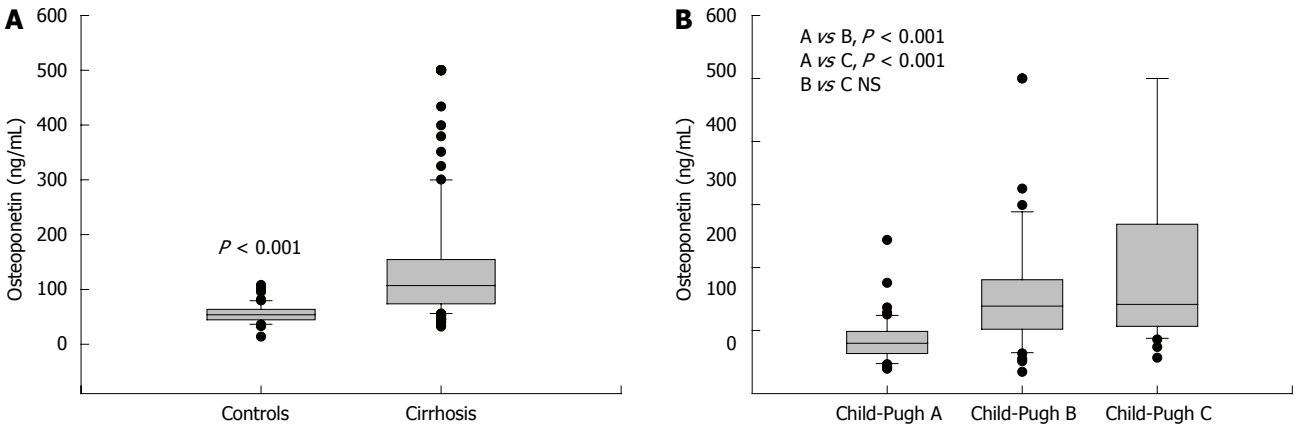

Figure 1 Plasma osteopontin in patients with cirrhosis and controls (A) and in patients with cirrhosis in Child-Pugh A, B, and C classes (B). Box-plot graphs, boxes correspond to the median value and interquartile range.

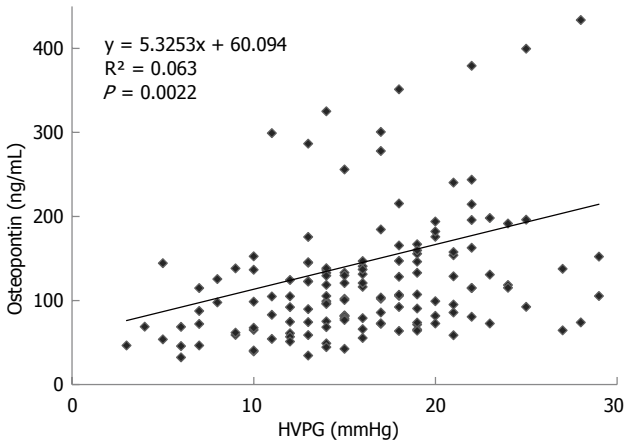

Figure 2 Relationship between hepatic venous pressure gradient and plasma osteopontin concentrations in patients with cirrhosis. HVPG: Hepatic venous pressure gradient.

(AUC, 0.392, 95%CI: 0.17-0.51), respectively; platelet count - 45%, 40% (AUC, 0.392, 95%CI: 0.3-0.62), respectively; AST/ALT ratio - 74%, 50% (AUC, 0.696, 95%CI: 0.53-0.89), respectively.

No relation of OPN or HVPG to ultrasound portal hemodynamic parameters or laboratory parameters (portal vein diameter, spleen size, platelet count, and serum concentration of albumin) was found, with the exception of portal vein flow velocity. Portal vein flow velocity correlated negatively with HVPG (P = 0.008, r = -0.356) and OPN levels (P = 0.002, r = -0.412). There was no relationship between plasma values of OPN and age, neither in patients (P = 0.9) nor in controls (P = 0.6). Under multivariate analysis performed with HVPG above/below 10 mmHg, OPN values still differed significantly in comparison with other commonly examined laboratory parameters of portal hypertension (Table 2).

Relationship of OPN to survival

The mean time of follow-up was 3.7 ± 2.6 years (a range of 1 mo to 7 years). During the follow-up, 62 patients died, 77 patients were alive at the time of evaluation (six of whom were transplanted), and 15 patients were lost for follow-up. The HVPG cut-off value of 10 mmHg (*i.e.,* the threshold for clinically significant portal hypertension) divided patients into two groups with significantly different probabilities of cumulative survival (39% for those with HVPG > 10 mmHg compared to 65% for those with HVPG ⩽ 10 mmHg; P = 0.0086, OR = 2.92, 95%CI: 1.09-7.76; Figure 3A). When survival probability was calculated as a function of OPN, the plasma cut-off value of OPN 80 ng/mL distinguished two different groups of patients with significantly different probabilities of cumulative survival (37% for those with OPN above 80 ng/mL compared to 56% for those with OPN below 80 ng/mL, P = 0.00035, OR = 2.23, 95%CI: 1.06-4.68; Figure 3B). This difference was significant, independent of HVPG value. Mortality in patients with at least one risk factor (HVPG > 10 mmHg or plasma OPN > 80 ng/L) was more than twice as high compared to patients without any risk factors (OR = 2.34); in those with both risk factors, mortality was more than five times as high (OR = 5.10) compared to patients without any risk factors (Table 3). When considering patients with compensated cirrhosis only (*i.e.,* without ascites), both the plasma cut-off value of OPN 80 ng/mL and the HVPG value of 10 mmHg divided patients into two groups with significantly different probabilities of cumulative survival (Figure 4A and B).

Table 2 Presence of clinically significant portal hypertension in relation to osteopontin and other parameters

Parameter	*P* value
Osteopontin	0.04
Platelet count	0.05
AST/ALT ratio	0.49

Multiple linear regression with hepatic venous pressure gradient (HVPG) > 10 mmHg as a variable carried out with OPN, platelet count, and AST/ALT ratio as independent variables. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

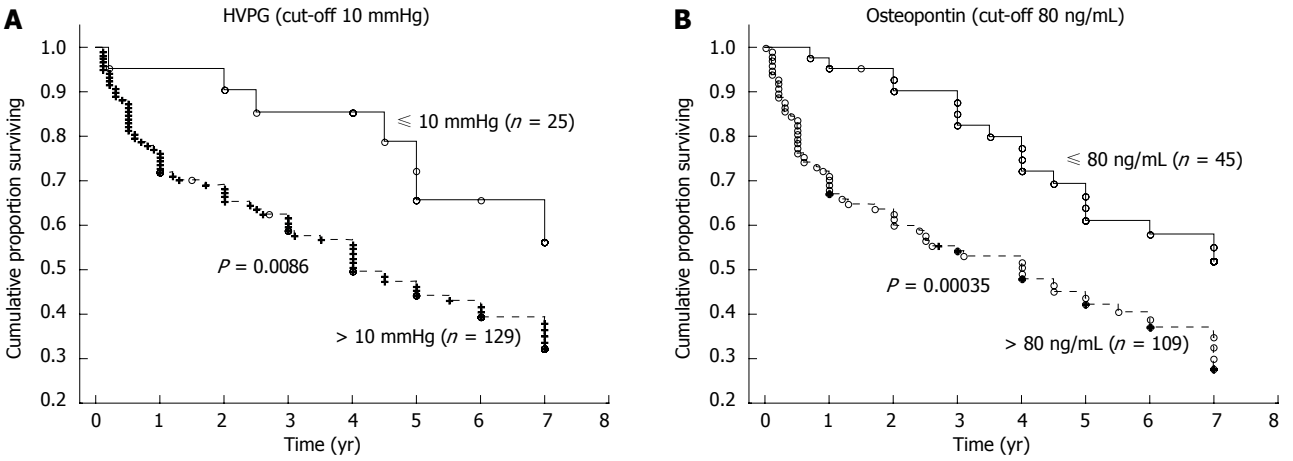

Figure 3 Cumulative proportion of surviving patients with hepatic venous pressure gradient values below and above 10 mmHg (A) and plasma osteopontin levels below and above 80 ng/mL (B) in the whole group of patients using the Kaplan-Meier method. HVPG: Hepatic venous pressure gradient; OPN: Osteopontin.

Table 3 Risk for death of patients with liver cirrhosis based on the cut-off values of hemodynamic evaluation of portal hypertension and osteopontin

Parameter	Odds ratio	95%CI	*P* value
HVPG > 10 mmHg	2.92	1.09-7.76	0.032
OPN > 80 ng/mL	2.23	1.06-4.68	0.034
HVPG or OPN above cut-off[1]	2.34	1.16-4.75	0.018
Both HVPG and OPN above cut-off[2]	5.09	1.29-20.15	0.020

[1]Only one parameter above cut-off values (10 mmHg for HVPG, 80 ng/mL for OPN); [2]Both parameters above cut-off. HVPG: Hepatic venous pressure gradient; OPN: Osteopontin.

Relation of OPN to other clinical parameters

Plasma values of OPN differed among the individual Child-Pugh groups of patients. The plasma values of OPN were 84.8 ± 34.9 ng/mL in Child-Pugh A, 158.8 ± 98.2 ng/mL in Child-Pugh B, and 205.2 ± 142.8 ng/mL in Child Pugh C. Significant differences were found between Child-Pugh A *vs* B and A *vs* C groups (Figure 1B). The same significance was found among Child-Pugh groups according to HVPG values (Table 4).

Plasma OPN concentrations correlated significantly with platelet count (r = 0.231; P = 0.009) and presence of ascites (P < 0.001) but not with the size of varices or history of variceal bleeding (Table 5). HVPG correlated with all of above-mentioned clinical parameters, including size of varices and history of variceal bleeding (Table 5). Information about the presence or absence of HCC during the follow-up period was available in the case of 81 patients, of whom HCC developed in six patients (7.4%). Neither plasma OPN levels nor HVPG values correlated with the occurrence of HCC.

DISCUSSION

The main finding of our study is that plasma concentration of OPN is in close relation to portal hypertension. Although there is much evidence available regarding the role of OPN in hepatic fibrogenesis, the relationship of OPN to portal hypertension (evaluated by HVPG measurement) has not been described previously. HVPG measurement is used in different clinical situations in routine praxis; distinguishing between patients with clinically significant portal hypertension (*i.e.*, HVPG > 10 mmHg) and those at risk of bleeding (HVPG > 12 mmHg)[17] is of most importance. Our data suggest that, in both these groups of patients, a single laboratory parameter, *i.e.*, plasma OPN levels, was sufficient to estimate values to quite a satisfactory sensitivity and specificity. From a clinical point of view, it is important to note that this differentiation was also observed in the group of compensated patients without ascites (which is usually the basic clinical parameter for showing the presence of clinically significant portal hypertension). The performance of OPN values in the discrimination of patients with clinically significant portal hypertension was better than those of common markers, such as platelet count/spleen size ratio, platelet count, and AST/ALT ratio.

In an experimental model of liver fibrosis, OPN was shown to serve as a key cytokine within the extracellular matrix protein network, contributing to scarring and liver fibrosis[6]. OPN also delays liver fibrosis resolution due to sustained fibrillar collagen-I deposition in mice after thioacetamide-induced fibrosis[7]. Recently, plasma OPN levels have been found to predict liver fibrosis in various chronic liver diseases, such as NASH[11], alcoholic liver disease[12], and chronic viral hepatitis B[13] and C[14]. Pereira *et al*[21] have also demonstrated that OPN secretion could be stimulated by *Schistosoma mansoni* and that serum OPN levels correlated with splenic vein pressure and liver fibrosis stage in patients with schistosomiasis.

Portal hypertension is pathogenically related to liver injury and fibrosis, which leads to major complication of cirrhosis, and has been evaluated to date by invasive measurement of portal pressure (HVPG)[15].

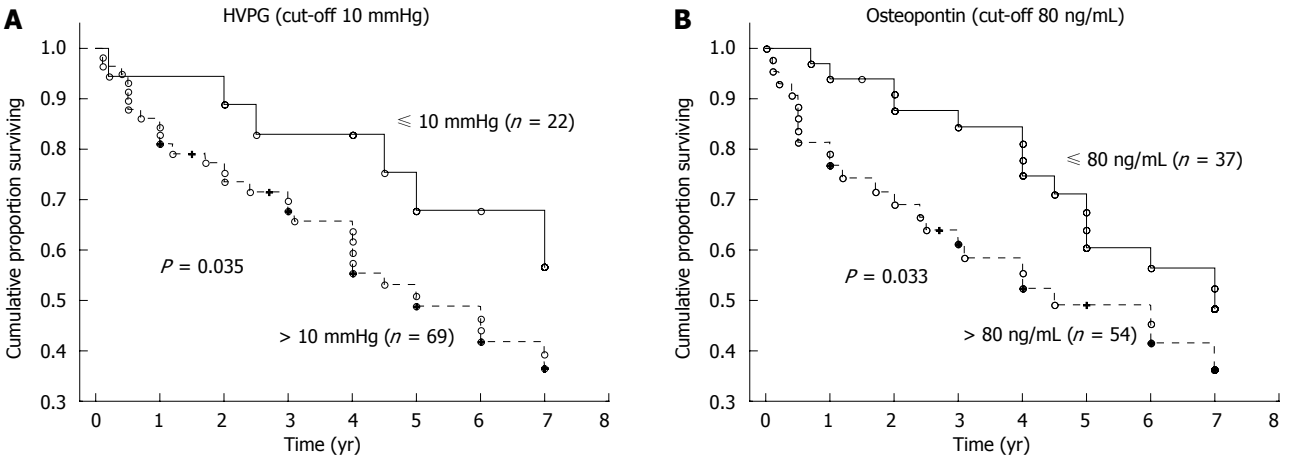

Figure 4 Cumulative proportion of surviving patients with hepatic venous pressure gradient values below and above 10 mmHg (A) and plasma osteopontin levels below and above 80 ng/mL (B) in compensated patients without ascites using the Kaplan-Meier method. HVPG: Hepatic venous pressure gradient; OPN: Osteopontin.

Table 4 Laboratory and clinical parameters in different Child-Pugh groups of patients

Parameter	Child-Pugh A	Child-Pugh B	Child-Pugh C	*P* value
Age (yr)	55.6 ± 10.7	52.9 ± 11.2	56.3 ± 10.7	NS
Osteopontin (ng/mL)	84.8 ± 34.9	158.8 ± 98.2	205.2 ± 142.8	A *vs* B, $P < 0.001$, A *vs* C, $P < 0.001$, B *vs* C, NS
HVPG (mmHg)	14.1 ± 5.1	17.1 ± 4.8	19.5 ± 4.5	A *vs* B, $P < 0.001$, A *vs* C, $P < 0.001$, B *vs* C, NS
Survival (yr)	4.6 ± 2.3	3.3 ± 2.5	2.5 ± 2.6	A *vs* C, $P < 0.001$, A *vs* B, NS, B *vs* C, NS

HVPG: Hepatic venous pressure gradient; NS: Not significant.

Table 5 Correlation of hemodynamic evaluation of portal hypertension and osteopontin to clinical parameters in patients with liver cirrhosis

Parameter	HVPG		Osteopontin	
	r	*P* value	*r*	*P* value
Survival	0.220	0.009	0.230	0.006
Platelet counts	0.247	0.005	0.231	0.009
Size of varices	0.352	< 0.001	0.009	0.927
History of bleeding from varices		< 0.001		0.506
Presence of ascites		< 0.001		< 0.001

r: Correlation coefficient; HVPG: Hepatic venous pressure gradient.

The HVPG is a prognostic factor for long-term survival in the case of cirrhosis[22] and can even reflect progression of the disease in the pre-cirrhotic stage. In fact, there is an association between the severity of hepatic inflammation and fibrosis and the HVPG even before cirrhosis develops[23]. Longitudinal studies are needed to assess whether OPN, as a key mediator of the alcohol-induced effects on hepatic stellate cell functions and liver fibrogenesis[24], could give similar information.

Another important finding from our study relates to the prognostic value of OPN.

Data are abound in the literature on the ability of HVPG to predict overall liver-related outcomes, in particular liver cirrhosis decompensation[25] and variceal hemorrhage[26]. In a study by Ripoll *et al*[25], patients with an HVPG < 10 mmHg had a 90% probability of not developing clinical decompensation after a 4-year median follow-up; however, the survival data were not clearly shown. A reduction in the HVPG to less than 12 mmHg or a reduction of more than 20% from the baseline value was associated with a decreased risk of variceal hemorrhage and improved survival[27,28]. In one study, the HVPG was shown to have better efficacy for predicting 1- and 2-year mortality in cirrhotic patients than that from results obtained using the MELD score[29]. Nevertheless, the clear relationship between single HVPG measurement and overall survival of patients with cirrhosis is supported by very few studies. We observed in our patients that a HVPG cut-off value of 10 mmHg, obtained during a single measurement, stratified cirrhotic patients into two groups with different prognoses regarding survival probability. Surprisingly, we found OPN to be a strong predictor of survival in patients with cirrhosis, with the same validity as the HVPG. A cut-off value of 80 ng/mL revealed two groups of cirrhotic patients with different probabilities of survival, even in the group of compensated patients, which suggests that OPN determination might benefit from being implemented in routine clinical settings. As described previously, common clinical parameters, such as platelet count or ultrasonographic parameters of portal hypertension, correlate with the degree of portal hypertension, but none of these simple parameters could be used for

staging portal hypertension or patient risk stratification. The most frequently used (and proved) prognostic parameters in cirrhosis are based on the evaluation of liver function (Child-Pugh classification, MELD score). The only prognostic parameter related to portal hypertension that is independent of other factors was shown to be the HVPG. Our study suggests that plasma OPN levels could stratify patients into two groups with different prognoses, similar to the HVPG. The survival of patients with plasma OPN concentrations below the cut-off value of 80 ng/mL was significantly longer compared to those patients with higher OPN levels (56% *vs* 37%).

The mean follow-up interval of our patients was 3.7 years, an interval sufficiently long to consider our data statistically significant and reliable for identification of survival differences.

Most patients in our study had cirrhosis of ethylic etiology (none of them presented with uncontrolled abuse of alcohol). The number of patients with other etiologies did not enable us to find a prognostic role, but based on literature data[26] we would not expect a significant difference.

As predicted, HVPG values correlated with different clinical parameters of portal hypertension, such as platelet count, size of esophageal varices, history of variceal bleeding, and presence of ascites. Surprisingly, plasma OPN levels correlated only with platelet count and presence of ascites but not with "variceal-related" parameters (size of varices or history of variceal bleeding).

However, it remains to be answered whether OPN plasma concentration reflects the actual value of HVPG and whether it in turn changes continuously with HVPG changes. If indeed it does, this would enable us, for example, to assess the effect of pharmacology treatment on portal hypertension or to evaluate other continuous changes in portal hypertension, otherwise made possible only by invasive HVPG measurement until now[30]. Further studies are needed to address these questions.

Another important issue is the relation of plasma OPN concentration and HCC. Using proteomic profiling of plasma from patients with cirrhosis and HCC, Shang *et al*[10] identified OPN to be significantly upregulated in HCC cases compared to cirrhosis controls. Subsequently, plasma concentrations of OPN measured in cirrhotic patients, with and without HCC, revealed significantly higher concentrations in individuals with HCC compared with those without tumors. Recently, Nabih *et al*[31] suggested OPN as a tumor marker, which could be used as a screening test for the diagnosis of HCC in patients with liver cirrhosis caused by the hepatitis C virus. No relationship of plasma OPN or HVPG levels to occurrence of HCC was found in our cohort of patients. This could be partly due to the limited number of patients with available clinical data with regard to HCC (81 of 154 patients) and partly due to the low incidence of HCC in our patients, which is in concordance with the generally low incidence of HCC in the Czech Republic[9].

Limitations

Some limitations of our study to consider are the lack of a validation cohort and the strong regional focus on enrolled patients in this study. The main influence within our examined region mainly pertains to the high percentage of alcoholic cirrhosis in our patients. Another limitation is the lack of liver stiffness measurement in our patients, which has been shown to perform well in the detection of clinically significant portal hypertension, especially in combination with the platelet count/spleen diameter ratio[32].

In conclusion, we report a close relationship between plasma concentrations of OPN and portal hypertension in cirrhotic patients, a fact not known until now. OPN could be used to detect significant portal hypertension even in compensated patients without ascites. Our results also indicate that OPN is an independent prognostic parameter of overall survival in cirrhotic patients and that it could be incorporated into prognostic models in patients with liver cirrhosis. The role of OPN in the evaluation of responses to portal hypertension treatments should be explored in future studies.

COMMENTS

Background

Portal hypertension leads to major complications of cirrhosis. Until now, invasive measurement of the hepatic venous pressure gradient (HVPG) has been the only method used for the exact evaluation of portal hypertension. Recently, osteopontin (OPN) has emerged as a new marker through its possible relation to fibrosis and cirrhosis.

Research frontiers

Although the relationship between OPN and liver fibrosis has been described previously, the relationship to portal hypertension has never been studied.

Innovations and breakthroughs

The close relation of OPN plasmatic levels to portal hypertension has never been described. OPN is a strong prognostic indicator in patients with liver cirrhosis and, similar to HVPG values, significantly determines survival probability even in compensated patients. Moreover, the combination of HVPG and OPN increases the validity of prognosis.

Applications

OPN could be used as a marker of clinically significant portal hypertension and a prognostic parameter in patients with cirrhosis.

Terminology

Clinically significant portal hypertension was defined as HVPG > 10 mmHg.

Peer-review

The authors provide interesting information on the value of OPN measurement as a non-invasive biomarker of portal hypertension. As noted by the authors, this association had not previously been described. The patient size is reasonably large, and the authors have made a good attempt to exclude indications associated with elevated circulation OPN, such as alcohol abuse and hepatocellular carcinoma, in their patient populations.

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ORIGINAL ARTICLE

Prospective Study

Comparison of 5-hydroxytryptophan signaling pathway characteristics in diarrhea-predominant irritable bowel syndrome and ulcerative colitis

Feng-Yan Yu, Shao-Gang Huang, Hai-Yan Zhang, Hua Ye, Hong-Gang Chi, Ying Zou, Ru-Xi Lv, Xue-Bao Zheng

Feng-Yan Yu, Hong-Gang Chi, Ying Zou, Ru-Xi Lv, Xue-Bao Zheng, Traditional Chinese Medicine Institute, Guangdong Medical University, Dongguan 524023, Guangdong Province, China

Shao-Gang Huang, Hai-Yan Zhang, University City Branch Hospital of Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou 510006, Guangdong Province, China

Hua Ye, Guangdong Key Laboratory of Natural Medicine Research and Development, Guangdong Medical College, Zhanjiang 524023, Guangdong Province, China

Author contributions: Yu FY, Huang SG, Zhang HY and Ye H performed the majority of experiments; Chi HG and Zou Y provided vital reagents and analytical tools and were also involved in editing the manuscript; Zheng XB coordinated and provided the collection of all of the human materials in addition to providing financial support for this work; Yu FY designed the study and wrote the manuscript.

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Correspondence to: Shao-Gang Huang, PhD, Professor, Director, University City Branch Hospital of Guangdong Provincial Hospital of Traditional Chinese Medicine, Inner Ring Road, Panyu District, Guangzhou 510006, Guangdong Province, China. huangshaogang@126.com
Telephone: +86-20-39318991
Fax: +86-20-34728881

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Abstract

AIM: To study differences in the visceral sensitivity of the colonic mucosa between patients with diarrhea-predominant irritable bowel syndrome (IBS-D) and those with ulcerative colitis (UC) in remission and to relate these differences with changes in the 5-hydroxytryptophan (5-HT) signaling pathway.

METHODS: Gastrointestinal symptoms were used to determine the clinical symptom scores and rectal visceral sensitivity of patients with IBS-D and patients with UC in remission. Blood levels of 5-HT and

5-hydroxyindoleacetic acid (5-HIAA) were measured using an HPLC-electrochemical detection system. The levels of 5-HT 3 receptor (3R), 4R, and 7R mRNAs in colonic biopsy samples were detected using reverse transcription-polymerase chain reaction. The protein expression of TPH1 was analyzed by Western blot and immunohistochemistry.

RESULTS: Abdominal pain or discomfort, stool frequency, and the scores of these symptoms in combination with gastrointestinal symptoms were higher in the IBS-D and UC groups than in the control groups. However, no significant differences were observed between the IBS-D and UC remission groups. With respect to rectal visceral sensitivity, the UC remission and IBS-D groups showed a decrease in the initial perception threshold, defecating threshold and pain threshold. However, these groups exhibited significantly increased anorectal relaxation pressure. Tests examining the main indicators of the 5-HT signaling pathway showed that the plasma 5-HT levels, 5-HIAA concentrations, TPH1 expression in the colonic mucosa, and 5-HT3R and 5-HT5R expression were increased in both the IBS-D and the UC remission groups; no increases were observed with respect to 5-HT7R expression.

CONCLUSION: The IBS-D and UC groups showed similar clinical symptom scores, visceral sensitivity, and levels of serotonin signaling pathway indicators in the plasma and colonic mucosa. However, the pain threshold and 5-HT7R expression in the colonic mucosa were significantly different between these groups. The results reveal that (1) IBS-D and UC are related to visceral sensitivity pathogenesis and the clinical manifestations of these conditions and (2) the observed differences in visceral hypersensitivity are possibly due to differences in levels of the 5-HT7 receptor, a component of the 5-HT signaling pathway.

Key words: Stomach type diarrhea; Diarrhea-predominant irritable bowel syndrome; Ulcerative colitis in remission; 5-Hydroxytryptophan

Core tip: Irritable bowel syndrome (IBS) is among the most common functional gastrointestinal disorders, but its pathogenesis is not understood. Ulcerative colitis (UC) is a chronic non-specific inflammatory gastrointestinal disease. Visceral hypersensitivity is the most well-known cause of abdominal pain related to diarrhea-predominant IBS (IBS-D) and UC. The 5-hydroxytryptophan (5-HT) signaling pathway is important for both sensory signal transduction in gastrointestinal motility and the development of visceral hypersensitivity. This study examined visceral sensitivity differences in the colonic mucosa between IBS-D and UC remission groups with respect to the 5-HT signaling pathway. We offer a new theoretical basis for Chinese medical treatment for the two types of common intestinal diseases related to 5-HT signaling pathways. These data will also provide new insights into future methods for the application of traditional Chinese medicine.

Yu FY, Huang SG, Zhang HY, Ye H, Chi HG, Zou Y, Lv RX, Zheng XB. Comparison of 5-hydroxytryptophan signaling pathway characteristics in diarrhea-predominant irritable bowel syndrome and ulcerative colitis. *World J Gastroenterol* 2016; 22(12): 3451-3459 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3451.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3451

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders, but its pathogenesis is poorly understood. Ulcerative colitis (UC) is a chronic non-specific inflammatory gastrointestinal disease. The pathogenesis of UC may be related to genetic factors, intestinal flora, immune disorders, dietary allergies, and anxiety.

Abdominal pain is an important symptom in UC patients, and visceral hypersensitivity is the most widely known cause of abdominal pain. The 5-hydroxytryptophan (5-HT) signaling pathway is important for both sensory signal transduction in gastrointestinal motility and the development of visceral hypersensitivity[1-4].

In this study, we determined the blood levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) using a high performance liquid chromatography (HPLC)-electrochemical detection system. We used reverse transcription-polymerase chain reaction (RT-PCR) to assay the transcriptional levels of 5-HT 3 receptor (3R), 4R, and 7R in biopsied colonic tissue samples. The protein expression of tryptophan hydroxylase 1 (TPH1) was determined using Western blot, and serotonin levels were detected *via* immunohistochemistry.

These assays were performed using samples from normal, diarrhea-predominant IBS (IBS-D), and UC patients. The results obtained will provide a new theoretical basis for the Chinese medical treatment of the two common intestinal diseases that are related to 5-HT signaling. These data will also provide new insights into future applications of traditional Chinese medicine.

MATERIALS AND METHODS

Diagnostic criteria

The diagnostic criteria for IBD were based on the Consensus Norms for Chinese Diagnosis and Treatment of Inflammatory Bowel Disease, which were issued in 2008 by the Inflammatory Bowel Disease Collaborative Group of the Chinese Medical Association Digest

Table 1 Comparison of age, sex and duration of remission

	Healthy group	UC remission group	IBS-D group
Cases	30	33	30
Age (yr), mean age	23-62, (42.5 ± 19.5)	22-56, (39 ± 17)	18-60, (39 ± 21)
Sex	15 females, 15 males	15 females, 18 males	16 males, 14 females
Duration (yr)	None	1.8 ± 18.2	1.6 ± 20.4

UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Credits. UC patients were not included in this group[5,6]. UC activity was assessed according to Mayo's Disease Activity Index (DAI), which considers the frequency of bowel movements, blood in the stool, colonic mucosal inflammation and the physician's overall evaluation. Cases of UC were judged to be in remission if the evaluation score was $\leqslant 2$.

Inclusion and exclusion criteria

Inclusion criteria were: (1) meeting the UC or IBS-D criteria; (2) being 19-60 years of age; and (3) signing an informed consent form. Exclusion criteria were: (1) having colon cancer; (2) having other autoimmune diseases, such as systemic lupus erythematosus and multiple sclerosis; and (3) having other tumors or digestive issues.

General information

We selected patients treated at University City Branch of Guangdong Provincial Hospital and Nanfang Hospital from June 2012 to January 2014. The normal control group consisted of healthy individuals. We selected 33 patients with UC in remission, 30 IBS-D patients and 30 healthy participants. There were 18 males and 15 females with UC in remission, and they were aged from 22 to 56 years. The average age was 39 ± 17 years, and the average duration of the condition was 1.8 ± 18.2 years. No significant differences were observed in age, sex, or duration of remission between the analyzed groups ($P > 0.05$) (Table 1).

Symptom score

The gastrointestinal symptoms rating scale (GSRS)[2] was used. For symptom rating, the considered symptoms/complaints included the degree of abdominal pain or discomfort, the frequency of stool passage, abnormal stool frequency, abnormal bowel movement frequency (bowel problems, defecation), and frequency of mucus in the stool. The severity was scored as follows: 0, symptom not present; 1, mild; 2, moderate; and 3, severe. Frequency rating was as follows: 0, symptom did not occur; 1, occasionally (symptomatic between 1% and 24% of the time); 2, often (symptomatic between 25% and 50% of the time); and 3, sustained (> 50% of the time).

Anorectal manometry

A Synectics Visceral Stimulator (CTD-Synectics Medical Company, Sweden) was used as a whole digestive tract detector. This device consists of an electronic pressure pump for monitoring gastrointestinal tension as well as a capillary perfusion digestive pressure monitoring system. The catheter used for anorectal manometry had an outer diameter of 0.8 cm, with a 10 cm × 8 cm air sac at the front end. The gas injection channel and the balloon pressure channel at the opening of the balloon were connected with an electronic pressure pump during the procedure. Four perfusion manometry channels (1 cm apart) were located 14 cm from the catheter tip and were connected with a PC Polygraf apparatus during testing. Patients undergoing anorectal manometry kept a normal diet and received a fecal enema a few days prior to testing. The patient was placed in the left lateral supine position, and the catheter was inserted following anal dilation until the four perfusion pressure measurement channels were in the high-pressure zone. The manometry catheter was then fixed. First, the anal sphincter resting pressure was recorded, and the patient was then asked to contract the anus for detection of maximum diastolic blood pressure and to defecate to detect sphincter diastolic blood pressure. We tested patient sensation and compliance using the electronic pressure pump. Gas was injected into a balloon using a 20 mL gas injection gradient at a rate of 38 mL/h. The patients' initial sensory thresholds, defecation thresholds and pain thresholds were observed. The pressure between the wall of the balloon and the intestine during gas injection was detected, and a compliance curve was made to determine the maximum compliance value.

Samples and detection reagents

Three milliliters of venous blood were obtained from all of the subjects for HPLC analysis. Colonoscopies were also carried out for all subjects. The specimen collection was approved by the hospital ethics committee. A polyclonal antibody for the serotonin transporter (SERT) was purchased from Beijing Bioss. The rabbit and mouse anti-human immunohistochemistry kits and the DAB reagent kit were purchased from Beijing Zhongshan Golden Bridge Company. A rabbit-anti-rat TPH1 antibody (Santa Cruz, United States), a mouse-anti-rat β2-actin antibody (Ab-cam, United States), mouse and rabbit secondary antibodies (KPL, United States) and TRIzol Reagent (Invitrogen, United States) were used. SDS-PAGE apparatus (BIO-RAD, United States), a DY2CZ-40B electrophoretic transfer tank (Beijing Liuyi, China) and image analysis system (UVP) were used for protein detection. RNA extraction was performed according to the kit's instructions, and OD260 was measured using a UV spectrophotometer (UV-1601). RT-PCR primers for the examined genes and the internal control (GAPDH) were synthesized by SANGON. A tissue RNA extraction kit was purchased

from Invitrogen. A reverse transcription kit was purchased from PROMEGA, and RNase AWAY was purchased from QIAGEN. All of these procedures were carried out according to the kits' instructions.

Indicator detection

5-HT and 5-HIAA in serum were detected using an HPLC-electrochemical detector system.

Determination of TPH1 protein in the colonic mucosa by Western blot

The specimens were cut into small pieces of 1 mm^3 and washed thoroughly with PBS. After the addition of lysis buffer, the samples were incubated for 2 h at 4 ℃. The samples were then centrifuged and denatured. The supernatant was immediately analyzed or stored at -80 ℃.

After electrophoresis, the proteins were transferred to a PVDF membrane using wet transfer. The membrane was blocked in 5% skim milk for 2 h at room temperature and then incubated with rabbit anti-TPH1 (1:200) or mouse anti-β2 actin (1:1000) antibody at 4 ℃ overnight. The membrane was incubated at room temperature with an anti- rabbit or mouse secondary antibody, as appropriate (1:1000 and 1:500) for 2 h. Photographs were taken after ECL coloration. Image analysis was carried out, and the gray ratio of TPH1 to β-actin was measured.

Immunohistochemical detection of serotonin in the colonic mucosa: Specimens were processed using conventional immobilization, embedding and sectioning. Serotonin levels were detected in each group using immunohistochemistry. Briefly, the SERT protein antigen was retrieved *via* hot fixation with EDTA (130-160 ℃, 1-2 min). PBS was employed as a negative control. Under a microscope (magnification × 200), three areas of positively stained cells were randomly selected for each slice to observe and calculate the average gray value. A digital image acquisition system and an HPIAS-1000 high-resolution, color pathology report analysis system were used for this procedure. The gray value was inversely proportional to the expression level, *i.e.*, the higher the gray value, the lower the level of expression.

Detection of 5-HT3R, 4R, and 7R expression in the colonic mucosa by RT-PCR

The obtained tissue blocks (approximately 100 mg) were homogenized, and TRIzol was added (approximately 2 mL TRIzol per 100 mg tissue). Frozen homogenized tissue was transferred to Eppendorf tubes and incubated for 5 min at 15-30 ℃. Chloroform was added (0.2 mL chloroform/1 mL TRIzol), after which the samples were shaken for 15 s and incubated for 2-3 min at 15-30 ℃. The samples were then centrifuged at 12000 r/min for 15 min at 4 ℃. The supernatant was transferred to a new Eppendorf tube, and isopropyl alcohol was added (0.5 mL isopropanol/1 mL TRIzol). The samples were incubated for 10 min at 15-30 ℃ and centrifuged at 12000 r/min (4 ℃) for 10 min. The supernatant was discarded, and 75% ethanol was used to wash the precipitate (at least 1 mL 75% ethanol/1 mL TRIzol). Then, the sample was centrifuged at 500 r/min for 5 min at 4 ℃, and the ethanol was removed. The precipitate was dried for 5-10 min in air (the sample was not allowed to dry completely). DEPC-treated water was added to dissolve the RNA, which was stored at -80 ℃. The RNA was reverse transcribed to cDNA. The cDNA was used as a template for PCR amplification with two pairs of primers. The first pair recognized the *5-HT3R* gene (forward: 5'-CAAGCCACCAAGACTGATGA-3'; reverse: 5'-AACCAGGGTGATGCTGTAGG-3'). The expected amplified fragment length was 290 bp. The second primer pair recognized GAPDH (forward: 5'-GAGTCAACGGA1TITI1GGTCGT-3'; reverse: 5'-CCATCCACAGTCTrCTGGGT-3'), and the expected amplified fragment length was 577 bp. The reaction conditions were as follows: 94 ℃ for 3 min; 30 cycles of 94 ℃ for 30 s, 59 ℃ for 30 s, and 72 ℃ for 1 min; and final extension for 5 min at 72 ℃. The primers for the *5-HT7R* gene were 5 -GCTCATCACGCTGCTGACGAT-3 (forward) and 5'-CGCCAGGGACACAATCAGG-3 (reverse), amplifying a 106-bp fragment. The specific steps were carried out according to kit instructions. Eight microliters of the PCR product and 2 μL DNA loading buffer were mixed to perform 2% agarose gel electrophoresis, and a 100-bp molecular weight standard was added as a control marker. The electrophoresis data were analyzed using a gel imaging system. The location of the desired product was determined, and the gray area densities on the gel images were used to represent gene expression. The mRNA levels of GAPDH and the *5-HT3R*, *4R*, and *7R* genes were determined in a semi-quantitative manner.

Statistical analysis

SPSS 13.0 software was used to analyze the results. The statistical data are expressed as the mean ± SD and were analyzed using single-factor analysis of variance (ANOVA).

RESULTS

Changes of gastrointestinal symptom scores in each group

The IBS-D and UC remission groups exhibited increased abdominal pain or discomfort, stool frequency, stool mucus integration and total gastrointestinal symptom scores compared with the normal control group. Various gastrointestinal symptom scores and the total symptom score of UC patients were higher than those for the IBS-D group, but no significant differences were observed between these two groups (Figure 1,

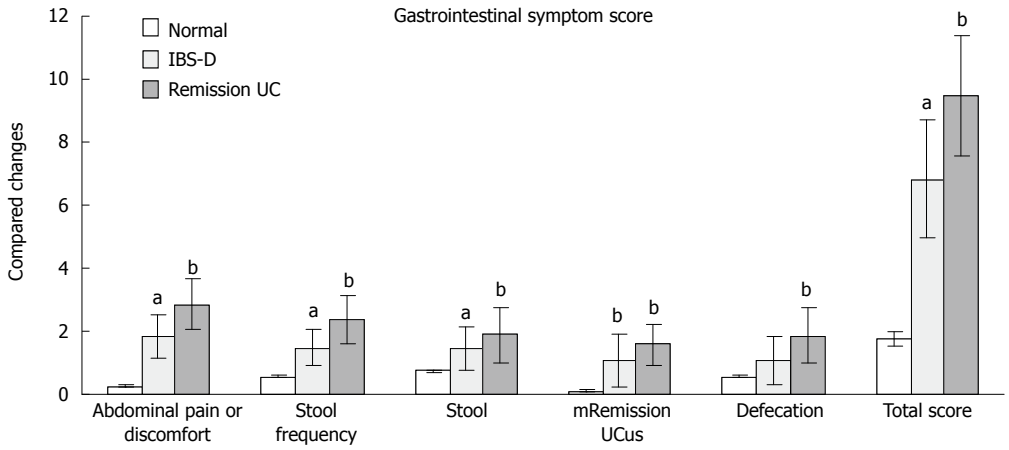

Figure 1 Changes in gastrointestinal symptom scores in each group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 2 Comparisons of changes in the gastrointestinal symptom scores of patients in each group (mean ± SD)

Symptom	Normal	IBS-D	UC in remission
Abdominal pain or discomfort	0.25 ± 0.05	1.84 ± 0.67[a]	2.84 ± 0.81[b]
Stool frequency	0.55 ± 0.05	1.46 ± 0.58[a]	2.34 ± 0.76[b]
Stool	0.75 ± 0.05	1.44 ± 0.67[a]	1.89 ± 0.87[b]
Mucus	0.10 ± 0.03	1.04 ± 0.84[b]	1.58 ± 0.67[b]
Defecation	0.55 ± 0.05	1.06 ± 0.74	1.86 ± 0.86[b]
Total score	1.75 ± 0.23	6.83 ± 1.86[a]	9.51 ± 1.91[b]

$^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 2).

Testing of anorectal motility and rectal visceral sensation

The anorectal pressure measurement results for each group are shown in Table 3. The initial sensory thresholds, defecation threshold and pain thresholds in the UC remission and IBS-D groups were significantly lower compared with those in the healthy control group ($P < 0.05$). In addition, anorectal relaxation pressure was significantly higher in both the UC remission and IBS-D groups compared to the control ($P < 0.05$). There were no significant differences in the initial sensory thresholds, defecation threshold or anorectal relaxation pressure between the UC remission and IBS-D groups, although these values were lower in the IBS-D group ($P > 0.05$). A significant difference was observed, however, for pain thresholds between these two groups ($P < 0.05$).

Measurements of 5-HT and 5-HIAA contents with an HPLC-electrochemical detector system

Plasma concentrations of 5-HT and 5-HIAA in patients with IBS-D and UC in remission were significantly increased relative to the controls ($P < 0.05$). The differences between the IBS-D and UC remission groups were not significant ($P > 0.05$) (Figure 2, Table 4).

Detection of TPH1 protein expression by Western blot

TPH1 expression was significantly increased in patients with IBS-D and UC in remission ($P < 0.05$) compared with the control group. The difference between the IBS-D group and the UC remission group was not significant ($P > 0.05$) (Figure 3, Table 5).

5-HT3R, 4R, and 7R mRNA detection

The *5-HT3R* expression levels in the colonic mucosa were significantly higher in both the IBS-D and UC remission groups compared with those in the normal control group, although there was no significant difference ($P > 0.05$) between the UC group and the IBS-D group. *5-HT7R* expression in the UC group was elevated compared with the IBS-D and normal control groups, and these differences were significant ($P < 0.05$). The difference in *5-HT7R* expression between the IBS-D group and the normal control group was not significant ($P > 0.05$). *5-HT4R* expression levels in the colonic mucosa of patients with IBS-D and UC in remission were significantly lower than those in the normal control group ($P < 0.05$), although there was no significant difference between the IBS-D group and UC remission group ($P > 0.05$) (Figure 4).

SERT protein expression

The SERT is widely expressed in the membrane and cytoplasm of cells in the colon. In this study, cells with positive expression were stained brown. Compared with the control group, the number of positive cells and staining density were both lower in the IBS-D and the UC groups. The PBS negative control group showed no positive staining. The gray values of each group fell within a normal distribution ($P = 0.2$). All of the results are shown in Table 6, and the gray values of the IBS-D and UC groups were significantly higher than those in the control ($P < 0.05$), while no statistical significance was observed between these two groups ($P > 0.05$) (Figure 5, Table 7).

Table 3 Testing of anorectal motility and rectal visceral sensation in each group

	Normal	IBS-D	UC in remission
Initial sensory threshold (mL)	50.18 ± 4.19	23.91 ± 10.15[a]	20.10 ± 9.17[b]
Defecation threshold (mL)	69.15 ± 10.75	35.17 ± 12.71[a]	21.28 ± 10.32[b]
Pain threshold (mL)	105.90 ± 20.15	70.10 ± 11.25[a]	50.33 ± 12.30[b]
Maximum compliance (mL/mmHg)	4.95 ± 1.81	5.02 ± 1.75	4.98 ± 1.91
Resting pressure (mmHg)	62.15 ± 15.21	65.54 ± 13.15	63.27 ± 10.15
Relaxation pressure (mmHg)	105.15 ± 60.15	140.50 ± 55.50[a]	138.69 ± 52.82[a]
Relaxation pressure (mmHg)	43.18 ± 22.50	45.15 ± 20.65	46.10 ± 19.97

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

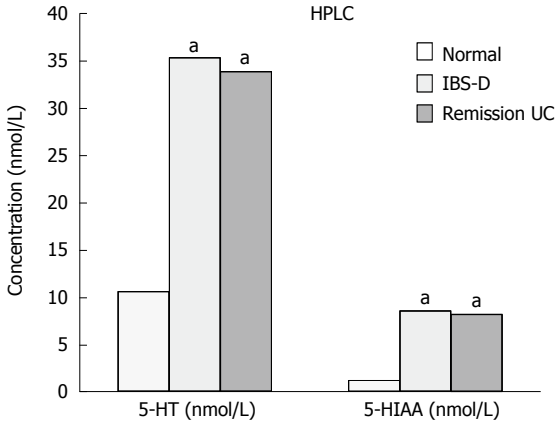

Figure 2 Comparison of 5-HT and 5-HIAA concentrations in each group. [a]$P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 4 Comparison of 5-HT and 5-HIAA concentrations in each group (mean ± SD)

	5-HT (nmol/L)	5-HIAA (nmol/L)
Normal	10.55 ± 1.35	1.15 ± 0.15
IBS-D	35.31 ± 2.15[a]	8.50 ± 1.50[a]
UC in remission	33.85 ± 1.55[a]	8.15 ± 1.35[a]

[a]$P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

DISCUSSION

IBS is one of the most common clinical functional gastrointestinal disorders, but the pathogenesis of IBS is unclear. UC is a chronic non-specific inflammatory gastrointestinal disease, and its pathogenesis may be related to genetic factors, intestinal flora, immune disorders, dietary allergies and anxiety. Abdominal pain is a frequent symptom in UC patients, and visceral hypersensitivity is currently the most widely used explanation for abdominal pain. Research[3] shows that 5-HT plays an important role in the formation of intestinal peristalsis, signal transduction and visceral hypersensitivity[4].

Ohman *et al*[7] used an enema containing acetic acid in SD rats. After 7 d, a histological examination of myeloperoxidase staining revealed normal results, but the rats showed a high sensitivity to intestine

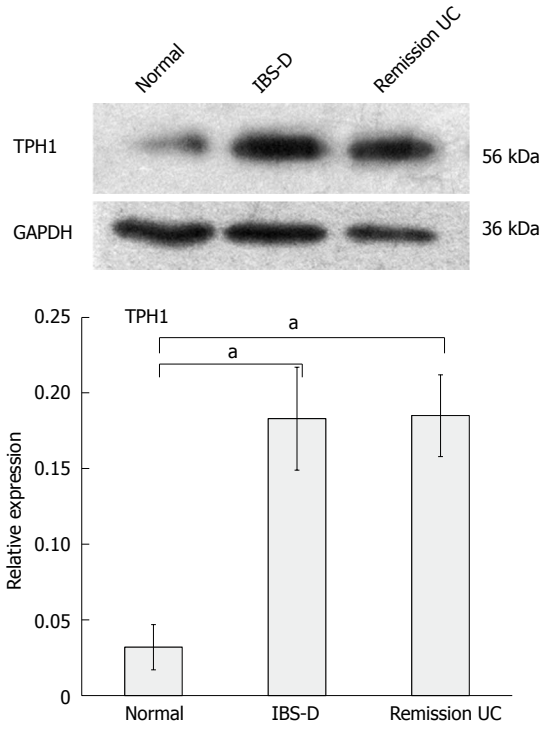

Figure 3 TPH1 protein expression in the colonic mucosa of patients from each group. [a]$P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 5 TPH1 protein expression in the colonic mucosa of patients in each group

Group	TPH1
Normal	0.032 ± 0.015
IBS-D	0.183 ± 0.034[a]
UC in remission	0.185 ± 0.027[a]

[a]$P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

expansion; after constant pressure stimulation, defecation was higher than that in the normal control group. This previous study revealed that both intestinal damage and high levels of intestinal smooth muscle tension were associated with high visceral sensitivity.

Another study found that visceral sensitivity was involved in UC disease activity, stage and involvement scope[8].

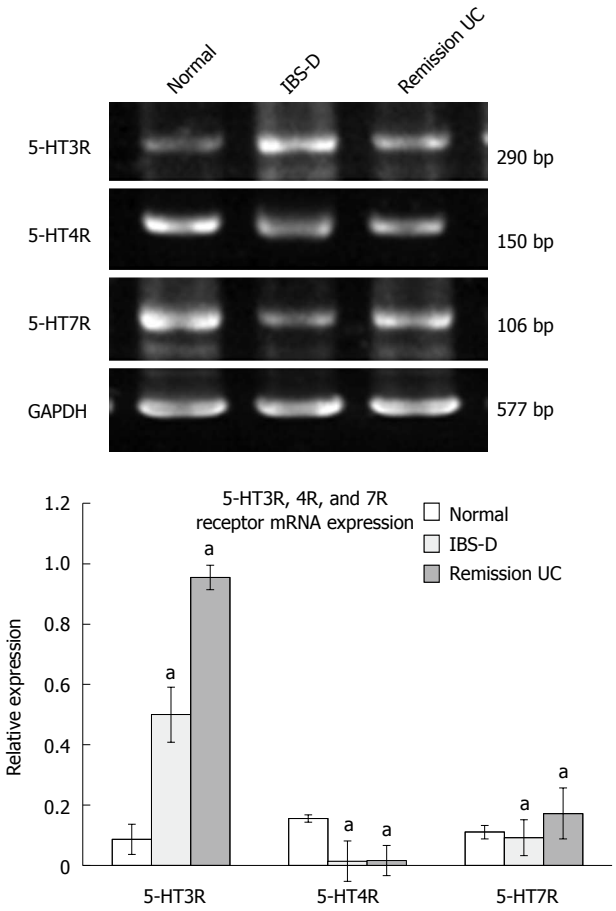

Figure 4 5-HT3R, 4R, and 7R receptor mRNA expression in each group. $^{a}P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 6 5-HT3R, 4R, and 7R receptor mRNA expression levels in each group

Group	5-HT3R	5-HT4R	5-HT7R
Normal	0.086 ± 0.05	0.155 ± 0.0913	0.1097 ± 0.0405
IBS-D	0.499 ± 0.012[a]	0.013 ± 0.067[a]	0.0911 ± 0.0501[a]
UC in remission	0.954 ± 0.022[a]	0.015 ± 0.059[a]	0.1711 ± 0.0845[a]

$^{a}P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

5-HT is both an important signaling molecule in the gastrointestinal tract and a neurotransmitter. Approximately 95% of 5-HT is in the intestine, where 90% is stored in enterochromaffin cells (ECs). The 5-HT transporter (SERT) is present in the central nervous system and digestive tract, where it is expressed to a high degree in neurons and intestinal epithelial cells of the intestinal tract. In these cells, SERT can mediate the effects of 5-HT by rapidly uptaking 5-HT. Abnormalities in the 5-HT signal system[9] can cause gastrointestinal motility problems, secretion function abnormalities and high visceral sensitivity. Moreover, abnormalities in this signaling pathway are closely related to abdominal pain, chronic

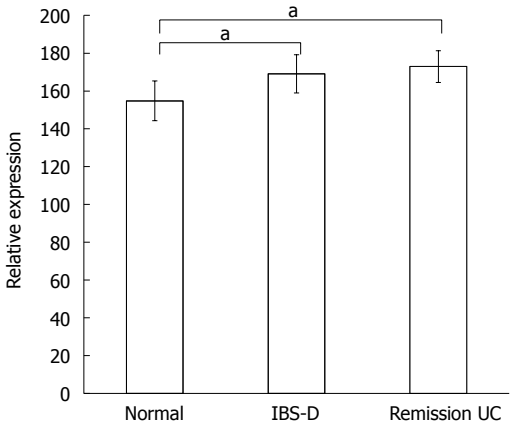

Figure 5 Comparison of serotonin transporter protein gray values. $^{a}P < 0.05$ *vs* normal. UC: Ulcerative colitis; IBS-D: Diarrhea-predominant irritable bowel syndrome.

Table 7 Comparison of serotonin transporter protein gray values

Group	Cases	Mean gray value
Normal	30	154.8 ± 10.5
IBS-D	30	169.1 ± 10.1[a]
Ulcerative colitis	33	173.0 ± 8.4[a]

$^{a}P < 0.05$ *vs* normal. IBS-D: Diarrhea-predominant irritable bowel syndrome.

constipation, diarrhea, IBS, functional dyspepsia and other diseases. SERT is the most important protein in mediating the biological activity of 5-HT. Selective serotonin reuptake inhibitors block 5-HT reuptake by acting on the SERT, increasing 5-HT concentrations in the synaptic cleft. We found that such drugs can effectively relieve some of the symptoms, especially abdominal pain, of IBS patients[10-16]. In summary, SERT plays an important role in the generation of visceral hypersensitivity. 5-HT functions through a variety of 5-HT receptors. The multiple types of 5-HT receptors in the intestine have different functions on smooth muscle cells. Among these receptors, both 5-HT3 and 5-HT4 are closely related to the pathogenesis of IBS-D. The 5-HT3 receptor is a ligand-gated anion channel that is expressed in the external sensory neurons of the intestinal tract. This receptor transmits injury signals to the central nervous system, acting as a rapid onset excitatory neurotransmitter on 5-HT neurons, and is closely related to the regulation of visceral sensitivity. The 5-HT4 receptor is a G protein-coupled metabotropic receptor. The opening of voltage-sensitive calcium channels stimulates the release of other neurotransmitters that play an important role in the gastrointestinal tract, such as calcitonin gene-related peptide and substance P. These neurotransmitters thereby affect gastrointestinal motility and visceral sensation[4,5]. This study shows that visceral hypersensitivity usually manifests as

hyperalgesia and allodynia, which have been linked to 5-HT1A, 5-HT2A, 5-HT3R, 5-HT4R, and 5-HT7R[16].

Compared with the healthy group, patients in both disease groups experienced abdominal pain or discomfort and passed stools frequently. The scores of these symptoms coupled with the gastrointestinal symptoms were higher in the IBS-D and UC groups than in the control group. However, there was no significant difference between the IBS-D and UC remission groups. In the rectal visceral sensitivity test, the UC remission and IBS-D groups showed decreases in the initial perception threshold, the defecating threshold and the pain threshold; however, these groups exhibited significantly increased anorectal relaxation pressure. With respect to the primary indicators of the 5-HT signaling pathway, the IBS-D and UC remission groups both exhibited increased levels of plasma 5-HT and 5-HIAA, elevated TPH1 expression in the colonic mucosa, and higher expression of 5-HT3R and 5-HT5R. No such increase was observed in the expression of 5-HT7R.

In summary, we have found that the IBS-D and UC groups showed similar changes in clinical symptom scores, visceral sensitivity and indicators of serotonin signaling pathway in the plasma and colonic mucosa. However, both the pain threshold and 5-HT7R expression in the colonic mucosa were significantly higher in UC patients. This study examined colonic visceral sensitivity differences between the IBS-D and UC remission groups with respect to the 5-HT signaling pathway. We offer a new theoretical basis for Chinese medicine for treatment of the two common intestinal diseases that are related to 5-HT signaling. These data will also provide novel insights into potential traditional Chinese medicine applications for the treatment of these conditions.

COMMENTS

Background

Irritable bowel syndrome (IBS) is one of the most common clinical functional gastrointestinal disorders. Ulcerative colitis (UC) is a chronic, non-specific inflammatory gastrointestinal disease, the pathogenesis of which is not fully understood. The present study demonstrates that visceral hypersensitivity is the most common cause of abdominal pain in patients with IBS-D and UC. 5-HT signaling pathways play an important role in both the sensory signal transduction of gastrointestinal motility and the development of visceral hypersensitivity.

Research frontiers

This study of IBS and UC reveals that these two diseases have the same pathogenic mechanism in terms of visceral sensitivity. Showing that the 5-HT signaling pathway is involved in visceral sensitivity is an extremely important result. This study reveals that both IBS and UP are related to visceral sensitivity, which represents both the pathogenic mechanism and clinical manifestation of these conditions. These results reveal how the 5-HT signaling pathway differs between these syndromes in terms of visceral hypersensitivity. These data will promote the exploration of visceral sensitivity and the evolution of gastrointestinal dynamics in the context of these two conditions.

Innovations and breakthroughs

This multi-level project shows that the 5-HT signaling pathway is involved in the pathogenic mechanism of visceral hypersensitivity in the two disease models. This study aims to make theoretical contributions to the field and to stimulate innovation with respect to understanding and treating the pathogenic mechanisms of the considered diseases.

Applications

The results suggest that patients with IBS-D and UC groups exhibit similar symptoms with respect to clinical symptom scores, visceral sensitivity, and indicators of the serotonin signaling pathway in the plasma and colonic mucosa. However, the pain threshold and 5-HT7R expression in the colonic mucosa were significantly higher for both groups relative to the control. The results reveal that both conditions are associated with visceral sensitivity and clinical manifestations, and evidence was provided showing that visceral hypersensitivity may be due to differences in levels of the 5-HT7 receptor, which is a component of the 5-HT signaling pathway.

Terminology

5-HT is an important signaling molecule in the central nervous system and is involved in a variety of physiological and psychological functions. There is a close relationship between 5-HT signaling and functional gastrointestinal diseases. Therefore, it is necessary to study the role of this signaling pathway in terms of its functional role in the pathogenesis of UC and for guiding clinical treatment.

Peer-review

This is a well-designed descriptive study in which the authors have analyzed the inhibitory effect of the 5-HT signaling pathway (especially 5-HT7R) and its important role in both the sensory signal transduction of gastrointestinal motility and the development of visceral hypersensitivity.

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Cost-effectiveness analysis of population-based screening of hepatocellular carcinoma: Comparing ultrasonography with two-stage screening

Ming-Jeng Kuo, Hsiu-Hsi Chen, Chi-Ling Chen, Jean Ching-Yuan Fann, Sam Li-Sheng Chen, Sherry Yueh-Hsia Chiu, Yu-Min Lin, Chao-Sheng Liao, Hung-Chuen Chang, Yueh-Shih Lin, Amy Ming-Fang Yen

Ming-Jeng Kuo, Department of Hepatogastroenterology, Tainan Municipal Hospital, Tainan 701, Taiwan

Hsiu-Hsi Chen, Graduate Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei 100, Taiwan

Chi-Ling Chen, Graduate Institute of Clinical Medicine, College of Medicine, and Graduate Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei 100, Taiwan

Jean Ching-Yuan Fann, Department of Health Industry Management, School of Healthcare Management, Kainan University, Taoyuan 338, Taiwan

Sam Li-Sheng Chen, Amy Ming-Fang Yen, School of Oral Hygiene, College of Oral Medicine, Taipei Medical University, Taipei 110, Taiwan

Sherry Yueh-Hsia Chiu, Department and Graduate Institute of Health Care Management, Chang Gung University, Taoyuan 333, Taiwan

Yu-Min Lin, Hung-Chuen Chang, Yueh-Shih Lin, Division of Gastroenterology, Department of Internal Medicine, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei 111, Taiwan

Chao-Sheng Liao, Yangder Health Clinics, Taipei 111, Taiwan

Author contributions: Kuo MJ, Chen HH, and Yen AMF designed the study and drafted the article; Kuo MJ and Yen AMF were responsible for model construction, collection of parameters, and interpretation of the results; Kuo MJ, Chen HH, Chen CL, Fann JCY, Chen SLS, Chiu SYH, Lin YM, Chang HC, and Liao CC made critical revision of the article for important intellectual content; and all authors approved the final version of the article.

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Correspondence to: Dr. Amy Ming-Fang Yen, School of Oral Hygiene, College of Oral Medicine, Taipei Medical University, No.250, Wu-Hsing Street, Xinyi District, Taipei 110, Taiwan. amyyen@tmu.edu.tw
Telephone: +886-2-27361661-5152
Fax: +886-2-27398004

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Abstract

AIM: To assess the cost-effectiveness of two population-based hepatocellular carcinoma (HCC) screening programs, two-stage biomarker-ultrasound method and mass screening using abdominal ultrasonography (AUS).

METHODS: In this study, we applied a Markov decision

model with a societal perspective and a lifetime horizon for the general population-based cohorts in an area with high HCC incidence, such as Taiwan. The accuracy of biomarkers and ultrasonography was estimated from published meta-analyses. The costs of surveillance, diagnosis, and treatment were based on a combination of published literature, Medicare payments, and medical expenditure at the National Taiwan University Hospital. The main outcome measure was cost per life-year gained with a 3% annual discount rate.

RESULTS: The results show that the mass screening using AUS was associated with an incremental cost-effectiveness ratio of USD39825 per life-year gained, whereas two-stage screening was associated with an incremental cost-effectiveness ratio of USD49733 per life-year gained, as compared with no screening. Screening programs with an initial screening age of 50 years old and biennial screening interval were the most cost-effective. These findings were sensitive to the costs of screening tools and the specificity of biomarker screening.

CONCLUSION: Mass screening using AUS is more cost effective than two-stage biomarker-ultrasound screening. The most optimal strategy is an initial screening age at 50 years old with a 2-year inter-screening interval.

Key words: Two-stage biomarker-ultrasound screening; One-stage abdominal ultrasonography screening; Markov model; Cost-effectiveness; Sensitivity analysis; Age

Core tip: Hepatocellular carcinoma (HCC) mortality could be reduced by early detection. Previous studies have investigated the cost-effectiveness of different surveillance intervals and screening modalities but were restricted to high risk populations. We conducted a cost-effectiveness analysis of mass screening for HCC with abdominal ultrasonography for the general population and compared it to the existing two-stage biomarker-ultrasound screening in an area with high HCC incidence. The findings suggest early detection of HCC with abdominal ultrasonography may be useful for the general population in an area with high HCC incidence not covered by hepatitis B vaccination.

Kuo MJ, Chen HH, Chen CL, Fann JCY, Chen SLS, Chiu SYH, Lin YM, Liao CS, Chang HC, Lin YS, Yen AMF. Cost-effectiveness analysis of population-based screening of hepatocellular carcinoma: Comparing ultrasonography with two-stage screening. *World J Gastroenterol* 2016; 22(12): 3460-3470 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3460.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3460

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. In Taiwan, HCC is the leading cause of cancer death, accounting for more than 7000 deaths annually[2]. Previously, we demonstrated that increasing incidence rather than poor survival accounts for the rapid rise in mortality rate from HCC in Taiwan[3]. Because the major cause of HCC is related to hepatitis B infection in Taiwan[4], a nationwide vaccination program was launched in 1984, resulting in a significant reduction in the incidence of childhood HCC[5]. Nonetheless, adults older than 30 years old are not covered by the nationwide vaccination program, and the incidence of HCC has been increasing in this population. Since the seroprevalence of hepatitis B virus and hepatitis C virus is nearly 20% in our country[6], a large number of individuals are at increased risk of developing HCC. Hence, a population-based screening program is needed for the early detection of HCC to facilitate a favorable survival rate. In many countries, abdominal ultrasonography (AUS)-based imaging technique (with or without α-fetoprotein, AFP) is used to detect early stage HCC[7,8]. Several studies on mass screening for high risk individuals have also demonstrated that screening for HCC using AUS-based tools resulted in improved survival compared with the unscreened control[9,10].

The conventional method for HCC screening is to first identify high-risk individuals by using a constellation of biomarkers. These high-risk individuals are further referred to undergo AUS. In previous studies, population-based two-stage liver cancer screening program for high risk individuals has proven efficacious, with a reduction of mortality up to 41% after adjusting for independent risk factors[10]. On the other hand, AUS, with or without AFP, has been proposed to screen HCC in high HCC endemic countries[11-14]. There are pros and cons for both two-stage biomarker-ultrasound and one-stage AUS. The two-stage method is efficient, but the AUS referral is a barrier for high-risk subjects identified at the first stage. One-stage AUS may dispense with the referral issue but with increased cost and false positive rate. In addition, its relative costs and effectiveness, particularly the long-term outcome, have yet to be estimated.

The choice of either a two-stage method or one-stage AUS screening is of great interest to health policy-makers in high endemic HCC areas. The primary aim of this study is to compare the cost-effectiveness of the two above mentioned strategies. The optimal initial age and inter-screening interval are also investigated.

MATERIALS AND METHODS

Model design and structure

We developed a Markov decision model as the frame-

work to evaluate the economics of two screening strategies for HCC prevention - two-stage biomarker-ultrasound method and mass screening using AUS - compared to no screening for a hypothetical cohort of 40-year-old residents in a high HCC incidence area, such as Taiwan.

Figure S1 shows a schematic representation of our four-state Markov model to represent the natural course of a hypothetical cohort with no screening. Parameters related to disease progress were based on Chen's model[10]. The population was divided into non-cirrhotic and cirrhotic groups. We utilized a Markov cohort simulation method to follow this hypothetical cohort from 40 to 79 years old or until death, whichever came first. The time cycle for our Markov model was 1 year. The health states were defined to capture the characteristics of HCC with considerations of screening and treatments. We used TreeAge Software for model construction and Winbugs software for parameters synthesis. Parameters, such as sensitivity and specificity of AUS screening and mortality rate from cirrhosis, were estimated based on the literature. The decision tree of the different screening strategies for HCC is also shown (Figure S2).

Intervention strategies

The following three different strategies for HCC screening were compared: (1) No intervention. The cohort received no organized screening program for HCC. The patients sought medical intervention only when they had HCC symptoms or signs. This group was not our comparator but provided a reference group to oppose the other two interventions; (2) Two-stage biomarker-ultrasound screening. In the first stage, high risk individuals were identified using fasting blood samples to test for hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV) antibody, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and AFP. In the second stage, AUS was used to screen those with at least one of the following findings: positive HBsAg or anti-HCV antibody, AST $\geqslant$ 40 U/L, ALT $\geqslant$ 40 U/L, or AFP $\geqslant$ 20 ng/mL; and (3) Mass screening using AUS. All residents in this high endemic area received AUS for HCC screening.

For strategies 2 and 3, the inter-screening interval was 1 year.

Clinical surveillance

Only those in the high risk group who had been diagnosed as either HBsAg or anti-HCV positive in the prevalent screen were tested for the three other markers (AST, ALT, and AFP) in the subsequent screen. People who had received AUS and were diagnosed with cirrhosis received three monthly abdominal sonogram surveillance. Under AUS, subjects suspected of malignant nodular lesions were referred to receive further confirmatory diagnosis with liver biopsy and subsequent pathological assessment. The confirmed HCC patients were referred for oncological treatment. Otherwise, those false positive patients returned to a regular screening program.

Input of parameters

Disease progression and mortality rate: Table 1 reports the incidence rates, transitional rates, and mortality rates of HCC used in the model for our cohort. The age-specific incidence rates of HCC were extracted from the annual report of the Cancer Registry of Taiwan[2]. The data sources for parameters used in the natural history estimation are our previous population-based studies and large studies on untreated HCC[10,13,15]. Population-based mortality rates for cirrhosis and non-cirrhosis adjusted for age were obtained from published Taiwan Vital Statistics and meta-analyses[2,16-19].

Test characteristics and prognosis of clinical practice: Parameters for test characteristics, such as sensitivity and specificity of biochemical examinations, in the first stage were derived from previous Taiwanese studies[13,20]. The sensitivity and specificity of AUS for both cirrhosis and HCC were extracted from two previous native studies and meta-analyses[9,12,21,22]. The prevalence of cirrhosis was derived from a two-stage screening program, the KCIS program[23]. The treatment outcomes of screening-detected HCC were estimated according to one large hospital-based cohort study in Taiwan[15]. The base-case estimate of 1 year survival was 75% for screening-detected HCC.

Attendance and compliance: A 60% attendance rate was assumed based on our previous experience with KCIS screening programs[24]. In addition, compliance rate and referral rate may vary with screening tools. Because AUS was a non-invasive screening tool, 80% of the referral rate was assumed based on our previous community-based studies[10,13] for individuals screened positive in the first-stage. The compliance rate of AUS was assumed to be 80%, which is comparable to the estimate of previous randomized trials[9,12,24].

Costs: The lifetime costs for HCC encompassed the initial costs (surgery, trans-catheter arterial chemoembolization, and chemotherapy), continuing costs (follow-up and treatment for recurrence), and the eventual cost of terminal care[25]. Costs for screening and confirmation were based on Medicare Payments by the Bureau of National Health Insurance in Taiwan. To acquire the data on costs of treatment, we reviewed the records of HCC patients under treatment in the National Taiwan University Hospital (NTUH). Indirect costs were calculated according to data from our index

Table 1 Base-case estimates and ranges used in sensitivity analysis

Variables	Base-case		Distribution of probabilistic sensitivity analysis		Ref.
Natural history and prognosis, per year					
Prevalence of cirrhosis	1.77%		B (1069, 59257)		[23]
Annual transition rates					
HCC incidence (1/yr)	NC	Cirrhosis	NC	Cirrhosis	
30-39 yr	0.00012	0.0024	Gamma (0.06, 510)	Gamma (0.23, 96)	[2,10,23]
40-49 yr	0.00036	0.0070	Gamma (0.33, 1190)	Gamma (1.2, 220)	
50-59 yr	0.00100	0.0200	Gamma (3.07, 3614)	Gamma (10, 632)	
60-69 yr	0.00210	0.0410	Gamma (19, 8928)	Gamma (67, 1640)	
70-79 yr	0.00430	0.0820	Gamma (79, 18280)	Gamma (269, 3280)	
PHCC to CHCC (Non-cirrhosis)	0.376 (0.157- 0.595)		Gamma (11.3, 30.1)		[10,15]
PHCC to CHCC (Cirrhosis)	0.637 (0.21-1.06)		Gamma (8.7, 13.6)		
CHCC to HCC death	1.05 (0.93-1.18)		Gamma (1.14, 1.09)		
Age-specific mortality rate of cirrhosis					
30-39 yr	0.0046		Gamma (0.2, 49.4)		[2,16-19]
40-49 yr	0.0086		Gamma (0.8, 92.3)		
50-59 yr	0.0170		Gamma (3.1, 182.4)		
60-69 yr	0.0380		Gamma (15.5, 407.7)		
70-79 yr	0.0980		Gamma (103, 1051.5)		
Survival rate of surveillance-detected PHCC (%)	75		Gamma (11.2, 15.8)		[15]
First-stage 5 markers screening characteristics					
Attendance rate (%)	60		B (23654, 18733)		[9,13,24]
Sensitivity to Cirrhosis (%)	80		B (62, 15)		[20]
Sensitivity to HCC	95		B (50, 1)		[13]
Specificity to HCC	70		B (9493, 4282)		[13]
Second-stage ultrasonography screening characteristics					
Compliance rate of ultrasonography (%)	80		B (16394, 3212)		[9,13,24]
Sensitivity to cirrhosis (%)	75		B (11, 3)		[21]
Sensitivity to HCC (%)	83		B (48, 10)		[9,12,22]
Specificity to HCC (%)	97		B (20137, 637)		[9,12,22]
Direct cost (USD)					
Biochemical test					
HBsAg	4.7				BNHI
HCVAb	7.4				BNHI
GOT	1.5				BNHI
GPT	1.5				BNHI
AFP	5.9				BNHI
Ultrasonography	26				BNHI
Confirmation (USD)					
Triple-phase abdominal CT	148				BNHI
Ultrasonic guidance for biopsy	38.3				BNHI
Liver puncture	36				BNHI
Specimen examinations of pathology	51.2				BNHI
Treatment (USD)					
Initial cost of HCC treatment	4892		Lognormal (8.28, 0.53)		NTUH
Continuing cost of HCC treatment	4266		Lognormal (8.18, 0.46)		NTUH
Incurable-cancer care (average)	5691		Lognormal (8.36, 0.81)		NTUH
Indirect cost (USD)					
Screening time (h)	0.5				[10,26]
Person accompanied for screening	0				[10,26]
Time spent for ultrasonography	4				[10,26]
Confirmation time (h)	8				NTUH, [26]
Person accompanied for confirmation	1				NTUH, [26]
Inpatient hospitalization (d)	15				NTUH
Inpatient recovered at home (d)	15				[26]
Person accompanied for inpatient care	1.69				[26]
Outpatient time per visit (h)	4				[26]
Outpatient visit per year	9.7				NTUH
Patient accompanied for outpatient visit	0.77				[26]
Inpatient of terminal care (d)	30				NTUH
Person accompanied for terminal care	1				[26]
Average work per month (h)	184				DGBAS
Production value per hour (USD)	7.6				DGBAS
Discount rate (%)	3				

NC: Non-cirrhosis; HCC: Hepatocellular carcinoma; PHCC: Preclinical hepatocellular carcinoma; CHCC: Clinical hepatocellular carcinoma; CT: Computed tomography; GNP: Gross national product; BNHI: Bureau of the National Health Insurance; NTUH: National Taiwan University Hospital; DGBAS: Directorate General of Budget, Accounting and Statistics.

hospital and previous studies[26,27]. All future costs and life-years were discounted to the present value at an annual rate of 3%. Base-case values and ranges used in the sensitivity analyses are summarized in Table 1.

Model assumptions

Several model assumptions were stated, as follows: (1) HCC incidence varied by age. The age-specific incidence rate of HCC was based on the Cancer Registry of Taiwan. The probability of HCC progression and survival was constant over time; (2) Clinically-detected HCCs often had large tumors and would be treated with palliative measures only, where the prognosis was poor. The survival rate of clinically-detected HCC was not dependent on the presence of liver cirrhosis; (3) False positive cases after referral confirmatory examination would undergo the ultrasonography surveillance at 3-mo intervals for 6 mo and would be resumed to the original screening strategy if negative screening results were found during surveillance; (4) The effect of antiviral therapy on cirrhosis progression and incidence of HCC was not modeled due to the uncertainty of its long-term effects; (5) Liver transplantation for treating HCC was not considered in the model due to the shortage of organ donations and the long waiting time in Taiwan; and (6) Based on the concept of the prevalence pool, the equilibrium state between cirrhosis and non-cirrhosis was stable. Hence, we did not model the transition between non-cirrhotic state and cirrhotic state. We believe this assumption had little influence on the outcome of the HCC estimation.

Cost-effectiveness analysis

We conducted analysis from a societal perspective. The effectiveness of any given screening program was evaluated by the life-year gained after converting mortality reduction as a result of each intervention. Costs are expressed in United States dollars (USD). The direct costs were associated with the screening itself, confirmatory tests, and treatment. The indirect costs were mainly derived from the loss of productivity. A 3% discount rate was used to convert future cost to present value. The results of comparisons between different screening strategies are presented by the incremental cost-effectiveness ratio (ICER). The ceiling ratio of ICER, the maximum amount of willingness to pay (WTP) per life-year saved, was set at the level of USD33000, approximately equivalent to two times per capita the gross national product (GNP) in Taiwan[28].

Sensitivity analyses

We performed one-way and probabilistic sensitivity analyses by varying key model parameters within a specified range in order to compare the main strategy and the reference strategy. Different initial ages and screening intervals were also compared by a series of acceptability curves and ICE scatter plots based on repeated Monte Carlo simulations. The statistical review of this study was performed by a biomedical statistician.

RESULTS

Cost analyses

In total, 158 HCC cases were sampled from the HCC cohort in our index hospital for cost estimation of initial and continuing care. According to the AJCC staging system of HCC, the numbers of patients with stage I to IV tumors were 62 (39%), 48 (30%), 33 (21%), and 15 (10%), respectively. After excluding subjects (n = 42) receiving either palliative treatments or no treatment, the primary modalities for HCC management in our sample were trans-arterial chemoembolization and hepatic resection, which accounted for up to 83% (96/116). The average costs of initial and continuing care were USD4892 (95%CI: USD3359-5936) and USD4266 (95%CI: USD3072-4685) for each individual patient. The average cost of terminal care, based on 93 patients who died within the enrolled year in our index hospital with a mean follow-up period of 4.2 mo, was USD5691 (95%CI: USD4327-7055). The detailed costs and their ranges used for model estimation are listed in Table 1.

Base-case analyses

The ICERs for screening strategies as compared to "No intervention" are listed in Table 2. Both screening strategies yielded more life-year gain and increased total costs compared with no intervention. The ICERs for two-stage screening and AUS screening were USD49733 and USD39825 per life-year gained, respectively. AUS screening was better than the two-stage method.

Sensitivity analyses

Results from the one-way sensitivity analyses are summarized to compare the two-stage method and AUS screening (Table S1). The results demonstrated that AUS screening was far more superior to the two-stage method. The superiority of AUS screening was sensitive to the specificity of the biochemical screening and the costs of biochemical screening and AUS. If the cost of biochemical screening was less than USD9.9 or the cost of AUS was greater than USD44.1, the two-stage method was better. Moreover, two-stage screening became more cost-effective if the specificity of biochemical screening was larger than 90%. When such parameters as sensitivity of AUS, cirrhosis prevalence, attendance rate of screening programs, and compliance rate for ultrasonography were varied within a reasonable range, their influence on the superiority of AUS screening was trivial.

Table 2 Simulated results for screening strategies to prevent hepatocellular carcinoma

Outcome	No intervention	Two-stage screening	Mass screening using ultrasonography
Cost per individual screened, USD	2755	3389	3359
Life-year gain[1] (yr)	20.4798	20.4926	20.4950
Comparing with ICER			
No screening as reference	-	49733	39825
Two-stage screening as reference	-	-	Dominant[2]

[1]Screening starting age was 40 years old, screening interval was 1 year; [2]More effective and less costly than reference strategy. ICER: Incremental cost-effectiveness ratio.

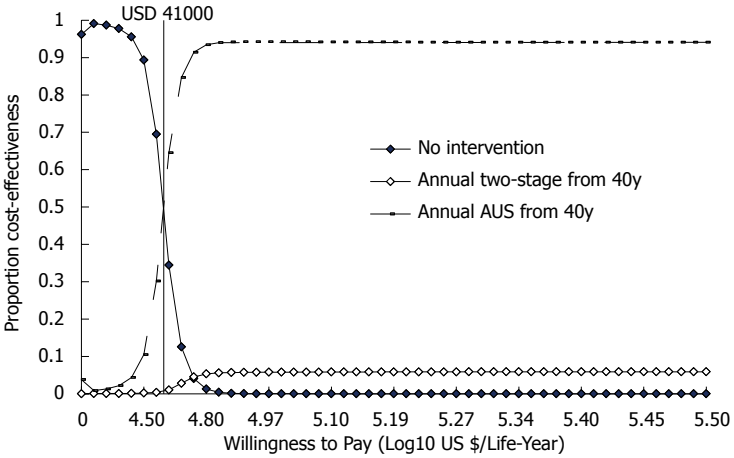

Figure 1 Results of sensitivity analysis: Cost-effectiveness acceptability curves.

In the probabilistic sensitivity test, with a maximum WTP of USD33000, AUS screening had an approximate 15% likelihood of being cost-effective. If the amount of WTP was raised to USD41000 or higher, the probability of AUS screening being cost-effective was over 50% (Figure 1).

Optimal initial screening age

The cost-effectiveness analyses at different initial ages of both screening programs at a given annual screening interval are shown in Figure 2A. The slope of the efficacy frontier showed the optimal ICER among different screening strategies. Other strategies internal to the efficacy frontier were less cost-effective based on the rules of extended dominance. AUS screening was more cost-effective than the two-stage method at any initial age. The most cost-effective strategy by using probabilistic sensitivity analysis was AUS screening with an initiated screening age of 50 years old (Figure S3A).

Inter-screening intervals

The efficacy frontier consisted of a combination of AUS screening with different screening intervals and no screening at a given initial screening age of 40 years (Figure 2B). The cost-effectiveness of both screening strategies with different inter-screening intervals was also evaluated by using 10000 replications from Monte Carlo simulation considering the acceptability curve (Figure S3B). The two-stage screening strategy was less cost-effective than AUS screening at all inter-screening intervals. The most favorable strategy was biennial AUS screening, followed by annual AUS screening.

Cost-effectiveness plane for ultrasonography screening

Because AUS screening has been shown to be superior based on its cost-effectiveness, we further compared different combinations of optimal and suboptimal inter-screening intervals and initial ages for AUS screening. Figure 3A-D illustrates the simulated results of 5000 ICER replicates plotted on a cost-effectiveness plane given the maximum amount of WTP per life-year saved (ceiling ratio) at the level of USD33000. If the ICER lies below the ceiling ratio, the strategy should be implemented. Compared to no screening, the probability of being cost-effective among the different strategies (*i.e.*, annual screening from 40 years, annual screening from 50 years, biennial screening from 40 years, and biennial screening from 50 years) was 15%, 45%, 55%, and 73%, respectively.

Model validation

The predicted age-specific incidence rate of HCC per 100000 individuals from our model was compared to the empirical data from the Cancer Registry in Taiwan (2007). The empirical figures were as follows: 40-44 years, 23; 45-49 years, 40; 50-54 years, 64; 55-59

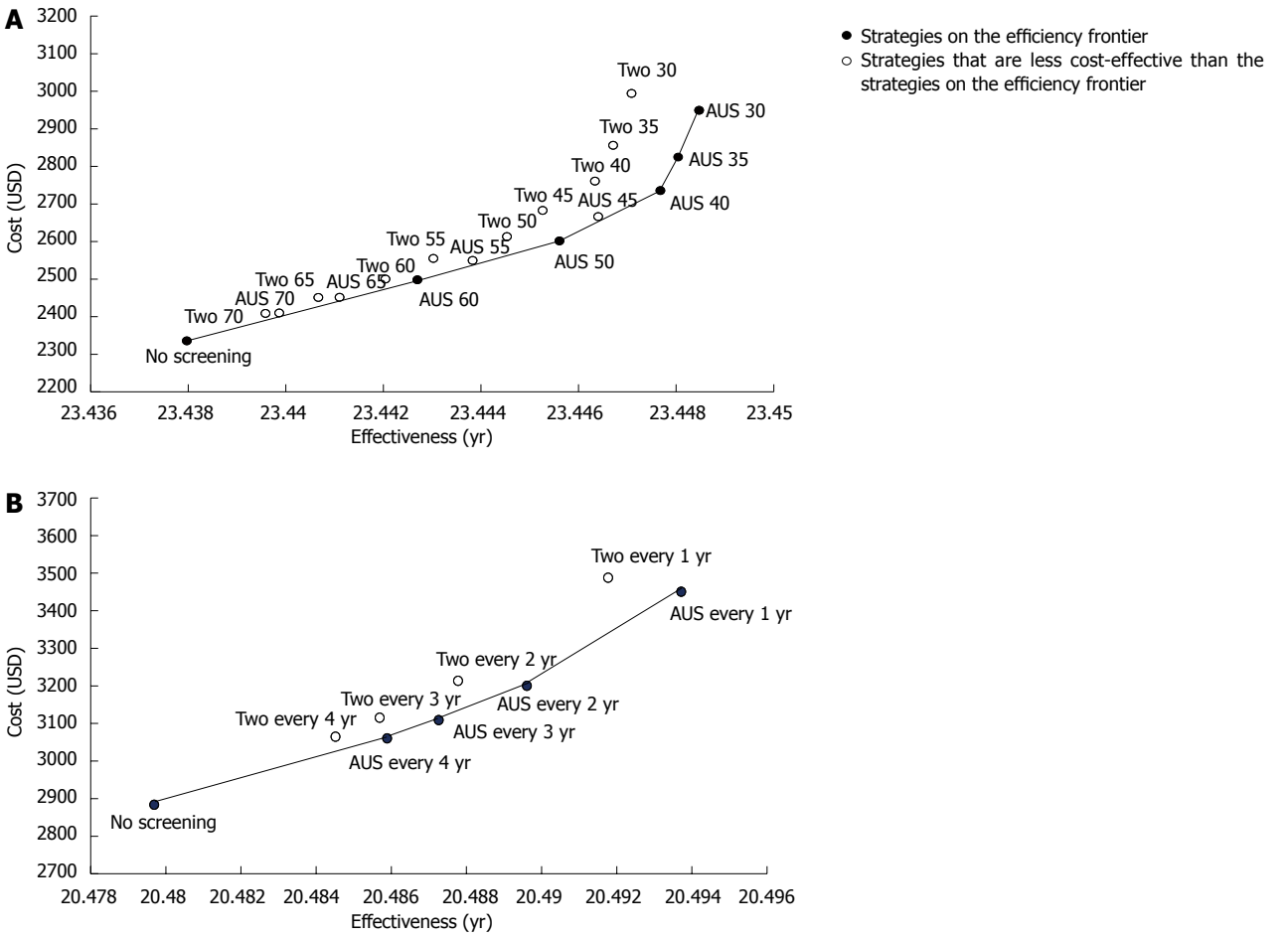

Figure 2 Cost-effectiveness of hepatocellular carcinoma screening with selected initial ages and selected screening intervals. The reference strategies were non-screening programs at the index initial ages. A: Strategies are labeled by the type and initial ages of screening; B: Strategies are labeled by the type and frequency of screening. AUS: Abdominal ultrasonography mass screening; Two: Two-stage biomarker-ultrasound screening.

years, 99; 60-64 years,146; 65-69 years, 197; 70-74 years, 232; and 75-79 years, 229. The predictive figures were as follows: 40-44 years, 30; 45-49 years, 41; 50-54 years, 81; 55-59 years, 102; 60-64 years,155; 65-69 years, 176; 70-74 years, 237; and 75-79 years, 233. There was no significant statistical difference as tested by χ^2 test ($\chi^2_{(7)} = 8.97$, $P = 0.26$), indicating fair model fitting.

DISCUSSION

Our study is the first to confirm the superiority of AUS screening for HCC (in terms of cost-effectiveness) compared to the conventional two-stage method in a hepatitis endemic area. The results showed that AUS screening was associated with an incremental cost-effectiveness ratio of USD39825 per life-year gained, whereas the two-stage method was associated with an incremental cost-effectiveness ratio of USD49733 per life-year gained, as compared with non-screening. If taking the maximum amount of WTP per life-year saved in our country into account (USD33000), neither AUS screening nor two-stage screening is more cost-effective than non-screening. However, the absolute cost-effectiveness still varies because the cost of management of HCC in different countries may vary, and the decision to implement any screening program also depends on the resources of any given country.

Both the deterministic and probabilistic modeling approaches revealed that AUS screening is more cost-effective than two-stage screening. Several factors accounted for such results. First, the difference of cost between AUS (USD27.6) and first stage biochemical tests (USD22.2) is relatively low in Taiwan. Two-stage screening became a better strategy if the costs of AUS were more expensive than USD44.1 or if the cost of the biochemical test was lowered to USD9.9. Second, the specificity of the five biochemical makers for HCC screening is low. Two-stage screening is superior only when the specificity of biochemical screening is greater than 90%. A low platelet value has recently been reported as a surrogate for cirrhosis[29]. It is necessary to further assess the optimal utility ratio based on the comparisons between different combinations of biochemical markers. However, at present, there is still no biomarker with relative good sensitivity and

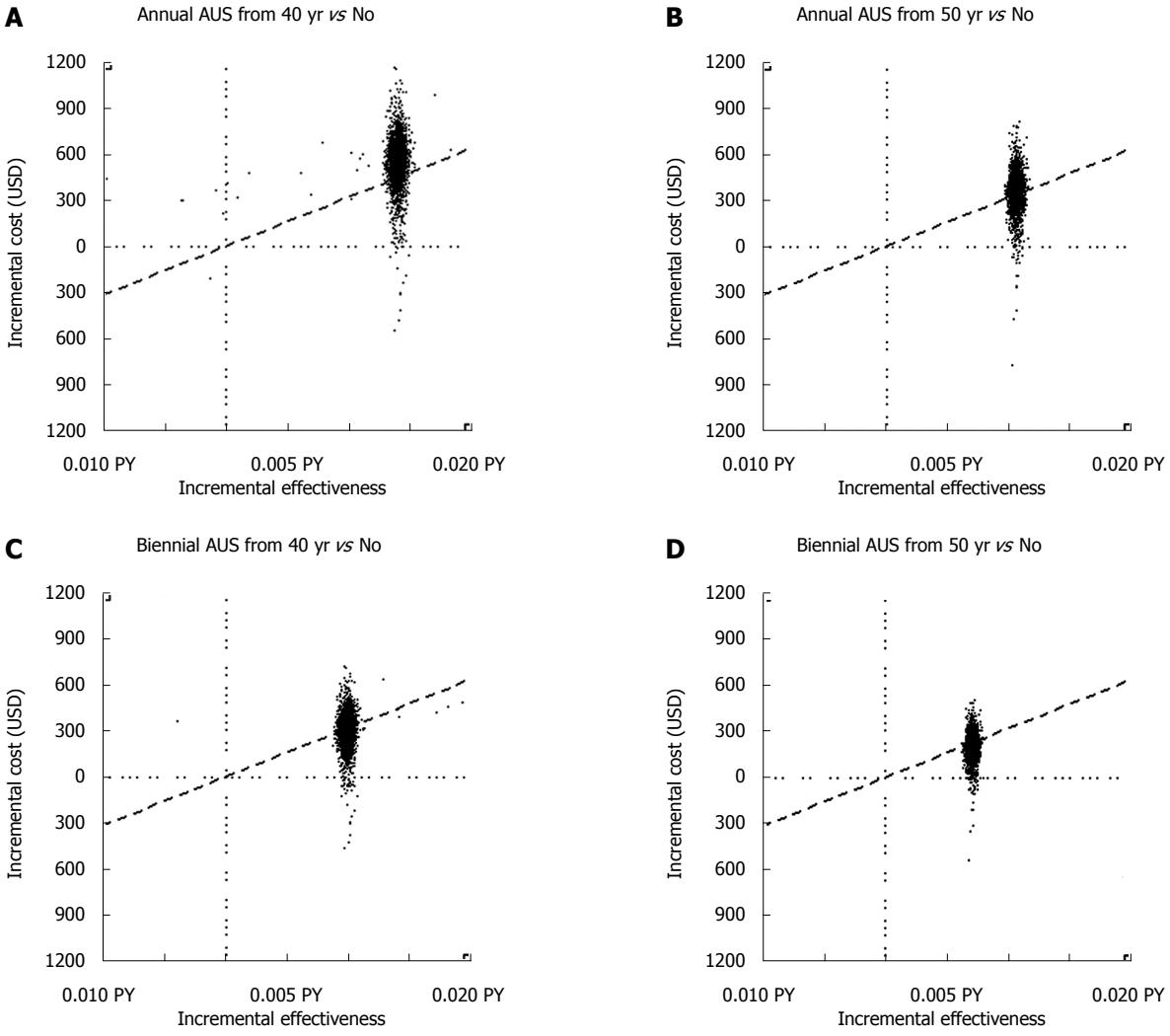

Figure 3 Cost-effectiveness plane for different combinations of optimal, suboptimal initial ages and inter-screening intervals of ultrasonography screening. The slope of the dashed line represents the ceiling ratio. A: Annual screening from 40 years *vs* no screening; B: Annual screening from 50 years *vs* no screening; C: Biennial screening from 40 years *vs* no screening; D: Biennial screening from 50 years *vs* no screening.

specificity for HCC surveillance[7,30,31].

Costs for initial, continuing, and terminal phases of care reported in previous cost-effectiveness studies of cancer care are summarized[25]. The average costs of initial care, continuing care, and terminal care are USD4892, USD4266, and USD5691, respectively. Initial care and terminal care costs are higher than continuing care costs for the treatment of HCC. These results are compatible with a previous study[27]. It should also be noted that our costs are based on those at a medical center, NTUH. As the leading hospital in Taiwan with a 118-year history, this hospital serves patients and accepts referrals evenly distributed from every part of Taiwan. Therefore, the patients of NTUH could represent all HCC patients in Taiwan without substantial bias, with perhaps a slight skew to severe cases. Costs estimated at community hospitals may be somewhat lower. Besides, it is difficult to distinguish asymptomatic and symptomatic cases by retrospective chart reviewing. We assume the same costs for initial treatment for clinically-detected and screen-detected cases. A sensitivity test with a wide range for cost of initial treatment between clinically- and screen-detected tumors did not change the superiority between screening strategies.

As far as the validity of the simulated model is concerned, there is evidence supporting our results. Firstly, the estimated parameters of natural history and variables were largely generated from two previous studies based on the same community cohort in Taiwan[10,13]. The heterogeneity among different studies could be overcome. Secondly, the predicted age-specific incidence rate of HCC was close to the observed one ($\chi^2_{(7)} = 8.97$, $P = 0.26$). Thirdly, by taking different time horizons into consideration, AUS screening was still more cost-effective than two-stage screening. This means that changes in time horizons had little effect on our results.

Our study was different from several previous decision analyses studies regarding HCC screening[32-37],

probably due to the following reasons. Firstly, our study included a four-state 'micro-simulation' model and community-based screening in Taiwan, a viral hepatitis endemic area. Previous studies were restricted to high risk populations, such as cirrhotic patients or patients waiting for liver transplantation. Secondly, the rates of incidence of HCC increased with age. We have used age-specific HCC incidence rates based on the Cancer Registry of Taiwan. The study by Arguedas *et al*[36] evaluated the incremental cost-effectiveness of no screening versus AUS and AFP every year in patients with cirrhosis. The results of base-case analyses were USD22500 per life year saved. Another study by Shih *et al*[37] from Taiwan that compared no screening and two-stage screening in patients with either hepatitis B or hepatitis C, reported an ICER of USD15600 per life year saved. Their ICERs were lower than our estimate. The difference was more likely attributed to the fact that we included indirect costs and our screening subjects were the general population rather than high risk individuals.

Taken together, our data support AUS screening for the general population in high HCC endemic areas. However, the success of this screening largely depends on a sufficiently well-trained staff to perform AUS. In some countries without this staff, it may be feasible to develop a risk-scoring system with subsequent referral for AUS[38]. On the other hand, in high-risk populations, such as those with advanced cirrhosis, where the detection rate for HCC by AUS is low, use of AUS in combination with biomarkers would be valuable.

There are some limitations in our model. Recently, the incidence of HCC was shown to be reduced with antiviral therapies[39]. However, we cannot model antiviral treatment effects on the natural history of HCC because the data on reversibility of advanced liver disease are not well established. On the other hand, liver transplantation is the optimal treatment for HCC because it simultaneously removes the tumor and underlying cirrhosis, thus reducing the risk of HCC recurrence[40]. This therapeutic option was excluded due to the shortage of organ resources in Taiwan and because decision making for liver transplantation was not based only on medical concerns.

In conclusion, AUS mass screening is more cost-effective than the two-stage method. Its relative cost-effectiveness may vary depending on the cost of the screening tools and the specificity of the biochemical test. We found that screening programs with initial screening at 50 years of age and subsequent biennial screening intervals were the optimal strategy.

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COMMENTS

Background

Two-stage biomarker-ultrasound method and mass screening using abdominal ultrasonography (AUS) have been proposed for the early detection of hepatocellular carcinoma (HCC). The cost-effectiveness of these two HCC screening strategies remains unclear, particularly regarding aspects such as the optimal initial age and inter-screening interval.

Research frontiers

This study contributes significantly to understanding the cost-effectiveness of mass screening the general population for HCC with AUS compared with the existing two-stage biomarker-ultrasound screening strategy in an area with high HCC incidence.

Innovations and breakthroughs

Mass screening using ultrasonography is more cost-effective than two-stage biomarker-ultrasound screening. The costs of screening tools and the specificity of biomarker screening play an important role in the relative cost-effectiveness of screening strategies.

Applications

Early detection of HCC with abdominal ultrasonography may be suggested for the general population in areas with a high incidence of HCC that had not been covered by hepatitis B vaccination. Optimal age to begin screening and inter-screening interval of HCC could be determined in clinical decision making for early diagnosis of HCC

Peer-review

It is a well written manuscript assessing the cost-effectiveness of two kinds of HCC screening programs.

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Glucose metabolic phenotype of pancreatic cancer

Anthony KC Chan, Jason IE Bruce, Ajith K Siriwardena

Anthony KC Chan, Ajith K Siriwardena, Regional Hepato-Pancreato-Biliary Surgery Unit, Manchester Royal Infirmary, Manchester M13 9WL, United Kingdom

Jason IE Bruce, Faculty of Life Sciences, University of Manchester, Manchester M13 9PL, United Kingdom

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Correspondence to: Ajith K Siriwardena, MD, FRCS, Professor, Hepato-Pancreato-Biliary Surgery Unit, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, United Kingdom. ajith.siriwardena@cmft.nhs.uk
Telephone: +44-161-2764250
Fax: +44-161-2764530

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Abstract

AIM: To construct a global "metabolic phenotype" of pancreatic ductal adenocarcinoma (PDAC) reflecting tumour-related metabolic enzyme expression.

METHODS: A systematic review of the literature was performed using OvidSP and PubMed databases using keywords "pancreatic cancer" and individual glycolytic and mitochondrial oxidative phosphorylation (MOP) enzymes. Both human and animal studies investigating the oncological effect of enzyme expression changes and inhibitors in both an *in vitro* and *in vivo* setting were included in the review. Data reporting changes in enzyme expression and the effects on PDAC cells, such as survival and metastatic potential, were extracted to construct a metabolic phenotype.

RESULTS: Seven hundred and ten papers were initially retrieved, and were screened to meet the review inclusion criteria. 107 unique articles were identified as reporting data involving glycolytic enzymes, and 28 articles involving MOP enzymes in PDAC. Data extraction followed a pre-defined protocol. There is consistent over-expression of glycolytic enzymes and lactate dehydrogenase in keeping with the Warburg effect to facilitate rapid adenosine-triphosphate production from glycolysis. Certain isoforms of these enzymes were over-expressed specifically in PDAC. Altering expression levels of HK, PGI, FBA, enolase, PK-M2 and LDA-A with metabolic inhibitors have shown a favourable effect on PDAC, thus identifying these as potential therapeutic targets. However, the Warburg effect on MOP enzymes is less clear, with different expression levels at different points in the Krebs cycle resulting in a fundamental change of metabolite levels, suggesting that other essential anabolic pathways are being stimulated.

CONCLUSION: Further characterisation of the PDAC metabolic phenotype is necessary as currently there

are few clinical studies and no successful clinical trials targeting metabolic enzymes.

Key words: Metabolism; Pancreatic cancer; Warburg effect; Metabolic inhibitor; Glycolysis; Krebs cycle

Core tip: Our systematic review constructs a global "metabolic phenotype" of pancreatic ductal adenocarcinoma (PDAC) reflecting tumour-related metabolic enzyme expression. We show that the Warburg effect is consistently demonstrated, with the over-expression of glycolytic enzymes and lacate dehydrogenase to facilitate rapid adenosine-triphosphate (ATP) production from glycolysis. We also show that the Warburg effect on mitochrondial oxidative phosphorylation in PDAC is more varied and not solely focused on ATP production, but also to stimulate other anabolic pathways for the purposes of tumourigencity. The metabolic phenotype provides an overview essential to elucidating the pathological changes that occur in PDAC.

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INTRODUCTION

Pancreatic cancer (PC), typically ductal adenocarcinoma (PDAC), is the 13th most common cancer[1] contributing to 250000 deaths annually worldwide and accounting for 3.6% of cancer deaths and 0.5% of all deaths[2]. Despite advances in diagnostic technology and treatment modalities, there has been no significant improvement over the last three decades, with five-year survival remaining below 7%. Recent progress in genetics has led to a renewed interest in the Warburg Effect described in 1956 by German physiologist Otto Warburg[3] who postulated that carcinogenesis was the result of "irreversible injuring of respiration". Eukaryotic cells utilise glycolysis to derive energy, where glucose is broken down over a series of enzymatic steps to produce adenosine-triphosphate (ATP) and pyruvate. In aerobic respiration, pyruvate is oxidised in the Krebs cycle to produce ATP and NADH (nicotinamide adenine dinucleotide hydride). Mitochondrial oxidative phosphorylation (MOP) then occurs *via* a series of redox reactions to generate more ATP from NADH. Overall, between 30 and 36 ATP are generated from 1 molecule of glucose. In the absence of an adequate oxygen supply, anaerobic fermentation occurs, reducing pyruvate to lactate and converting NADH into NAD^+ (nicotinamide adenine dinucleotide) for use in further glycolysis reactions. The energy released per glucose molecule in anaerobic respiration is only 2 ATP; per mole, this is 18-fold less than aerobic respiration but at a much faster rate of several hundred times[4]. The ratio of MOP and anaerobic fermentation is reduced in cancer cells[5-7], such as the Henrietta Lacks (HeLa) cervical cancer cell line where approximately 80% of glucose uptake undergoes glycolysis, and only 5% enters the Krebs cycle[8]. Warburg proposed that this "morphological inferiority" would change highly differentiated cells into undifferentiated cells that can divide, grow and lead to cancer.

Hypoxia is one stress factor in the tumour micro-environment that is thought to lead to this switch[9]. Hypoxia-inducible factor 1 (HIF-1) is an important regulator of cellular oxygen homeostasis[10], but is also up-regulated in many cancers, including pancreatic, gastric, lung, breast and hepatic cancers[11-14]. HIF-1 up-regulates most glycolytic enzymes, including hexokinase Ⅱ, the first enzyme in the glycolysis pathway[15], and reduces MOP by up-regulating pyruvate dehydrogenase kinase I, responsible for inactivating the pyruvate dehydrogenase complex that subsequently stops pyruvate decarboxylation for entry into the Krebs cycle[16]. HIF-1 also up-regulates other genes including vascular endothelial growth factor (VEGF, a known promoter of tumour angiogenesis[17]) and the glucose transporter protein, Glut-1, facilitating glucose influx[11].

The Warburg Effect is likely a result of mutations in oncogenes and tumour suppressor genes with several pathways contributing to this "metabolic switch"[18]. This study undertakes a systematic literature review of changes in enzyme expression and the resulting metabolite levels in both the glycolytic and MOP pathways in PDAC in order to construct a 'metabolic phenotype' of this disease. New potential therapeutic targets can be identified within this phenotype for further study as novel treatments for PDAC.

MATERIALS AND METHODS

Literature search strategy

A systematic review of the literature was performed using OvidSP and the PubMed database. Search terms for individual glycolytic enzymes (hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase and lactate dehydrogenase) and Krebs cycle enzymes (pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl-CoA synthase, succinic dehydrogenase, fumarase and malate dehydrogenase) were combined with key words "PC" and the Boolean "AND" operator (*e.g.*, "Hexokinase and *PC*"). Human and animal *in vivo* studies, as well as *in vitro* studies involving cell

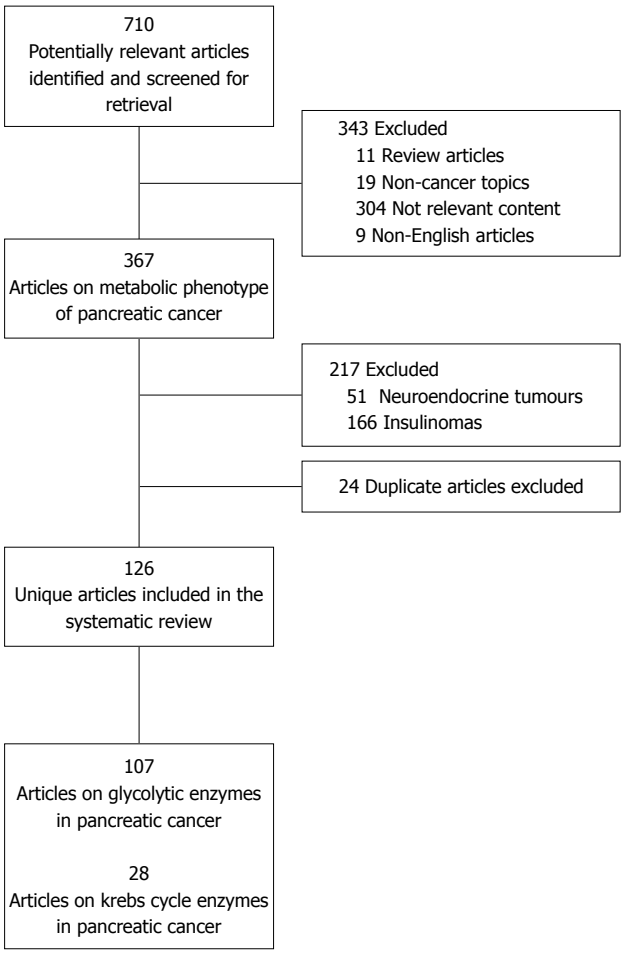

Figure 1 Preferred reporting items for systematic reviews and meta-analyses flowchart of articles found and screened in the systematic review.

lines, were included. The initial search yielded 710 results, and after excluding review articles, non-cancer articles and those with non-relevant content, 367 articles were analysed. A further 217 articles describing pancreatic cancers of histology other than PDAC, such as carcinoid and other neuroendocrine tumours were excluded. Finally, duplicate articles (24) were identified and excluded. One hundred and twenty-six publications were identified as meeting the inclusion criteria of this systematic review looking at the metabolic phenotype of PDAC (Figure 1). Of these, 107 unique articles describe glycolytic enzymes (Table 1) and 28 unique articles describe the MOP pathway (Table 2) in PDAC.

Eligibility, data extraction and analysis

Studies describing the biochemical mechanisms involved in the physiological, onco-pathological or manipulation of metabolic pathways and/or individual metabolic enzymes in PDAC were included. For quality assurance, information was extracted following a predefined protocol from the text of each article, including changes in expression of metabolic enzymes and/or in the metabolic pathways and their overall effect on normal and PDAC phenotype. The effect on cell viability and growth was also noted, particularly if a metabolic inhibitor was used, as was evidence implicating known oncogenic pathways. The data were collated (Tables 1 and 2) using Microsoft Excel (Microsoft, Richmond, United States) for analysis. A PDAC "metabolic phenotype" was constructed using these data (Tables 3-5), and the results presented as the hexose and triose stage of glycolysis, anaerobic fermentation and aerobic respiration (Krebs Cycle).

RESULTS

Hexose stage of glycolysis

Hexokinase: Hexokinase (HK) is the first enzyme in glycolysis, and exists as 4 isoforms (HKI - Ⅲ, and glucokinase). It phosphorylates glucose into glucose-6-phosphate (G6P), and represents the rate-limiting step in glycolysis[19]. G6P is transported into mitochondria and immediately used for ATP production *via* glycolysis, or for nucleic acid synthesis *via* the pentose-phosphate shunt[20]. Three HK isoforms (Ⅰ, Ⅱ and Ⅲ) are competitively inhibited by 2-Deoxy-D-glucose, whereas 3-bromopyruvate (3-BP) selectively inhibits HK-Ⅱ[21].

Several studies have shown HK to be over-expressed in PDAC[22,23] with expression levels varying by tumour histology. Ductal tumours, for example, take up more glucose and express HK-Ⅰ and Ⅱ more than acinar variants[24]. Direct inhibition with 3-BP reduces cell survival and increase necrosis in PDAC[25,26] and Panc-1 cell lines[27]. Treatment of Panc-1 with 3-BP also affects cell signalling, significantly reducing the expression of the GTPase signal transduction KRas (Kirsten rat sarcoma) pathway, as well as the Akt (protein kinase B) and mTOR (mammalian target of rapamycin) pathways, to induce cell necrosis[27]. Inhibiting the mTOR pathway in Panc-1 using everolimus reduces the expression of HK-Ⅱ and glycolysis, which inhibits cell proliferation and induces apoptosis[28].

The up-regulation of HK is partly due to the HIF-1 pathway in hypoxic conditions[29]. An increase in extracellular glucose is responsible for increasing HIF-1 expression (and subsequently intracellular ATP) whilst inhibiting mitochondrial activity in MiaPaCa2 cells[30]. An interaction between insulin and HIF-1 has also been suggested[31], with type 2 diabetes mellitus associated with the development of PDAC in patients who have a particular HK Ⅱ (genotype R844K) or glucokinase (ICS1+9652C>T) variant[32,33]. Clinically, a high level of HK-Ⅱ expression is associated with longer survival, with different variants of HK Ⅱ (such as N692N) associated with differing clinical outcomes[33].

Phosphoglucose isomerase: Phosphoglucose isomerase (PGI) reversibly catalyses glucose-6-phosphate to fructose-6-phosphate. Previously identified as the autocrine motility factor (AMF)[34], PGI binds to the gp78/AMFR receptor[35] to stimulate cell migration and metastasis[36]. Under hypoxic conditions, PGI expression

Table 1 Systematic review of the literature involving glycolytic enzymes and pancreatic ductal adenocarcinoma

Glycolytic enzyme	*In vitro* studies		*In vivo*/clinical studies	
	Ref.	Summary	Ref.	Summary
Hexokinase (HK)	[23-30,32,33,153-155]	Induced by hypoxia. Higher expression in PDAC than acinar cells. Suppresses mitochondrial ATP production	[31,153,156,157]	HK-2 expression suggests an unfavourable clinical outcome
Phosphoglucose isomerase (PGI)	[29,38-40,158]	Stimulates cell migration and metastatic potential. Induced by hypoxia. β-1 integrins are stimulated by PGI	[39]	Over-expression contributes to a more aggressive PDAC phenotype
Phosphofructokinase (PFK)	[11,48]	Upregulated in PDAC epithelia.		
Aldolase	[49,52,54]	Overexpressed in PDAC. Induced by hypoxia. Delays apoptosis.	[52,53]	Highly expressed in PDAC where HIF-1α is constitutively expressed. Inhibition prolongs survival
Triosephosphate isomerase (TPI)	[55,56]	Overexpressed in PDAC		
Glyceradehyde-3-Phosphate Dehydrogenase (G3PD)	[27,50,55,57,58,61,159]	Overexpressed in PDAC. Increases PDAC metabolic activity, and disrupts downstream apoptotic capases	[50,58]	Increased expression. Possible biomarker candidate.
Phosphoglycerate kinase (PK)	[62-66,160]	Overexpressed in PDAC. Angiogenesis promoter	[63]	No significant increase in expression in a murine model, but fivefold increase in activity.
Phosphoglycerate mutase (PGM)	[67,68]	M-isoform is under-expressed and B-isoform is over-expressed in murine models		
Enolase	[55-58,69-83]	Overexpressed in PDAC. Promotes cell migration and metastasis. Biomarker candidate	[58,69,76,77,161-163]	Increased expression. Induced antibodies to enolase-alpha correlates to a better outcome
Pyruvate kinase (PK)	[108,109,117,121,122, 157,164-166]	Overexpressed in PDAC. Dimeric form favours synthesis; Tetrameric form favours energy production; PK-M2 used as a tumour marker	[110,112,115,119,120, 157,167,168]	M2 isoform levels correlate to tumour metastasis. HK-2 and M2 expression indicates an unfavourable clinical outcome
Lactate dehydrogenase (LDH)	[28,89,123-138,164, 169-179]	Overexpressed in PDAC. Down regulated by graviola	[153,180-183]	Down regulated by graviola to reduce tumorigenicity and metastasis

HIF: Hypoxia-inducible factor; PDAC: Pancreatic ductal adenocarcinoma.

Table 2 Systematic review of the literature involving Krebs cycle enzymes and pancreatic ductal adenocarcinoma

Glycolytic Enzyme	*In vitro*/*In vivo*/clinical studies	
	Ref.	Summary
Pyruvate dehydrogenase	[30,109,131,132,140,154,164,170,171,173,177, 179,180,184-188]	Inhibition of pyruvate dehydrogenase kinase (the activity of which is regulated by pyruvate dehydrogenase) stimulates the Krebs cycle and reverses the Warburg effect.
Pyruvate carboxylase	[141]	Over expressed in human and murine PDAC
Citrate synthase	[67,124,179]	Over expressed in PDAC. Activity of citrate synthase also higher in PDAC compared to normal tissue
Aconitase	[67]	Mitochrondrial isoform under-expressed
Isocitrate dehydrogenase	[67,158,189,190]	Regulated by HuR (an RNA-binding protein) in PDAC.
a-ketoglutarate dehydrogenase	[67,187]	Marginally increased in murine PDAC
Succinyl-CoA synthetase	[67]	Reduced expression
Succinic dehydrogenase	[67,128]	Over expressed in human and murine PDAC
Fumarase	[30]	HIF-1α increases fumarate by inhibiting distal mitochondrial metabolisms
Malate dehydrogenase	[50,64,67]	Over expressed in human and murine PDAC. Possible candidate biomarker

HIF: Hypoxia-inducible factor; PDAC: Pancreatic ductal adenocarcinoma.

is regulated in part by the HIF pathway[29,37] and has been found to be over-expressed in the Capan-2 cell line. PGI increases the metastatic potential of PDAC[38] and MiaPaCa-2 cells transfected with PGI grow more aggressively with an increase in tumour mass[39]. Down-regulation of E-cadherin expression - a protein involved in cell adhesion - also occurs. HIF-1 expression in PDAC can be inhibited by 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole, which subsequently reduces PGI mRNA expression; this has the effect of reducing overall cell viability and increasing apoptosis rates[40,41]. Herceptin has

Table 3 Summary of changes in glycolysis in pancreatic ductal adenocarcinoma

Glycolytic enzyme	Encoding gene	Change in PDAC	Implicated pathways	Known inhibitors	Inhibitor effect in PDAC
Hexokinase			HIF	2-Deoxy-D-glucose	
Hexokinase Ⅰ	10q22	Up	mTor		
Hexokinase Ⅱ	2p13	Up	K-ras	3-BP[21]; Lonidamine, Everolimus	Reduced PDAC survival/induces PDAC necrosis
Hexokinase Ⅲ	5q35.2	-	Akt		
Glucokinase	7p15.3-p15.1				
Phosphoglucose Isomerase (also known as Autocrine Motility Factor)	19q13.1	Up	HIF; Apoptosis	Insulin-like growth factor binding protein-3; Herceptin; 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole	Reduces overall cell viability and increases apoptosis rates
Phosphofructokinase			HIF	Aurintricarboxylic acid[191]	Inhibits fatty acid synthesis in rat hepatocytes
PFK-M (muscle type)	12q13.3	Down			
PFK-L (liver type)	21q22.3	Up			
PFK-P (platelet type)	10p15.3-p15.2	Up			
Aldolase			HIF	3-fluro-D-glucose; 4-fluro-D-glucose	
Aldolase-A	16p11.2	Up		3-[2-hydroxyethyl(methyl)amino]-2-quinoxalinecarbonitrile 1,4-dioxide	Reduces PDAC proliferation and tumour volume
Aldolase-B	5q22	Down			
Aldolase-C	17cen-q12	Up			
Triose Phosphate Isomerase	12p13	Up		2-phosphoglycolate; D-glycerol-1-phosphate[192]	
Glyceraldehyde Phosphate Dehydrogenase			HIF; p53	Iodoacetate[27]; gossypol[21]	Reduces cell survival and induces necrosis. No effect on K-ras
GAPDHS	12p13	Up			
GAPDHS (testes-specific)	19q13.12				
Phosphoglycerate Kinase			HIF	1,3-bisphosphoglycerate[193]	
PGK1	Xq13.3	Up			
PGK2	6p12.3	?			
Phosphoglycerate Mutase			p53	Inositol hexakisphosphate[194]	
PGM-B	1p31	Up			
PGM-M	4p14	Down			
Enolase			c-Myc	Sodium fluoride[21]; D-tartonate; 3-aminoenolpyruvate 2-phosphate[195]	
ENO1 (alpha)	1p36.2	Up			
ENO2 (gamma, neuronal)	12.p13	Up			
ENO3 (beta, muscle)	17pter-p11	-			
Pyruvate Kinase			Tyrosine kinase		
Isoform PK-M1 (liver/RBC)	1q21	-	Akt/c-Myc		
Isoform PK-M2 (muscle)	15q22	Up		L-phospholactate; M2-PK-binding peptide aptamers[196]	Anti-cancer effects in animal models
Dimeric form					
Tetrameric form					

HIF: Hypoxia-inducible factor; PDAC: Pancreatic ductal adenocarcinoma.

Table 4 Summary of changes in anaerobic fermentation in pancreatic ductal adenocarcinoma

Glycolytic enzyme	Encoding gene	Change in PDAC	Implicated pathways	Known inhibitors	Inhibitor effect in PDAC
Lactate dehydrogenase				Oxamate	Inhibits PDAC growth
Lactate dehydrogenase A	11p15.4	Up	c-Myc; mTor	Aryl-substituted N-hydroxyindole-2-carboxylates; Everolimus (by blocking the mTor pathway[28])	Inhibits PDAC growth
Lactate dehydrogenase B	1p12.2-p12.1	Up			
Lactate dehydrogenase C	11p15.1	-			

PDAC: Pancreatic ductal adenocarcinoma.

been shown to inhibit the expression of PGI and potentiate the effects of other PGI inhibitors[42]. Beta-1 integrins (receptors that facilitate binding between neighbouring cells) are stimulated by PGI, up-regulating cell adhesion, invasion and metastasis possibly by a signalling pathway involving protein kinase C[43]. Beta-1 integrins have been shown to be highly expressed in several PDAC cell lines[44] which may explain its high metastatic potential. PGI has also been shown to regulate the expression of apoptotic

Table 5 Summary of changes in aerobic respiration in pancreatic ductal adenocarcinoma

Glycolytic Enzyme	Encoding gene	Change in PDAC	Implicated pathways	Known inhibitors	Inhibitor effect in PDAC
Pyruvate dehydrogenase complex	11p13		HIF-1	Dichloroacetate (inhibits PDC regulator, pyruvate dehydrogenase kinase)	Stimulates Krebs Cycle and reverses Warburg effect; Reduces PDAC proliferation and viability
Pyruvate dehydrogenase				Stimulator: Lipoic acid	Reduces cancer cell viability
Pyruvate dehydrogenase α	Xp22.1	Down			
Pyruvate dehydrogenase β	3p21.1-p14.2	Down			
Pyruvate carboxylase	11q13.4-q13.5	Up/down[1]		Avidin	
Citrate synthase	12q13.2	Up		Succinyl-CoA	
Aconitase				Flouroacetate	
Aconitase 1 (soluble)	9p21.1				
Aconitase 2 (mitochondrial)	22q13.2	Down			
Isocitrate dehydrogenase					
ICD (soluble)	2q33.3				
ICD (mitochondrial)	15q26.1	Down			
Oxoglurate (α-ketoglutarate) Dehydrogenase	7p14-p13	Up			
Succinyl-CoA synthetase		Down			
Succinic dehydrogenase		Up/down[1]			
Fumarase	1q42.1			D-malic, trans-aconitic, citrate, glycine	
Malate dehydrogenase					
MD (soluble)	2p13.3				
MD (mitochondrial)	7cen-q22	Up			

[1]Under-expressed in animal PDAC. PDAC: Pancreatic ductal adenocarcinoma.

protease activating factor 1 (Apaf-1) and caspase-9 genes involved in apoptosis[45].

Phosphofructokinase: Phosphofructokinase (PFK) phosphorylates fructose-6-phosphate into fructose 1,6-bisphosphate[19]. It has been referred to as the "pacemaker of carbohydrate metabolism" as it also regulates a wide range of sugars, including fructose and galactose that can feed into glycolysis[21]. The activity of PFK is regulated by fructose-2,6-bisphosphate, which itself is regulated by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases, encoded by 4 genes (PFKFB1-4)[46]. PFKFB expression (particularly isoenzyme 3 which exhibits the highest kinase/bisphosphatase activity) is altered in lung, gastric and pancreatic cancers[11,47,48]. Hypoxia also up-regulates the expression of PFKFB-3 and -4 in Panc-1 cells *via* the HIF-1α dependent pathway[11].

Fructose bisphosphate aldolase: Fructose bisphosphate aldolase (FBA) splits fructose 1,6-biphosphate into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate. Three different isoenzymes exist (A, B and C) and are encoded by 3 different genes[21]. FBA-A is overexpressed in PDAC and pancreatic cystadenoma[49], and proteome analysis of murine PDAC has also shown an overexpression of FBA-C[50]. Overexpression of FBA delays apoptosis, as does the addition of the end product G3P, by suppressing caspase-3 activity[51]. Under hypoxic conditions, PDAC cells expressing HIF-1 also express FBA-A and Glut-1 (glucose transporter 1) more highly, making the cell more resistant to apoptosis[52]. The hypoxic cytotoxin 3-[2-hydroxyethyl(methyl)amino]-2-quinoxaline-carbonitrile 1,4-dioxide inhibits the expression of FBA-A together with HIF-1, which subsequently reduces the proliferation of PDAC cells *in vivo* and reduced tumour volumes in models of *in vitro* murine cancer[53]. Suppression of HIF-1 also reduces the overexpression of FBA-A in PDAC and subsequently reduces *in vivo* tumorigenicity[54].

Triose phosphate isomerase: Triose phosphate isomerase (TPI) reversibly isomerises dihydroxyacetone into G3P. TPI is overexpressed in pancreatic cancer in several studies[55,56], but no correlation, however, is found between TPI-1 expression and tumour staging.

Triose stage of glycolysis

Glyceraldehyde phosphate dehydrogenase: Glyceraldehyde phosphate dehydrogenase (GAPDH) reversibly catalyses G3P into 1,3-biphosphoglycerate, and is over-expressed in PDAC[50,55,57,58], as well as other adenocarcinomas such as prostate[59] and breast[60]. GAPDH and Glut transporter over-expression is thought to be partially responsible for an increase in PDAC metabolic capacity[61]. Inhibition with iodoacetate on Panc-1 cells reduces survival and induces necrosis[27] but interestingly, did not significantly reduce the induction of signalling pathways K-ras, Akt and mTOR, as seen with 3-BP.

Phosphoglycerate kinase: Phosphoglycerate kinase (PGK) reversibly dephosphorylates 1,3-biphosphoglycerate into 3-phosphoglycerate, and is over-expressed in human PDAC[62]. In contrast, a study

involving hamster PDAC cell lines demonstrated no overall increase in expression levels, but did show a significant fivefold increase in PGK activity[63]. Clinically, its use as a diagnostic marker has been suggested due to its high antibody reactivity and high accuracy in distinguishing between cancer and non-cancer[64-66].

Phosphoglycerate mutase

Phosphoglycerate mutase (PGM) reversibly catalyses 3-phosphoglycerate into 2-phosphoglycerate, and exists as two isoenzymes (M and B). Expression levels of PGM-M have been found to be under-expressed, and PGM-B over-expressed, in rat PDAC[67]. PGM expression is regulated by p53[68].

Enolase

Enolase reversibly catalyses 2-phosphoglycerate into phosphoenolpyuvate, and is over-expressed in PDAC, particularly α-enolase[55-58,69-72]. It is also seen in pancreatic atypical hyperplasia[73] and intraepithelial neoplasia[74]. Its presence on the PDAC cell surface allows the promotion of cell migration and metastasis, but also induces a strong T-cell response leading it to be recognised as a tumour antigen[69,75,76]. As such, an increase in circulating enolase autoantibodies has been observed[76]. Animal studies have also shown good humoral and cellular immune responses against PDAC following inoculation with an α-enolase-encoded plasmid[77]. The resulting IgG response resulted in slower tumour progression and significantly improved survival. Several case reports have also clearly shown an over-expression of γ-enolase in serous microcystic adenomas[78,79], and solid-papillary[80], solid-pseudopapillary[81-86], solid-cystic papillary[87-102], and serous cystic[103,104] pancreatic tumours as well as PDAC[71].

Pyruvate kinase

Pyruvate kinase (PK) is the last enzymatic step in glycolysis, dephosphorylating phosphoenolpyruvate into pyruvate and converting ADP to ATP. Pyruvate then fuels the Krebs cycle. PK exists as 2 isoforms (M1 and M2); PK-M2 expressed exclusively in cancer cells (favouring anaerobic respiration) whereas the PK-M1 is predominately expressed in normal cells (favouring increased oxidative phosphorylation)[50,105,106]. Expression of the oncogenic tyrosine kinase receptor pathways inactivate PK to disrupt the pathway between glycolysis and MOP to perpetuate the Warburg effect[107]. Furthermore, two forms of PK-M2 have been identified - a tetrameric form favouring ATP production and a dimeric form that channels glucose into synthesis. The hypoxic and acidified tumour microenvironment staved of glucose favours the dimeric form of PK-M2[108]. Inhibition of the Akt/c-Myc (myelocytomatosis) pathway inhibits the activity of PK-M2 to down-regulate glycolysis[109]. TLN-232/CAP-232 (amino acid peptides targeting M2-PK) has also been shown to have anti-cancer effects in animal models, and clinical trials are underway to ascertain its effectiveness in pancreatic cancer[21].

Several studies have shown an increase in serum PK-M2 in patients with metastatic PDAC[110], and other pancreatic conditions such as chronic pancreatitis[111]. Its presence has also led suggestions that it should be used as a tumour marker for diagnosis, prognosis[112] and surveillance[113,114] in both pancreatic and other gastrointestinal cancers[115-117], particularly in combination with another marker, such as Ca19-9[118] or CEA[119], to increase its specificity value[120]. Surprisingly, PK-M2 over-expression is not found immunohistochemically in premalignant or PDAC tissue samples[64,121]. It is also weakly expressed in some cell lines such as SK-PC-1[122].

Anaerobic fermentation - lactate dehydrogenase

Lactate dehydrogenase (LDH) reversibly catalyses pyruvate to lactate, and is a target gene of the c-Myc regulator[123]. There are five isoforms, with LDH-A being the primary and over-expressed isoform in PDAC[124-128], including cell lines Capan-1[129] and SW-1990[130]. Mass spectrometry studies on LDH from PDAC have shown differential methylation to the LDH from normal ductal cells[131]. PDAC LDH-A acetylation, which normally inhibits LDH-A and prepares it for lysosomal degradation is also reduced[132]. Forced expression and inhibition of LDH-A increases and reduces the rate of growth respectively[133]. The activity of LDH-A and subsequent lactate production can be inhibited by blocking the mTor pathway using everolimus. The transcription factor Forkhead box protein M1 (FOXM1) over-expresses LDH-A to increase tumorigenicity[134].

Other inhibitors of LDH-A, such as derivatives of aryl-substituted N-hydroxyindole-2-carboxylates, have also been shown to inhibit the growth of PDAC[135]. Oxidative stress and a subsequent reduction in ATP may be responsible for this inhibition[136]. Lactate in the PDAC microenvironment has also been shown to be immunosuppressive by directly inhibiting Natural Killer cells and preventing an innate response to tumour cells[137]. Clinically, a raised serum LDH levels can also be a poor prognostic indicator in PDAC[138].

Aerobic respiration - Krebs cycle

The pyruvate dehydrogenase complex (PDC) consists of pyruvate dehydrogenase (PDH), dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase, and is the first enzymatic reaction that converts pyruvate into acetyl Co-A for the Krebs cycle. PDC itself is regulated and inhibited by pyruvate dehydrogenase kinase (PDK). Three isoenzymatic forms of PDK have been found in eukaryotic cells, with PDK2 being the major form that regulates PDC[139]. Inhibition of PDK by dichloroacetate in Panc-1 cells has been shown to stimulate metabolism *via* the Krebs cycle and away

from glycolysis, with the effect of reducing Panc-1 proliferation and viability[140].

Expression levels of other Krebs cycle enzymes are also changed in PDAC (Table 5). Malate dehydrogenase has been shown to be over-expressed in both human[64] and animal PDAC[50] cell lines, as has succinic dehydrogenase in mice PDAC[128], and pyruvate carboxylase[141]. The activity of citrate synthase was found to be higher in PDAC tissue than in normal pancreatic tissue by up to 20%[67]. The expression levels of mitochondrial citrate synthase and α-ketoglutarate dehydrogenase were found to be marginally increased in recent rat PDAC mRNA microarray studies by Yabushita *et al*[67]; pyruvate carboxylase, aconitase, isocitrate dehydrogenase, succinic dehydrogenase, succinic-CoA ligase and malate dehydrogenase expression were found to be reduced. Transcriptomic profiling from the same dataset also showed an increase in anaerobic glycolysis and nucleotide degradation and a reduction in Krebs cycle activity.

There is evidence that the mitochondrial oxidative phosphorylation (MOP) pathway can be used as a therapeutic target[142,143]. In glucose-limiting conditions, MOP inhibitors have been shown to be cytotoxic to Panc-1 cells. Momose *et al*[144] report that treatment with efrapeptin F (a fungal toxin that acts as a potent ATPase inhibitor) was cytotoxic to Panc-1 cells if they were cultured in nutrient-deficient media and, importantly, in glucose limiting conditions. Panc-1 cells grown in nutrient-deficient media were found to have reduced levels of ATP, and were sensitive to MOP inhibitors, suggesting that mitochondrial oxidative phosphorylation contributes to intracellular ATP production.

DISCUSSION

This detailed overview demonstrates that the metabolic phenotype of pancreatic cells is altered in PDAC where the Warburg effect increases glucose utilisation to fuel the pathological metabolic requirements of neoplastic cells. Enzymes at almost every stage of glycolysis are over-expressed. The rate at which glucose is transported into cells is also increased, and it has long been known that an over-expression of glucose transporters (particularly subtypes Glut-1 and Glut-3 in PDAC[145]) is associated with cancer progression and poor tumour prognosis[145-148]. Glut-1 expression in pancreas neoplasia correlates to tumour size and histological grading, from low grade PanIN 1A dysplastic lesions which are devoid of Glut-1, to PDAC where most cases showed some degree of GLUT-1 expression[149]. Serous cystadenomas consistently exhibit Glut-1 expression[150]. With such differences in expression between normal and cancerous tissue, the Glut family represents a potential therapeutic target. 2-Deoxyglucose, a metabolic inhibitor of Glut, hexokinase and phosphoglucose isomerase, inhibits growth by as much as 59% in Panc-1 cells and over 95% in MIAPaCa-2 after 2 d of treatment[151]. A similar but less profound effect was seen when the cells were treated with oxamate, a competitive metabolic inhibitor of lactate dehydrogenase at the final step of glycolysis. The reduction of cell proliferation is more profound under hypoxic conditions, where Glut-1 expression levels were increased and were more sensitive to inhibition.

The enzyme changes in metabolic phenotype of PDAC is complex, with variable changes in expression levels. Altering expression levels of HK, PGI, FBA, enolase, PK-M2 and LDA-A with metabolic inhibitors have shown a favourable effect on PDAC, thus identifying these as potential therapeutic targets.

To date, there are no clinical trials involving metabolic inhibitors and PDAC. There is, however, progress in using metabolic inhibitors in cell types other than PDAC which show a translation to *in vivo* treatment. Ko *et al*[148] have reported the use of 3-BP as an anticancer drug tested on the highly glycolytic hepatocellular cancer cell line AS-30D, where it was shown to rapidly and completely deplete intracellular ATP levels whilst not affecting levels in normal hepatocytes. Cell viability was ultimately affected, dropping to 10% with the ATP depletion. The *in vivo* effects of 3-BP in an animal model confirmed this anti-cancer effect. More recently, Ko *et al*[152] published a case study of a 16 year old male with primary hepatocellular cancer treated with 3-BP. The patient responded to treatment with tumour destruction (confirmed by computed tomography) and went on to survive 2 years. Clearly, the use of metabolic inhibitors as a treatment for cancer is at an early stage. By "blocking" glycolysis, there will be a global and unpredictable effect on all cellular functions, and present as an obstacle when translating *in vitro* cell line research into clinical practice. By using a metabolic phenotype to identify pathologically over-expressed enzymes, a more targeting approach can differentiate a patient's normal enzymes to that of PDAC.

In summary, the Warburg Effect has been long been recognised to occur in cancer cells, and describes a "metabolic switch" in the way cells use glucose to produce ATP. This overview highlights the extensive changes that in PDAC to produce a distinct metabolic phenotype. This phenotype has potential clinical correlates in that distinct components may be amenable to therapeutic manipulation.

COMMENTS

Background

Pancreatic cancer, typically ductal adenocarcinoma (PDAC), is an insidious cancer with poor outcomes that has seen no significant improvement over the last three decades. Recent progress in genetics has lead to a renewed interest in the Warburg effect, which describes the pathological switch from mitochondrial phosphorylation to glycolysis for rapid ATP production, thus presenting new potential therapeutic targets. The primary aim of this review is to provide a comprehensive overview of the metabolic phenotype of PDAC, particularly where metabolic inhibitors have shown a favourable response. The second aim is to identify steps in the metabolic pathways where there is little or

no research evidence to show its oncological importance.

Research frontiers

The Warburg effect was described in 1956 by German physiologist Otto Warburg who postulated that carcinogenesis was the result of "irreversible injuring of respiration". Data from high throughput techniques, such as genomic and metabolomic studies, have shown the Warburg effect is much more complex and extends to biomass synthesis for tumour proliferation.

Innovations and breakthroughs

There have been numerous studies looking at individual metabolic enzymes and the effect of inhibiting these enzymes on tumour cell physiology. Retrieved manuscripts concerning the metabolic enzyme and their role in PDAC were reviewed by the authors, and the data extracted to construct an overall "metabolic phenotype".

Applications

The metabolic phenotype of PDAC illustrates the role of individual enzymes, and the known effects on PDAC. Inhibiting certain enzymes - hexokinase, phosphoglucose isomerase, aldolase, pyruvate kinase and lactate dehydrogenase - has been shown to interfere with PDAC cell function. The review also highlights areas (and thus unidentified therapeutic targets) in the pathways, particularly in mitochondrial oxidative phosphorylation, where there is little research into the effects of metabolic inhibition.

Terminology

The Warburg effect describes the pathological switch from mitochondrial phosphorylation to glycolysis for rapid ATP production. The change in enzyme expression at certain points of these pathways facilitating the Warburg effect may be potentially exploitable as a therapeutic target.

Peer-review

Simply "blocking" major pathways such as glycolysis in an *in vitro* cell line environment may yield successful in reducing cell growth or tumourigenicity, but would also have a non-specific effect on normal cells leading to as yet unknown toxic effects. This presents a challenge of using such broad inhibitors in an *in vivo* or clinical setting. A detailed metabolic phenotype is therefore useful in identifying and targeting specific oncological changes and so would theoretically have less effect on normal cells.

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META-ANALYSIS

Helicobacter pylori eradication therapy for functional dyspepsia: Systematic review and meta-analysis

Li-Jun Du, Bin-Rui Chen, John J Kim, Sarah Kim, Jin-Hua Shen, Ning Dai

Li-Jun Du, Bin-Rui Chen, John J Kim, Sarah Kim, Jin-Hua Shen, Ning Dai, Department of Gastroenterology, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310020, Zhejiang Province, China

John J Kim, Division of Gastroenterology, Loma Linda University, Loma Linda, CA 92354, United States

Author contributions: Du LJ and Dai N designed the research; Du LJ and Chen BR performed the research; Du LJ and Shen JH analyzed the data; Du LJ and Kim JJ wrote the paper; Kim JJ, Kim S and Dai N provided critical feedback and revised the manuscript.

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Correspondence to: Ning Dai, MD, Professor, Department of Gastroenterology, Sir Run Run Shaw Hospital, Zhejiang University, No. 3 East Qingchun Road, Hangzhou 310020, Zhejiang Province, China. ndaicn@yahoo.com
Telephone: +86-571-86006144
Fax: +86-571-86044817

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Abstract

AIM: To evaluate whether *Helicobacter pylori* (*H. pylori*) eradication therapy benefits patients with functional dyspepsia (FD).

METHODS: Randomized controlled trials (RCTs) investigating the efficacy and safety of *H. pylori* eradication therapy for patients with functional dyspepsia published in English (up to May 2015) were identified by searching PubMed, EMBASE, and The Cochrane Library. Pooled estimates were measured using the fixed or random effect model. Overall effect was expressed as a pooled risk ratio (RR) or a standard mean difference (SMD). All data were analyzed with Review Manager 5.3 and Stata 12.0.

RESULTS: This systematic review included 25 RCTs with a total of 5555 patients with FD. Twenty-three of these studies were used to evaluate the benefits of *H. pylori* eradication therapy for symptom improvement; the pooled RR was 1.23 (95%CI: 1.12-1.36, $P < 0.0001$). *H. pylori* eradication therapy demonstrated symptom improvement during long-term follow-up at $\geqslant$ 1 year (RR = 1.24; 95%CI: 1.12-1.37, $P < 0.0001$) but not during short-term follow-up at < 1 year (RR = 1.26; 95%CI: 0.83-1.92, $P = 0.27$). Seven studies showed no benefit of *H. pylori* eradication therapy on quality of life with an SMD of -0.01 (95%CI: -0.11 to 0.08, $P = 0.80$). Six studies demonstrated that *H. pylori* eradication therapy reduced the development of peptic ulcer disease compared to no eradication therapy (RR = 0.35; 95%CI: 0.18-0.68, $P = 0.002$). Eight studies showed that *H. pylori* eradication therapy increased the likelihood of treatment-related side effects compared to no eradication therapy (RR = 2.02; 95%CI: 1.12-3.65, $P = 0.02$). Ten studies demonstrated that patients who received *H. pylori* eradication therapy were more likely to obtain histologic resolution of chronic gastritis compared to those who did not receive eradication

therapy (RR = 7.13; 95%CI: 3.68-13.81, $P < 0.00001$).

CONCLUSION: The decision to eradicate *H. pylori* in patients with functional dyspepsia requires individual assessment.

Key words: Functional dyspepsia; *Helicobacter pylori* eradication; Symptom improvement; Quality of life; Peptic ulceration; Meta-analysis

Core tip: The decision to eradicate *Helicobacter pylori* (*H. pylori*) in patients with functional dyspepsia requires individual assessment. This meta-analysis suggests that *H. pylori* eradication therapy is beneficial for symptom relief, reduces the development of peptic ulceration, and leads to histologic resolution of chronic gastritis but does not improve the quality of life and may even result in adverse events. Otherwise, other validated treatment such as acid suppression, prokinetics, and psychiatric treatment should also be considered.

Du LJ, Chen BR, Kim JJ, Kim S, Shen JH, Dai N. *Helicobacter pylori* eradication therapy for functional dyspepsia: Systematic review and meta-analysis. *World J Gastroenterol* 2016; 22(12): 3486-3495 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3486.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3486

INTRODUCTION

Functional dyspepsia (FD) is a common gastrointestinal disorder and affects as many as 21%[1] of the population worldwide and 2%-24%[2,3] of the Chinese population. Characterized by epigastric pain, postprandial fullness, and early satiation without organic causes, FD adversely impacts the patient's quality of life. FD is diagnosed by Rome Ⅲ criteria, which are symptom-based criteria[4]. Although the pathophysiology is not well established, gastro-duodenal motility dysfunction[5,6], visceral hypersensitivity[7,8], and psychological disturbance[9] may play a role in the pathogenesis of FD. *Helicobacter pylori* (*H. pylori*) infection is more common in patients with dyspepsia (OR = 2.3; 95%CI: 1.9-2.7) in comparison to healthy controls[10]. However, the effects of *H. pylori* eradication therapy in FD are inconsistent in previously published randomized trials and meta-analyses.

Previous meta-analyses mainly focused on symptom improvement after *H. pylori* eradication therapy; their findings (whether or not to eradicate) were not consistent because of variable study designs and follow-up durations[11-13]. One meta-analysis conducted by Moayyedi *et al*[14] provided an economic evaluation and suggested that *H. pylori* eradication therapy is the most cost-effective treatment method. We carried out this meta-analysis not only to evaluate benefits of *H. pylori* eradication therapy for symptom relief, but also to discuss the effects on the quality of life, adverse events, and the risk of subsequent peptic ulcer disease. We performed a more comprehensive meta-analysis than previous studies in order to assess the overall clinical impact of *H. pylori* eradication therapy in this population.

MATERIALS AND METHODS

Search strategy

A standard protocol, based on current PRISMA guidelines, was implemented for study inclusion, data extraction, and data analysis. PubMed, EMBASE, and The Cochrane Library were searched for published randomized controlled trials (RCTs) in English from 1988 to 2015. The main search strategies were as follows: "*Helicobacter pylori* OR *Campylobacter* OR *Campylobacter pylori* OR *C. pylori* OR *Helicobacter* infection" AND "treat OR eradication OR eradicating OR therapy OR anti-" AND "dyspepsia OR functional gastrointestinal disorder OR non-ulcer dyspepsia OR functional dyspepsia."

Inclusion and exclusion criteria

Studies were considered eligible if they met the following criteria: (1) RCTs; (2) study population of patients with dyspepsia (symptom-based criteria including ROME Ⅰ, Ⅱ, or Ⅲ) and *H. pylori* infection (^{13}C breath test, histology, or rapid urease teat); (3) *H. pylori* eradication regimens (dual, triple, quadruple, and sequential therapies) as intervention for treatment group and placebo or other drugs known not to eradicate *H. pylori* (no antibiotics or bismuth) as intervention for control groups; and (4) age above 17 years old. Studies were excluded if they were available only as abstracts, review studies, case reports, or if predefined outcome data required for analyses were lacking.

Data extraction and quality evaluation

Two investigators (Du LJ, Chen BR) reviewed all the titles and abstracts independently. Data was extracted from eligible full-text studies. The data included study population, demographical characteristics, year of publication, country, age, gender, *H. pylori* eradication regimens, duration of follow-up, *H. pylori* eradication rate, and study outcomes. The individual study quality was assessed according to the Cochrane collaboration's tool for risk of bias, which contains random sequence generation, allocation concealment, blindness, incomplete outcome data, selective outcome reporting, and other biases. Any disagreement was resolved by a third investigator (Dai N).

Study endpoints

The primary outcome for this study was the pooled risk ratio (RR) of successful treatment (presence

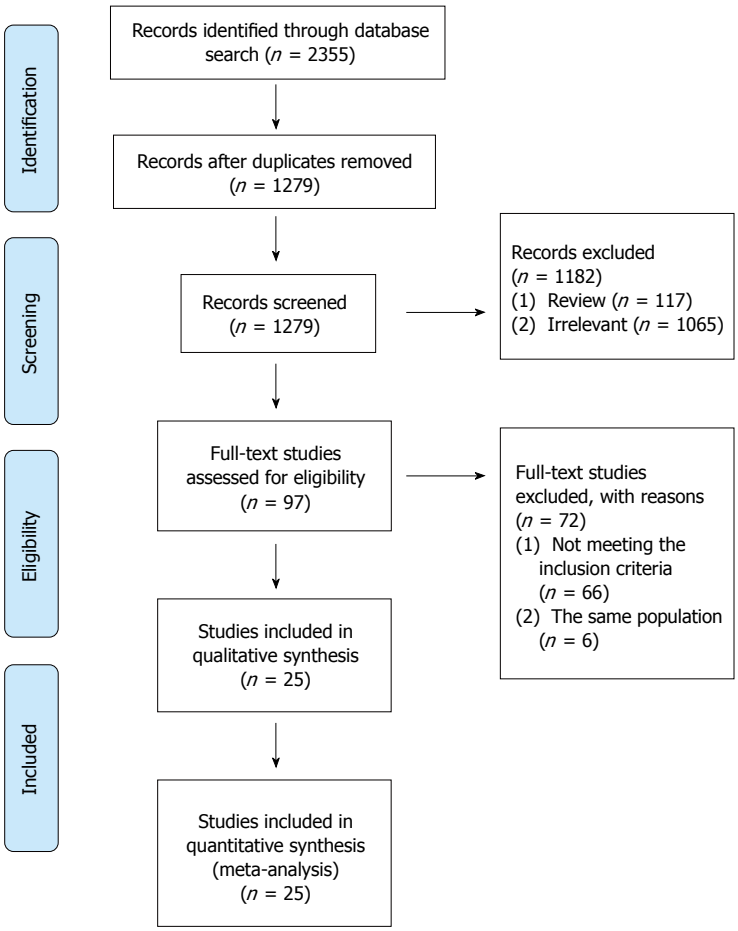

Figure 1 Study flow diagram.

of no more than mild pain or discomfort after treatment) with a 95%CI. The secondary outcomes were the pooled RR of improvement of dyspepsia at short-term (< 1 year) and long-term (≥ 1 year) follow-up, standard mean difference (SMD) of improvement in quality of life (SF-36), pooled RR of incidence of peptic ulceration during follow-up, pooled RR of development of treatment-related adverse events, and pooled RR of histologic resolution of chronic gastritis. If the studies were homogeneous (I^2 < 50%), the fixed-effects model was used; otherwise (I^2 > 50%), the random-effects model was chosen. Intervention was considered statistically significant when a *P*-value was < 0.05. If the studies were heterogeneous, a sensitivity analysis was performed. Publication bias was assessed by the funnel plot. All data were analyzed with RevMan 5.3 and Stata 12.0. The statistical methods of this study were reviewed by professor Yun-Xian Yu from Department of Epidemiology and Health Statistics of Zhejiang University.

RESULTS

Literature search and description of included studies

According to the search strategy, 2355 citations were identified from three databases. After removing the duplicates (*n* = 1076), two reviewers screened the titles and abstracts of potentially relevant studies (*n* = 1279) independently. Out of 97 full-text studies that were reviewed, 66 did not meet the inclusion criteria. Twenty-five RCTs with a total of 5555 people Which met the inclusion criteria were included in this systematic review (Figure 1)[15-39]. The assessment on the quality of the individual study is shown in Figure 2. The demographic data, eradication regimens, and eradication rates are listed in Table 1.

Benefits of H. pylori eradication therapy on symptom improvement

Twenty-three of 25 studies reported information on treatment success. Eradication therapy groups were treated with antibiotics, proton pump inhibitors, and bismuth, while control groups were treated with placebo, prokinetics, and/or proton pump inhibitors. Primary analysis demonstrated that 1183 (40%) of 2939 patients in the eradication therapy group and 795 (32%) of 2468 in the control groups had no or mild symptoms during the last follow-up visit (pooled RR = 1.23; 95%CI: 1.12-1.36, *P* < 0.0001). Although there was no significant heterogeneity (I^2 = 42%) among the selected studies, the asymmetry in the funnel plot (Figure 3) indicated existing publication bias. *H. pylori* eradication therapy demonstrated

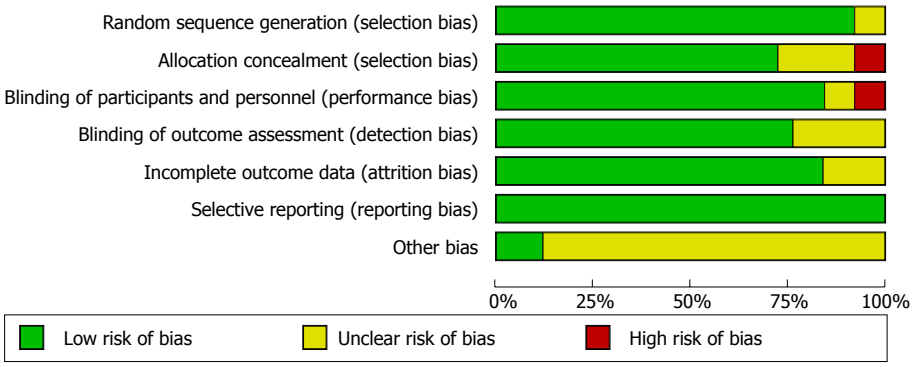

Figure 2 Risk of bias graph. The Cochrane collaboration's tool was used to evaluate risk of bias.

Table 1 Characteristics of studies included in the meta-analysis

Study	Sample (M/F)	Age, mean	Country	Last visit (mo)	Intervention	*Helicobacter pylori* eradication
Ang, 2006	130 (47/83)	38.0	Singapore	12	LAC	73.2%
Blum, 1998	328 (136/192)	47.0	Global	12	OAC	79%
Chiba, 2002	394 (148/246)	49.5	Canada	12	OMC	75%
Dhali, 1999	62 (44/18)	32.5	India	12	BMTe	87.5%
Froehlich, 2001	144 (64/80)	44.6	Switzerland	12	LAC	75%
Gisbert, 2004	50 (15/35)	41.5	Spain	12	OAC	76%
Greenberg, 1999	100 (31/69)	46.5	United States	12	OC	70.5%
Gwee, 2009	82 (38/44)	40.4	Singapore	12	OCT	68.3%
Hsu, 2001	161 (78/83)	50.9	China	12	LMTe	78%
Koelz, 2003	181 (74/107)	47.5	Switzerland	6	AO	51.7%
Koskenpato, 2001	151 (52/99)	51.7	Finland	12	AMO	82%
Lan, 2011	195 (89/106)	47.4	China	3	RAC	85.7%
Malfertheiner, 2003	800 (380/420)	46.2	Germany	12	LAC	63.9%
Mazzoleni, 2006	89 (20/69)	41.3	Brazil	12	LAC	91.3%
Mazzoleni, 2011	404 (86/318)	46.0	Brazil	12	OAC	88.6%
McColl, 1998	318 (155/163)	42.1	United Kingdom	12	AMO	88%
Miwa, 2000	85 (40/45)	51.5	Japan	3	OAC	85.4%
Naeeni, 2002	157 (47/110)	32.5	Iran	9	ABM	52.6%
Sodhi, 2013	519 (169/350)	44.5	India	12	OAC	69.9%
Talley, 1999	293 (133/160)	46.4	United States	12	LCA	80%
Talley, 1999 (ORCHID)	275 (98/177)	50.0	Australia	12	OAC	85%
Varannes, 2001	253 (112/141)	51.0	France	12	RaAC	69%
Varasa, 2008	48 (21/27)	37.0	Spain	12	RA	81.4%
Xu, 2013	396 (135/261)	40.0	China	12	ACE	76.36%
Zanten, 2003	157 (72/81)	48.0	Canada	12	LCA	82%

M/F: Male/female; A: Amoxillin; B: Bismuth salt; C: Clarithromycin; E: Esoprazole; F: Furazolidone; L: Lansoprazole; M: Metronidizole; O: Omeprazole; R: Rabeprazole; Ra: Ranitidine; T: Tinidazole; Te: Tetracycline.

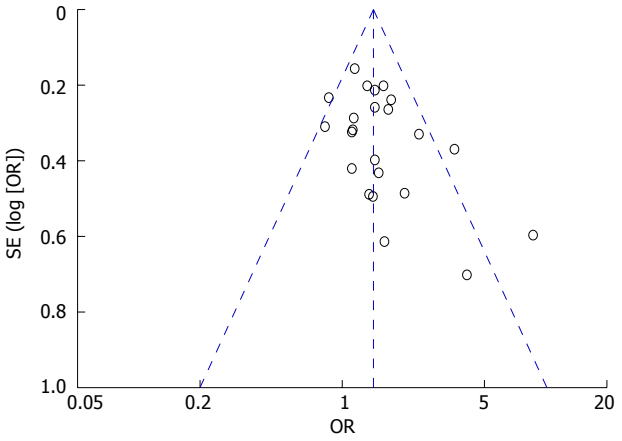

Figure 3 Funnel plot of included studies for potential publication bias. The funnel plot was not absolutely symmetrical.

symptom improvement at long-term (≥ 1 year) (RR = 1.24; 95%CI: 1.12-1.37, $P < 0.0001$) but not at short-term (< 1 year) (RR = 1.26; 95%CI: 0.83-1.92, $P = 0.27$) follow-up. The studies that reported short-term outcomes demonstrated significant heterogeneity ($I^2 = 64\%$). The forest plot and sensitivity analysis are shown in Figures 4 and 5, respectively.

Benefits of H. pylori eradication therapy on quality of life

Seven studies reported data on quality of life both at baseline and at the last visit required for the meta-analysis. Five trials used the SF-36, one used the general well-being index, and one used QoL-PEI. A fixed effect model ($I^2 = 0\%$) was performed on all seven studies. Overall, *H. pylori* eradication therapy had no significant benefit on quality of life, with an

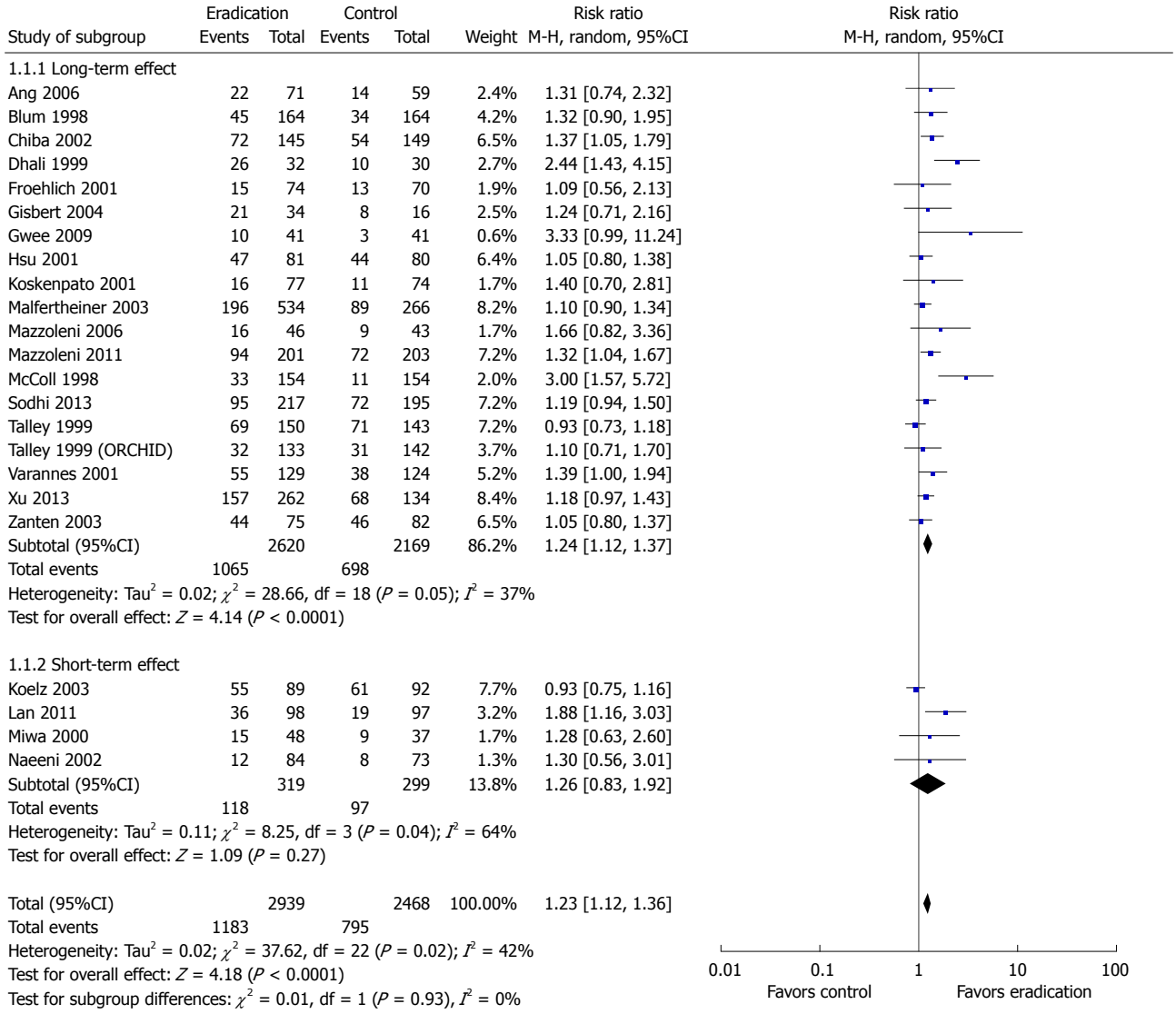

Figure 4 Forest plot of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on symptom relief. Twenty-three studies were included. The random effect model (Mantel-Haenszel method) was applied.

SMD of -0.01 (95%CI: -0.11 to 0.08, *P* = 0.80). Detailed information is shown in Figure 6.

Benefits of H. pylori eradication therapy on long-term peptic ulceration

Six studies reported endoscopic data at the last visit to evaluate for the development of peptic ulcer disease. *H. pylori* eradication therapy compared to no eradication therapy reduced the development of peptic ulcer disease (RR = 0.35; 95%CI: 0.18-0.68, *P* = 0.002). There was no significant study heterogeneity (I^2 = 0%). Detailed information is shown in Figure 7.

H. pylori eradication therapy on the development of adverse events

Eight studies provided data on development of common side effects associated with the intervention. Patients who received *H. pylori* eradication therapy were more likely to have side effects compared to controls (RR = 2.02; 95%CI: 1.12-3.65, *P* = 0.02). The random effect model was used because significant study heterogeneity (I^2 = 94%) was detected. The forest plot and sensitivity analysis are shown in Figures 8 and 9.

Other outcomes comparing H. pylori eradication therapy and control groups

One study provided outcome data on the cost of interventions such as medication, diagnostic tests, and physician consultation and did not demonstrate a difference between eradication therapy and the control[38]. However, the cost of intervention from this study was derived from utilization of healthcare services rather than the actual cost. Ten studies reported histological outcomes following intervention (Figure 10). Patients who received *H. pylori* eradication therapy were more likely to obtain histologic resolution of chronic gastritis compared to control (RR = 7.13; 95%CI: 3.68-13.81, *P* < 0.00001).

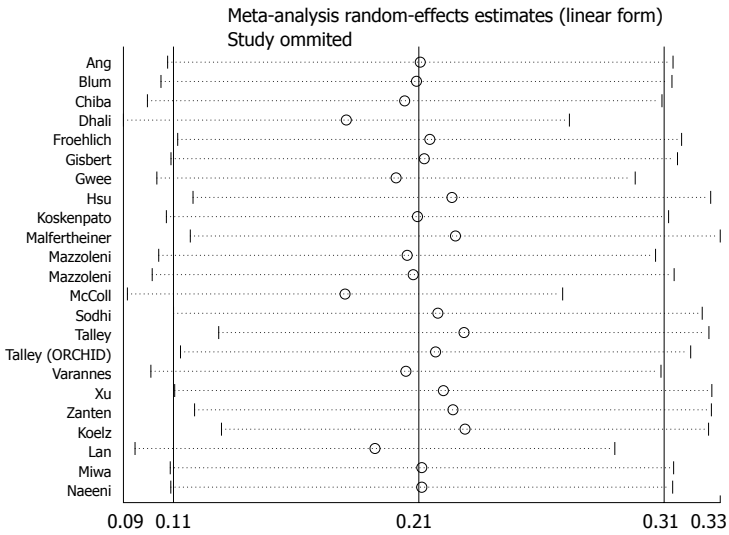

Figure 5 Sensitivity analysis of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on symptom relief.

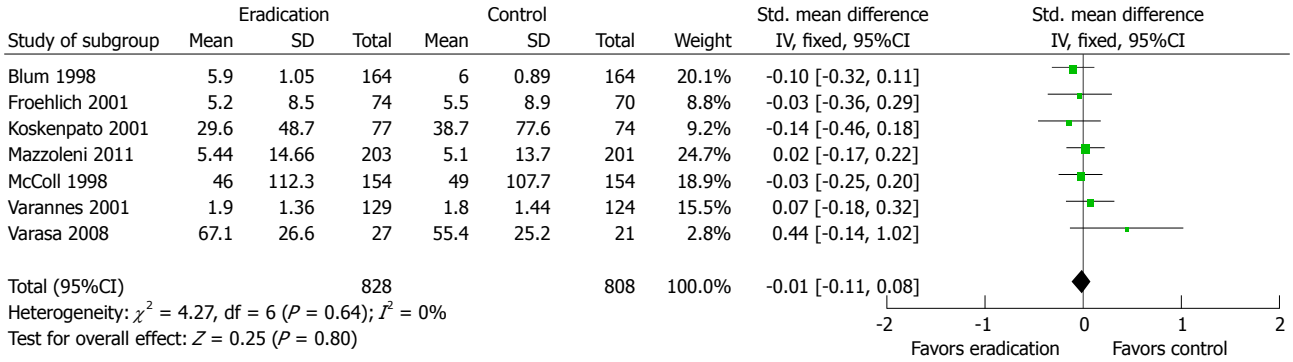

Figure 6 Forest plot of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on quality of life. Seven studies were included. The fixed effect model (Inverse Variance method) was applied.

DISCUSSION

Our meta-analysis based on well-designed RCTs demonstrated that the effect size of symptom relief from *H. pylori* eradication therapy in patients with FD was small (RR = 1.23; 95%CI: 1.12-1.36, *P* < 0.0001) with an undetectable short-term benefit. Eradication therapy was nearly three times more likely to reduce the development of peptic ulcer disease compared with no eradication therapy. Furthermore, histologic findings of chronic gastritis were more likely to resolve after *H. pylori* eradication therapy compared to controls. However, *H. pylori* eradication therapy did not improve the quality of life for patients with FD compared to anti-acids, prokinetics, or placebo therapy. Eradication therapy was also more likely to be associated with side effects (RR = 2.02; 95%CI: 1.12-3.65, *P* = 0.02) compared to control.

H. pylori infection is more prevalent in Asia than in Western countries with high prevalence observed in China and South Korea[40]. Eradication therapy appears to be more effective in Asian populations as shown by the meta-analysis conducted by Jin and Li[13] on the Chinese population. Their study showed that *H. pylori* eradication therapy compared to controls increased the likelihood of improvement in dyspeptic symptoms by 3.6-fold. Another meta-analysis performed by Zhao *et al*[12] found that *H. pylori* eradication therapy compared to no eradication therapy was beneficial for improvement of dyspepsia in European (OR = 1.49; 95 CI% 1.10-2.02) and American populations (OR = 1.43; 95%CI: 1.12-1.83).

H. pylori is strongly associated with many diseases, including functional dyspepsia, gastric or duodenal ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma[41,42]. However, *H. pylori*-induced gastritis is the most important risk factor for development of peptic ulcer disease[43]. Most patients with *H. pylori* infection have asymptomatic gastritis, and experience variable clinical symptoms depending on bacteria, host, and environmental factors. Whether *H. pylori* infection delays gastric emptying is unclear[44,45], but *H. pylori* appears to alter gastric acid production by changing gastrin and somatostatin secretion[46]. Abnormal gastric acid secretion causes mainly dysmotility-like, dyspeptic symptoms[47]. Duodenal acid exposure indirectly induces fullness, bloating, and epigastric pain by suppressing antral

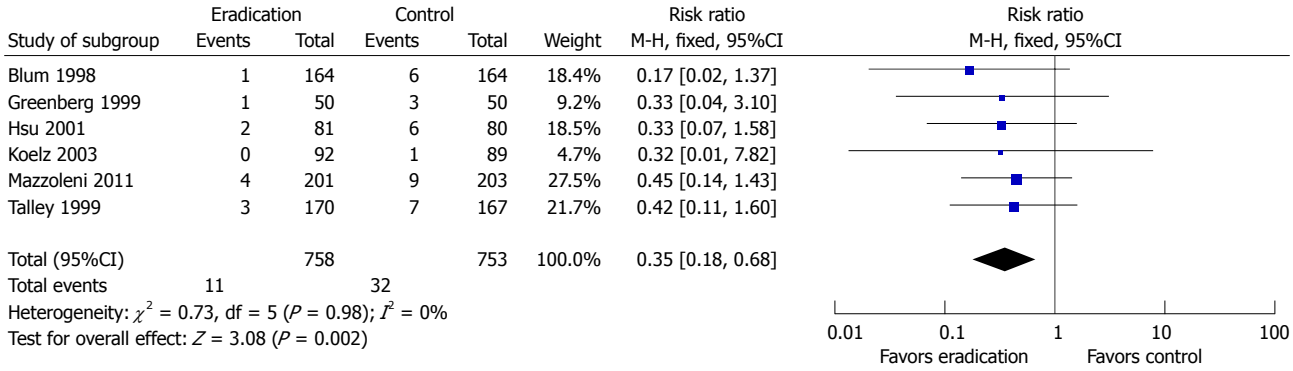

Figure 7 Forest plot of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on long-term peptic ulceration. Six studies were included. The fixed effect (Inverse Variance method) model was applied.

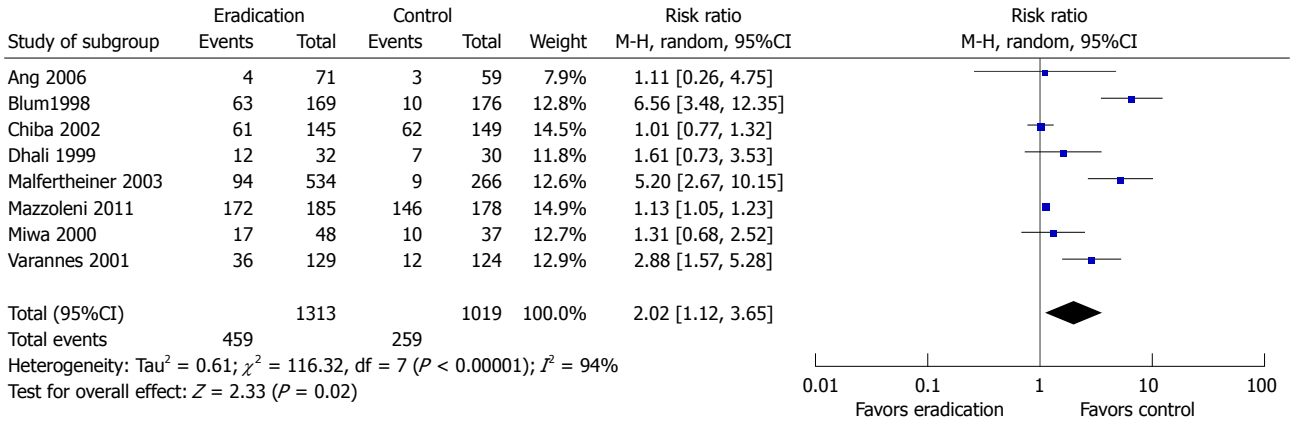

Figure 8 Forest plot of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on adverse events. Eight studies were included. The random effect model (Mantel-Haenszel method) was applied.

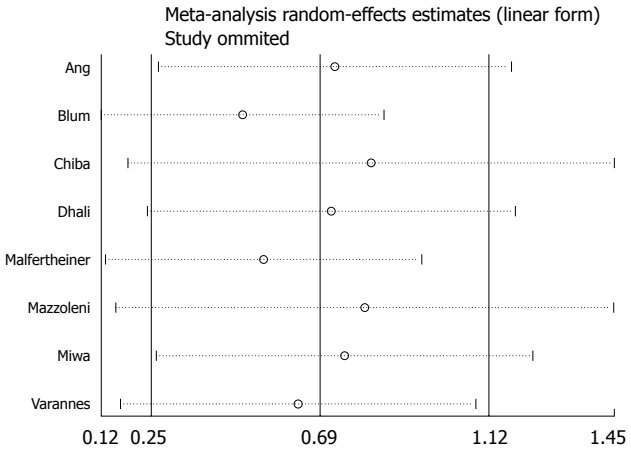

Figure 9 Sensitivity analysis of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on adverse events.

contractions, which may contribute to delayed gastric emptying[48,49].

According to the results of this meta-analysis, decision to eradicate *H. pylori* may be influenced by several key points. First, eradication therapy may be preferable among patients with risk factors for peptic ulcer disease or gastric cancer. Our study showed long-term benefits such as reduction in incidence of future peptic ulcer disease and resolution of gastritis, which are associated with gastric cancer[50,51]. Second, because of apparent adverse effects associated with eradication therapy, alternative validated therapy for FD such as acid suppression, prokinetics, or lifestyle changes for mild dyspeptic symptoms should also be considered. A large study of 1425 patients showed that *H. pylori* infection was a significant risk factor for

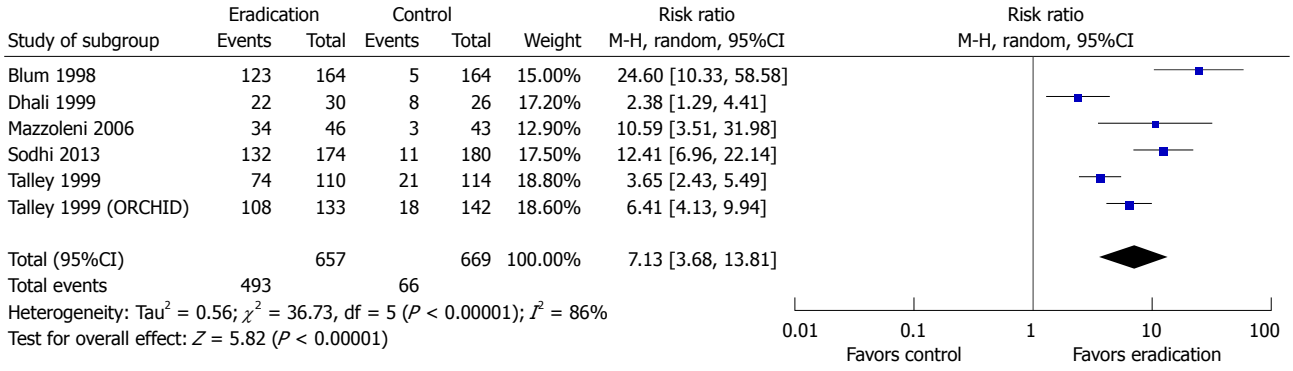

Figure 10 Forest plot of the effects comparing *Helicobacter pylori* eradication therapy *vs* control on histologic resolution of chronic gastritis. Six studies were included. The random effect model (Mantel-Haenszel method) was applied.

dyspepsia. However, other factors such as NSAIDs use, unemployment, and heavy smoking demonstrated larger magnitude of association compared to *H pylori* infection[52]. Furthermore, rising prevalence of antibiotics resistance[53] and *H. pylori* reinfection[54] cannot be ignored. Third, it has been well established that the presence of psychiatric disorders, such as anxiety disorder, is more common in patients with functional gastrointestinal disorders than in the general population[55,56]. Psychiatric treatment with antidepressants is helpful in the reduction of dyspeptic symptoms[57]. Anxiety and depression are considered to be the best predictors of quality of life[58]. Cognitive-behavioral therapy (CBT), psychotherapy, anxiolytics, and antidepressants can also relieve dyspeptic symptoms[59,60].

The strength of this meta-analysis includes a comprehensive analysis of high-quality studies with evaluation of various FD outcomes other than symptom improvement alone. There are some limitations to this meta-analysis. First, some well-designed studies were excluded because they were published in non-English language. Second, the random effect model was chosen to evaluate the short-term symptom improvement and development of adverse events in the presence of significant study heterogeneity resulting from different study designs and methods. Third, there is a possibility of publication bias as we excluded some RCTs that did not have sufficient data for meta-analysis or were not published in manuscript form at the time of submission.

In conclusion, *H. pylori* eradication therapy compared to no eradication therapy has a statistically significant but small magnitude of benefit for symptom relief and can also reduce the development of peptic ulcer disease. However, *H. pylori* eradication therapy was associated with higher incidence of adverse events during the treatment and failed to demonstrate any effect in improving the quality of life. In addition to *H. pylori* eradication therapy, alternative therapies such as acid-suppression, prokinetics, psychotherapy, and anxiolytics should also be considered after an individualized assessment.

ACKNOWLEDGMENTS

We would like to thank professor Yun-Xian Yu for reviewing the statistical methods of this study.

COMMENTS

Background

Functional dyspepsia (FD) is a common gastrointestinal disorder and affects as many as 21% of the population worldwide. *Helicobacter pylori* (*H. pylori*) infection is one of the most important factors for development of dyspeptic symptoms.

Research frontiers

Benefits of *H. pylori* eradication therapy in patients with FD are not consistent. Relying on antibiotics may lead to an increased rate of drug resistance, which may consequently lead to an increased rate of eradication failure.

Innovations and breakthroughs

Compared to previous studies, the current meta-analysis included additional clinical outcomes on the benefits of *H. pylori* eradication therapy other than symptom relief such as quality of life, adverse events, and development of peptic ulceration.

Applications

According to the current meta-analysis, *H. pylori* eradication therapy should be considered after individual assessment. The authors have highlighted that *H. pylori* eradication therapy was significantly beneficial for symptom relief and reduced the risk of development of peptic ulceration in patients with functional dyspepsia. However, *H. pylori* eradication therapy failed to improve the quality of life and was associated with higher likelihood of treatment-related adverse effects. Otherwise, alternative validated therapies such as acid suppression, prokinetics, and psychiatric treatment should also be considered.

Peer-review

The conclusions are warranted by the results, and it is a useful meta-analysis.

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CASE REPORT

Pancreatic metastasis from mycosis fungoides mimicking primary pancreatic tumor

Paola Ceriolo, Valentina Fausti, Elisa Cinotti, Silvia Bonadio, Lizzia Raffaghello, Giovanna Bianchi, Giulio Fraternali Orcioni, Roberto Fiocca, Franco Rongioletti, Vito Pistoia, Giacomo Borgonovo

Paola Ceriolo, Course in Paediatric Science, Fetal-Perinatal and Paediatric Pathology, University of Genoa, 16132 Genoa, Italy

Paola Ceriolo, Pathology Unit, Istituto Giannina Gaslini, 16132 Genoa, Italy

Valentina Fausti, Elisa Cinotti, Franco Rongioletti, Section of Dermatology, IRCCS AOU San Martino-IST, 16132 Genoa, Italy

Silvia Bonadio, Giulio Fraternali Orcioni, Pathology Unit, IRCCS AOU San Martino-IST, 16132 Genoa, Italy

Lizzia Raffaghello, Giovanna Bianchi, Vito Pistoia, Laboratory of Oncology, Istituto Giannina Gaslini, 16147 Genoa, Italy

Roberto Fiocca, Pathology Unit, IRCCS AOU San Martino-IST and University of Genoa, 16132 Genoa, Italy

Giacomo Borgonovo, Surgery Unit, IRCCS AOU San Martino-IST and University of Genoa, 16132 Genoa, Italy

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Correspondence to: Paola Ceriolo, MD, PhD, Course in Paediatric Science, Fetal-Perinatal and Paediatric Pathology, University of Genoa, 16132 Genoa, Italy. paolettac@gmail.com
Telephone: +39-33-95794156
Fax: +39-0103075010

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Abstract

Mycosis fungoides (MF) is a cutaneous T-cell lymphoma that can undergo local progression with possible systemic dissemination. We report a case of a patient affected by MF with a pancreatic mass that was a diagnostic challenge between primitive tumor and pancreatic metastasis from MF. Clinical setting findings and imaging studies raised the suspicion of a pancreatic primary neoplasm. A diagnostic clue was provided by the combined histomorphologic/immunohistochemical study of pancreatic and cutaneous biopsies, which revealed a pancreatic localization of MF. Considering the rarity of metastatic localization of MF to the pancreas, we next investigated whether chemokine-chemokine receptor interactions could be involved in the phenomenon to provide new insight into the possible mechanisms underlying metastatic localization of MF to the pancreas. Histological analyses of archival pancreatic tissue demonstrated that glucagon-secreting cells of the pancreatic islets expressed the CCL27 chemokine, which may have attracted in our case

metastatic MF cells expressing the complementary receptor CCR10.

Key words: Differential diagnosis; Pancreatic mass; Extracutaneous localization; Mycosis fungoides; CCL27, CCR10

Core tip: We believe that this clinical case is very interesting both for its rarity (mycosis fungoides metastasized to the pancreas) and the complexity of the differential diagnosis. In addition, the investigations performed provide new insight into the possible mechanisms underlying metastatic localization of mycosis fungoides to the pancreas. Indeed, histological analyses of archival pancreatic tissue demonstrated for the first time that glucagon-secreting cells of the pancreatic islets express the CCL27 chemokine, which may have attracted, in our case, metastatic mycosis fungoides cells expressing the complementary receptor CCR10.

Ceriolo P, Fausti V, Cinotti E, Bonadio S, Raffaghello L, Bianchi G, Orcioni GF, Fiocca R, Rongioletti F, Pistoia V, Borgonovo G. Pancreatic metastasis from mycosis fungoides mimicking primary pancreatic tumor. *World J Gastroenterol* 2016; 22(12): 3496-3501 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3496.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3496

INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) includes a group of mature (peripheral) T-cell neoplasms characterized by clonal expansion of a mature CD4-positive T-cell, putatively belonging to a skin homing subset of memory T-cells[1]. According to World Health Organization (WHO) criteria, mycosis fungoides (MF) is the most common form of CTCL and accounts for almost 50% of all primary cutaneous lymphomas[2].

MF is an indolent disease initially limited to the skin that undergoes variable cutaneous progression with possible systemic dissemination in late stages. Although spread of MF to the gastrointestinal tract has been described previously, there have been only two reports on symptomatic pancreatic involvement[3,4].

Herein, we report a case of MF in anaplastic transformation with pancreatic involvement, mimicking primary pancreatic tumor.

CASE REPORT

A 64-year-old man presented with erythematous and nodular lesions on the trunk and extremities (erythematous patches on the trunk, red-brown sharply demarcated plaques on the extremities, and violaceous nodules and exophytic tumor lesions on the occipital region, right forearm, and gluteus) (Figure 1A). Erythematous skin lesions manifested 6 years before admission and followed an indolent course until 1 year ago when the full aggressive clinical picture described above became apparent.

Since the lesions were increasing in number and unresponsive to the conventional topical therapy, a nodule on the right arm of the patient was biopsied. The histopathological evaluation led to the diagnosis of CTCL, MF-type. The patient was studied with abdominal computed tomography (CT) that did not show any visceral or lymph node involvement.

Initially, the patient was treated with narrow-band ultraviolet B therapy (three sessions a week on nonconsecutive days, initial dose 0.1 J/cm^2, increment of the dose 0.1 J/cm^2 per session) followed 2 mo later by Interferon α2b, 3 million units subcutaneously three times a week. Interferon α2b therapy was temporarily stopped because of side effects (nausea, gastric pyrosis, diarrhea, neutropenia, and anemia) and restarted 3 mo later with the addition of oral bexarotene (75 mg twice a day for 10 d, subsequently three times a day for another 10 d and then four times a day for 3 mo) and local radiotherapy of the occipital region (36 Gy five times a week for 2 mo). The patient responded with partial resolution of the skin lesions, especially the nodular ones. Five months later, the patient complained of persistent abdominal pain, but blood chemistry and abdominal ultrasonography did not reveal any abnormality.

Eight months later, the patient presented with jaundice, asthenia, and worsening of abdominal pain.

Blood exams and gastrointestinal ultrasonography were initially performed; thereafter, a percutaneous ultrasound guided pancreatic core biopsy and a cutaneous biopsy were performed for a combined histomorphologic and immunohistochemical study. In particular, immunohistochemical staining for CD3, CD4, CD30, CD45, glucagon, somatostatin, pancreatic polypeptide, CCL27, and CCR10 was carried out.

Blood chemistry disclosed abnormal levels of amylase (625 U/L; n.v. 5-53 U/L), lipase (9228 U/L; n.v. 114-286 U/L), aspartate aminotransferase: 105 U/L; n.v. 0-40 U/L), alanine aminotransferase (171 U/L; n.v. 0-40 U/L), alkaline phosphatase (1539 U/L; n.v. 98-280 U/L), gamma-glutamyltransferase (675 U/L; n.v. 11-50), total bilirubin (1.36 mg/dL; n.v. 0.2-1.2 mg/dL), and direct bilirubin (1 mg/dL; n.v. 0.0-0.4 mg/dL). These results were indicative of biliary stasis.

Thereafter, gastrointestinal ultrasonography was performed showing a mass measuring 70 mm × 48 mm × 68 mm and involving the head of pancreas with peripheric vascularization pattern and Wirsung duct dilation. An abdominal CT with contrast agent showed an irregular pancreatic mass involving the head of the pancreas (Figure 2A, arrow), with dilation of the

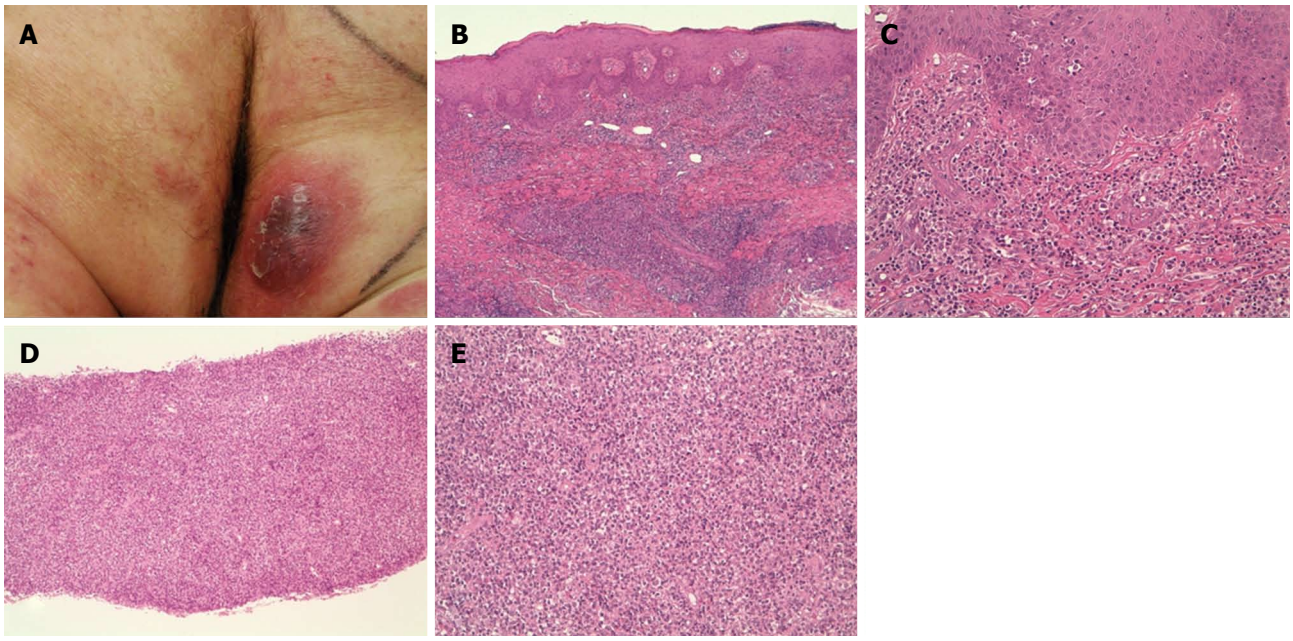

Figure 1 Macroscopic aspect of cutaneous lesions (A-C) and pancreatic lesion (D and E). Hematoxilyn and eosin staining, magnification × 4 (B), × 10 (D), × 20 (C and E).

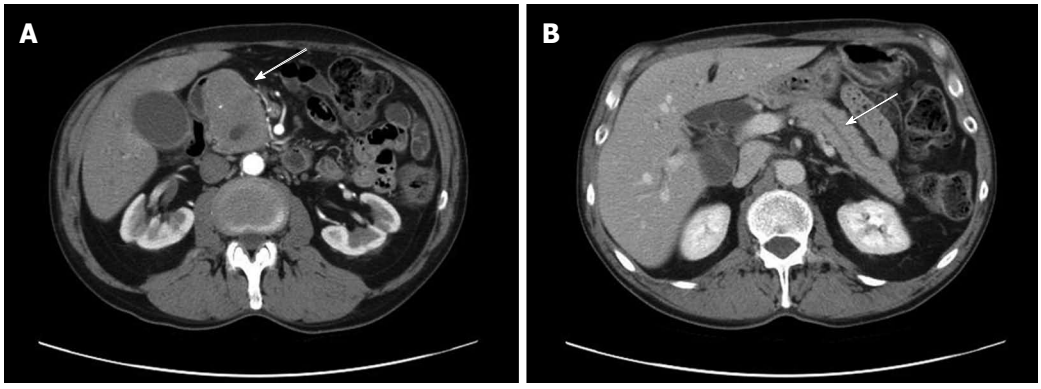

Figure 2 Abdominal computed tomography with contrast agent.

intrahepatic and the common bile ducts (Figure 2B, arrow) in the absence of further pancreatic lesions. No involvement of spleen, liver, kidney, or lymph node was detected. Imaging studies suggested the presence of a pancreatic primary neoplasm. A diagnosis of pancreatic adenocarcinoma was unlikely because of the homogeneous contrast enhancement and the regular margins of the mass. Other potential diagnoses included papillary neoplasm, neuroendocrine tumors, or lymphoma. Genetic analysis of K-ras, P16, and p53, which are highly mutated in pancreatic cancer, was not performed. The patient was subjected to percutaneous ultrasound guided core biopsy of the pancreatic mass, which suggested a metastatic localization of MF but did not allow drawing definitive conclusions due to the paucity of the material.

Considering also the extreme rarity of this occurrence, the pancreatic needle biopsy was repeated in parallel with a cutaneous biopsy to compare the histological features of the two specimens. A lymphomatous infiltrate consistent with MF was detected in both bioptic specimens.

The cutaneous specimen (Figure 1B and C) revealed a dermal nodular infiltrate mainly composed of small-medium size cells with focal evidence of epidermotropism. Neoplastic lymphocytes were positive for CD45 and CD4 and showed a heterogeneous expression of CD3. A minority (< 25%) of $CD30^+$ larger elements and an eosinophilic component were also observed.

The pancreatic parenchyma was completely replaced by a neoplastic infiltrate (Figure 1D and E) showing features overlapping with those detected in the cutaneous specimen.

These findings led us to diagnose peripheral T cell lymphoma, MF type. The medium-large $CD30^+$ component suggested a tendency toward histological transformation[2].

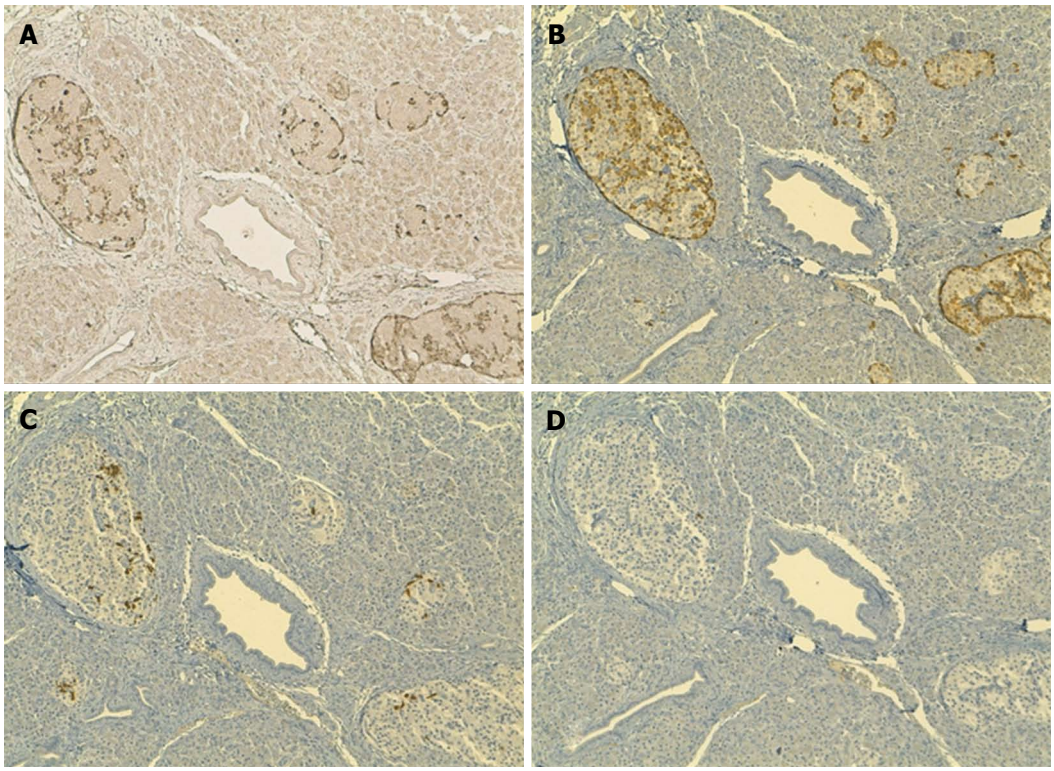

Figure 3 Immunohistochemical staining of pancreatic archival tissue sections. A: CCL27; B: Glucagon; C: Somatostati; D: Pancreatic polypeptide. A-D: Magnifications × 10.

DISCUSSION

Extracutaneous localizations of MF are rare and occur in long-standing MF with lymph node involvement. The literature reports involvement of lungs, spleen, liver, kidney, thyroid gland, bone marrow, oral cavity, larynx, heart[5], breast[6], central nervous system[7], esophagus[8,9], stomach, small intestine[10] and pancreas[3,4].

The diagnosis *ante mortem* of an extracutaneous manifestation of MF is rare and limited to few case reports. This is in contrast with autopsy findings, showing a high rate of extracutaneous involvement in lungs (75%), spleen (60%), liver (53), kidney (44%), and pancreas (41%)[11].

To date, only two patients with clinically detectable pancreatic metastasis from MF have been reported[3,4]. In these cases, intrapancreatic metastases displayed an infiltrative pattern without any gross tumor, a feature associated with most neoplasms that metastasize to the pancreas[4].

Pancreatic involvement is rare not only in MF but also in other T-cell cutaneous lymphomas. Only two such cases have been reported in association with disseminated pagetoid reticulosis[12] and adult T-cell leukaemia/lymphoma[13].

Chemokines and their receptors have been associated with tumor metastasis, invasion of lymphatic vessels, and trafficking of lymphoma cells[1]. Due to the extraordinary rarity of metastatic localization of MF cells to the pancreas, we investigated whether chemokine-chemokine receptor interactions could be involved in the phenomenon. We focused on the CCL27-CCR10 axis that has a primary role in T cell-mediated skin inflammation[14] and cutaneous T cell malignancy[15]. Thus, $CCR10^+$ $CD4^+$ T lymphocytes are attracted to the skin where the CCR10 ligand CCL27 is secreted by keratinocytes of the basal layers of the epidermis in normal skin, immobilized on extracellular matrix, and displayed on the surface of endothelial cells[14,15].

By immunohistochemical analysis, primary MF cells from our patient expressed CCR10, consistent with previous reports[1,15]. We hypothesized that a subset of MF cells circulating in the peripheral blood had upregulated CCR10 and was attracted by CCL27 expressed in the pancreas. However, CCR10 expression in metastatic MF cells and CCL27 expression in infiltrated pancreas could not be investigated due to the minute amount of pancreatic tissue obtained from the biopsy. We found no evidence in the literature for CCL27 expression in normal pancreas. Nonetheless, upon staining of five different archival pancreatic tissue samples from three patients with pancreatic ductal adenocarcinoma and two patients with papillary adenocarcinoma of ampulla (one with a concomitant well differentiated neuroendocrine tumor), we discovered that the islets of the pancreas, but not the exocrine portion, tested positive for CCL27 expression. Immunohistochemical staining on serial sections demonstrated that glucagon-secreting, but not somatostatin - or pancreatic polypeptide (PP) - secreting, pancreatic endocrine cells expressed CCL27 (Figure 3). These results point to the attraction of

CCR10^{+} malignant lymphocytes by glucagon-secreting insulae expressing CCL27 as a potential mechanism driving localization of MF cells to the pancreas in our patient. To the best of our knowledge, this is the first demonstration of CCL27 expression in the pancreas. It is important to note, however, that these results on CCL27 expression were derived from archival pancreatic tissue samples from patients with primary malignancies, and the possibility that CCL27 may be abnormally upregulated in these conditions cannot be excluded.

The CCL27/CCR10 axis is not the only one that has been implicated in MF pathogenesis. Recent studies have highlighted the pivotal role of the CCL20/CCR6 axis in cutaneous T cell lymphoma invasiveness and metastasis[16]. Thus, our findings may have disclosed only one of the possible mechanisms promoting MF cell metastasis to the pancreas.

In conclusion, our case posed a clinical diagnostic challenge between pancreatic primitive tumor and a pancreatic metastasis from MF. In the clinical setting, the patient had: (1) jaundice and blood exams indicative of bile stasis; and (2) imaging studies raising the suspicion of a pancreatic primary neoplasm. A diagnostic clue came from the combined histomorphologic and immunohistochemical study of pancreatic and cutaneous biopsies, which revealed a pancreatic localization of MF. Although the prognosis of MF patients that develop lymph node or visceral metastasis (stage Ⅳ disease) is poor[17], our patient is still alive after a 3 year follow up.

COMMENTS

Case characteristics

A 64-year-old man with a previous history of erythematous skins lesions and a diagnosis of cutaneous T cell lymphoma, mycosis fungoides (MF) type, presented with jaundice, asthenia, and severe abdominal pain.

Clinical diagnosis

Persistent abdominal pain combined with jaundice suggested a disorder of bile ducts or pancreas.

Differential diagnosis

Pancreatic adenocarcinoma, papillary neoplasm, neuroendocrine tumor, lymphoma.

Laboratory diagnosis

Abnormally increased levels of amylase, lipase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, total bilirubin, and direct bilirubin.

Imaging diagnosis

Gastrointestinal ultrasonography showed a mass involving the head of the pancreas and Wirsung duct dilation. An abdominal computed tomography (CT) with contrast agent showed an irregular pancreatic mass involving the head of the pancreas, with dilation of the intrahepatic and the common bile ducts.

Pathologic diagnosis

Percutaneous biopsy of the pancreatic mass showed a lymphomatous infiltrate consistent with metastatic localization of MF.

Treatment

The patient was subjected to palliative endoscopic retrograde cholangiopancreatography.

Related reports

Extracutaneous localizations of MF are rare and occur in long-standing MF with lymph node involvement. The literature reports involvement of lungs, spleen, liver, kidney, thyroid gland, bone marrow, oral cavity, larynx, heart, breast, central nervous system, esophagus, stomach, small intestine, as well as pancreas (two cases).

Term explanation

MF is the most common form of cutaneous T-cell lymphoma and accounts for almost 50% of all primary cutaneous lymphomas. Primary pancreatic tumors may involve the exocrine or the endocrine portion.

Experience and lessons

This case posed a clinical diagnostic challenge between pancreatic primitive tumor and a pancreatic metastasis from MF. A diagnostic clue came from the combined histomorphologic and immunohistochemical study of pancreatic and cutaneous biopsies, which revealed a pancreatic localization of MF.

Peer-review

This manuscript described a rare case with the diagnosis of pancreatic metastasis from MF. It further provides interesting study on the mechanism.

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CASE REPORT

Ileus caused by cholesterol crystal embolization: A case report

Shunjiro Azuma, Maiko Ikenouchi, Takuji Akamatsu, Takeshi Seta, Shunji Urai, Yoshito Uenoyama, Yukitaka Yamashita

Shunjiro Azuma, Maiko Ikenouchi, Takuji Akamatsu, Takeshi Seta, Shunji Urai, Yoshito Uenoyama, Yukitaka Yamashita, Department of Gastroenterology, Japan Red Cross Society Wakayama Medical Center, Wakayama-city, Wakayama 640-8558, Japan

Author contributions: Azuma S designed the report; Azuma S, Ikenouchi M, Akamatsu T, Seta T, Urai S, Uenoyama Y and Yamashita Y were the attending doctors; and Azuma S wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Japan Red Cross Society Wakayama Medical Center Institutional Review Board.

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Correspondence to: Shunjiro Azuma, MD, Department of Gastroenterology, Japan Red Cross Society Wakayama Medical Center, 4-20 Komatsubaradori, Wakayama-city, Wakayama 640-8558, Japan. aaazzzuuummmaaa1984@gmail.com
Telephone: +81-73-4224171
Fax: +81-73-4261168

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Abstract

Cholesterol crystal embolization (CCE) is a rare systemic embolism caused by formation of cholesterol crystals from atherosclerotic plaques. CCE usually occurs during vascular manipulation, such as vascular surgery or endovascular catheter manipulation, or due to anticoagulation or thrombolytic therapy. We report a rare case of intestinal obstruction caused by spontaneous CCE. An 81-year-old man with a history of hypertension was admitted for complaints of abdominal pain, bloating, and anorexia persisting for 4 mo. An abdominal computed tomography revealed intestinal ileus. His symptoms were immediately relieved by an ileus tube insertion, and he was discharged 6 d later. However, these symptoms immediately reappeared and persisted, and partial resection of the small intestine was performed. A histopathological examination indicated that small intestine obstruction was caused by CCE. At the 12-mo follow-up, the patient showed no evidence of CCE recurrence. Thus, in cases of intestinal obstruction, CCE should also be considered.

Key words: Cholesterol crystal embolization; Ileus; Intestinal obstruction; Vascular manipulation; Catheter manipulation

Core tip: Cholesterol crystal embolization (CCE) is a rare systemic embolism caused by formation of cholesterol crystals from atherosclerotic plaques. This case report details a rare case that was not only a spontaneous occurrence but also caused a focal intestinal lesion.

CCE usually carries a serious prognosis, but this case had a good prognosis.

Azuma S, Ikenouchi M, Akamatsu T, Seta T, Urai S, Uenoyama Y, Yamashita Y. Ileus caused by cholesterol crystal embolization: A case report. *World J Gastroenterol* 2016; 22(12): 3502-3505 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3502.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3502

INTRODUCTION

Cholesterol crystal embolization (CCE) is a rare systemic embolism caused by occlusion of the peripheral arteries by cholesterol crystals from atherosclerotic plaques and may show multiorgan involvement, leading to renal failure, cutaneous manifestations, and gastrointestinal disease. CCE is usually an iatrogenic event occurring either after vascular surgery or endovascular catheter manipulations, or in the course of anticoagulation or thrombolytic therapy. Here, we report a rare case of intestinal obstruction caused by CCE in the absence of any causative medication. To our knowledge, this is the first reported case of single intestinal obstruction caused by CCE.

CASE REPORT

An 81-year-old man with a clinical history of hypertension, benign prostatic hyperplasia, pulmonary tuberculosis, and an oropharyngeal benign tumor was admitted to the hospital for complaints of abdominal pain, bloating, and anorexia persisting for the past 4 mo. Physical examination revealed severe abdominal distension. Chemical laboratory examination showed a slightly elevated white blood cell count (9100/μL; normal, 4000-7000/μL), a high serum creatinine level (1.21 mg/dL; normal, 0.60-1.10 mg/dL), and a low eosinophil value (1.3%; normal, 2.0%-4.0%). An abdominal computed tomography scan revealed focal thickening of the wall of the small intestine, intestinal dilatation of the oral intestine, and intraperitoneal lymphadenopathy (Figure 1). We diagnosed his condition as small intestinal obstruction and prioritized conservative treatment. We promptly inserted an ileus tube, owing to which, the abdominal symptoms alleviated rapidly. Contrast-enhanced imaging of the ileus tube showed that the tube had advanced beyond the lesion site on the third day of admission, and therefore, the patient was discharged on the sixth day of admission. However, his symptoms reappeared immediately after discharge, and he was readmitted to the hospital. An abdominal computed tomography scan at this point revealed focal thickening of the wall of the small intestine, similar to that seen earlier.

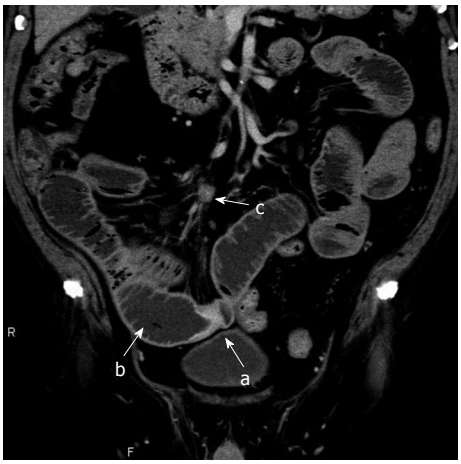

Figure 1 Abdominal computed tomography-scan showed focal thickening of the wall of the small intestine (a), **intestinal dilatation of the oral intestine** (b), **and intraperitoneal lymphadenopathy** (c).

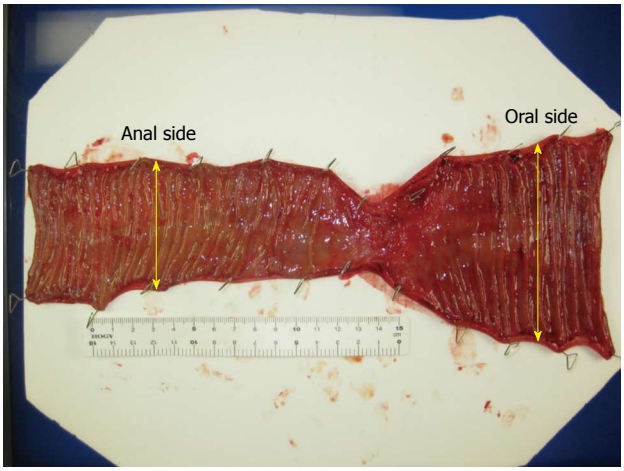

Figure 2 Resected specimen showed a 5-6 cm tumor. The oral side lumen was 1.5-fold bigger lumen than the anal side.

A double-balloon endoscopy of the small intestine was considered at this point; however, it was not performed owing to the presence of severe abdominal bloating. Ultimately, surgery was performed considering the possibility of small intestine cancer. Laparotomy showed a 5-6 cm tumor that had formed at a distance of 220 cm from the Treitz ligament and 180 cm from the terminal ileum. The small intestine has a 1.5-fold bigger lumen on the oral side than on the anal side. The tumor was resected from both the oral and anus sides to ensure a margin of 10 cm. We performed the vertical incision at the mesenteric contralateral anal side of the small intestine and the end-to-end anastomosis. On naked-eye observation of the sample, a narrowing of the intestinal tract caliber of 5-6 cm was observed, and the intestinal wall was found to be slightly thickened (Figure 2). Histologically, we found thickened portions in the wall, muscle plate thickening, fat reduction, fibrosis, and cholesterol clefts, all of which indicated that needle-like cholesterol

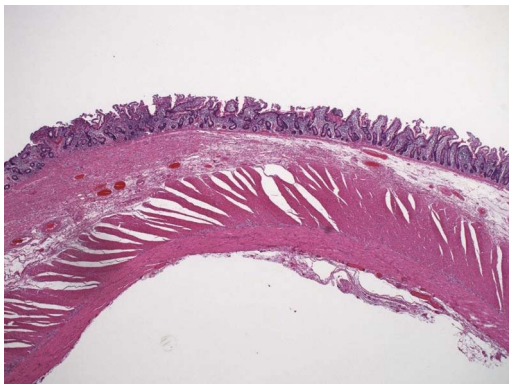

Figure 3 Histologically, we found thickened portions in the wall, muscle plate thickening, fat reduction, fibrosis. The left half of the specimen is lesion area and the right half is normal area (HE × 10).

thrombi were present in the small arteries of the lumen (Figure 3). Further, giant cells were observed in the vicinity, which could have developed due to a foreign-body reaction (Figure 4).

There was no relapse after surgery, and the patient was discharged 8 d later. Up to the last follow-up at the 12 mo after discharge, the patient showed no recurrence.

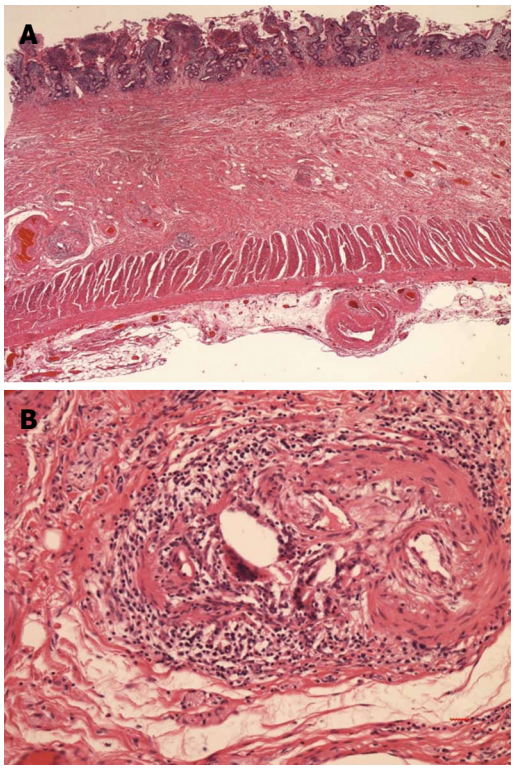

Figure 4 Cholesterol clefts were detected in the thickened portions in the wall. A: × 20; B: × 200.

DISCUSSION

CCE was first reported by Peter Ludvig Panum in 1862 and is a rare systemic embolism caused by cholesterol crystals from atherosclerotic plaques. CCE may manifest as a systemic disease, characterized by constitutional symptoms (fever, myalgia, and anemia) and clinical signs due to single or multiorgan involvement (renal failure, gastrointestinal ischemia, central nervous system infarcts, or retinal disturbances)[1]. The risk factors are male sex, age > 60 years, diabetes mellitus, hypertension, atherosclerotic vascular disease, and ischemic nephropathy[2]. Approximately 80% of the cases of CCE reported thus far have occurred because of catheter manipulation and anticoagulation therapy[3]. The present patient had no history of endovascular catheter manipulation, anticoagulation, or thrombolytic therapy, and his symptoms were caused by an embolism. The symptoms could vary according the amount and range of embolization, from acute symptoms caused by arterial embolization to chronic symptoms caused by foreign body reaction to cholesterol crystals. There is no established treatment for CCE[3]. In addition, high-pressure therapy, low-density cholesterol apheresis, steroids, and plasma exchange have been reported as causes of CCE. According to Scolari *et al*[4] following the kidneys and skin, the digestive system is most susceptible to CCE.

With respect to lesions in the gastrointestinal tract, lesions occur most frequently the large intestine, followed by the small intestine, and finally the stomach[5]. The characteristics of the lesions vary in terms of redness, erosion, ulcer, stricture, obstruction, and symptoms caused such as abdominal pain, diarrhea, and gastrointestinal bleeding[6,7]. Such lesions may also lead to perforation by intestinal ischemia[8]. In our case, the patient was not under any type of treatment including anti-thrombotic therapy and vascular catheterization. Since the arteriosclerosis was very pronounced, it may have been a risk factor, along with hypertension, for CCE. The lesion was confined to one area of the small intestine, and atherosclerosis was observed in the large blood vessels in the vicinity. Thus, the cholesterol emboli could have led to vessel occlusion. Embolism caused by cholesterol crystals differs from thrombosis in that the former does not usually completely occlude the vessel. Since an embolism caused gradual ischemic changes, it may have caused advanced fibrosis, which could further lead to intestinal stenosis. Although this condition has a poor prognosis[9], at present, the current patient is healthy without recurrence.

In conclusion, intestinal obstruction caused by spontaneous CCE is very rare. Although CCE usually has a serious prognosis, this patient is presently healthy without recurrence. In cases of unexplained intestinal obstruction, it may be useful to think of CCE as a potential differential diagnosis.

COMMENTS

Case characteristics

An 81-year-old man with a clinical history of hypertension, benign prostatic

hyperplasia, pulmonary tuberculosis, and an oropharyngeal benign tumor complained abdominal pain, bloating, and anorexia.

Clinical diagnosis
Physical examination revealed severe abdominal distension.

Differential diagnosis
Intestinal obstruction, ascites, peritonitis, Intra-abdominal tumor.

Laboratory diagnosis
Inflammatory response and renal dysfunction were showed.

Imaging diagnosis
An abdominal computed tomography revealed focal thickening of the wall of the small intestine, intestinal dilatation of the oral intestine, and intraperitoneal lymphadenopathy.

Pathological diagnosis
Cholesterol clefts in small intestine.

Treatment
Complete surgical excision of lesion.

Related reports
Cholesterol crystal embolization (CCE) is a rare systemic embolism caused by occlusion of the peripheral arteries by cholesterol crystals from atherosclerotic plaques.

Term explanation
CCE is a rare systemic embolism caused by formation of cholesterol crystals from atherosclerotic plaques. Cases were confined to the gastrointestinal tract is little.

Experiences and lessons
Although the main cause of intestinal obstruction is adhesion or tumor, in cases of unexplained intestinal obstruction, it may be useful to think of CCE as a potential differential diagnosis.

Peer-review
In their work, the Authors describe a rare case of intestinal obstruction caused by spontaneous CCE. This rare form of systemic embolism may attract the attention of the readers. The work is well written with a good iconography.

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CASE REPORT

Primary splenic angiosarcoma with liver metastasis: A case report and literature review

Kai-Feng Yang, Yong Li, De-Long Wang, Jun-Wu Yang, Sen-Yan Wu, Wei-Dong Xiao

Kai-Feng Yang, Yong Li, De-Long Wang, Jun-Wu Yang, Sen-Yan Wu, Wei-Dong Xiao, Department of General Surgery, The First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Author contributions: Yang KF and Li Y designed the report; Li Y, Wang DL, Wu SY and Xiao WD had the surgery; Yang JW has helped the bibliography search; Yang KF has done the data collection and then finished the report.

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Institutional review board statement: The research was verified by the First Affiliated Hospital of Nanchang University Institutional Review Board, Nanchang, China.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors announced that there is no conflict of interest exists in manuscript, included personal, political, and others.

Correspondence to: Yong Li, MD, Professor, Department of General Surgery, The First Affiliated Hospital of Nanchang University, No. 17, Yongwai Zhengjie, Nanchang 330006, Jiangxi Province, China. yfyly@163.com
Telephone: +86-791-88694131
Fax: +86-791-88694131

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Abstract

Primary splenic angiosarcoma (PSA) is an unusual and highly malignant vascular tumour with a high rate of metastatic. Moreover, the research on prognosis of the disease is poor. The epidemiology, etiology, clinical diagnosis and treatment of the disease remain challenging, because case reports of the disease are few in number. In accordance with other malignant tumors, PSA is very aggressive, and the majority of patients in which this disease is found are at an advanced stage. Almost all patients die within 12 mo of diagnosis irrespective of treatment. We report here a woman who had complained of upper bellyache and anorexia for 10 d. Magnetic resonance imaging showed enlargement of the spleen with multiple heterogeneous masses in the lower pole of the spleen. A hand-assisted laparoscopic splenectomy was performed which allowed histopathologic diagnosis. The patient was diagnosed with PSA and liver metastasis, and succumbed to the disease 35 d after surgery. The literature was finished combined with the clinical features, diagnosis and management of PSA.

Key words: Angiosarcoma; Immunohistochemistry; Spleen; Splenectomy; Metastasis

Core tip: Primary splenic angiosarcoma (PSA) is an unusual tumor originating from the blood vessel. To date, very few cases of PSA have been reported. We report a woman who had PSA after splenectomy, and liver metastasis was also detected. The patient

died 35 d after surgery. We review the literature and conclude that early diagnosis followed by splenectomy is beneficial for better survival of the patients.

Yang KF, Li Y, Wang DL, Yang JW, Wu SY, Xiao WD. Primary splenic angiosarcoma with liver metastasis: A case report and literature review. *World J Gastroenterol* 2016; 22(12): 3506-3510 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i12/3506.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i12.3506

INTRODUCTION

Primary splenic angiosarcoma (PSA) is an unusual and highly malignant non-hematolymphoid tumor of the spleen. It was described for the first time by Theodor Langhans in 1879[1], and the incidence of SPA has been reported between 0.15 and 0.26 per million people[2,3]. Although it occurs primarily in older patients, individuals of any age can be affected[2,4-8], from 14 mo to 89 years. It has a slight gender difference, such that the ratio of rates in males and females is 4:3[3]. We report a 46-year-old female PSA patient with hepatic metastasis. In addition, we summarize the features and outcomes of PSA according to the clinical characteristics.

CASE REPORT

A woman was referred to our hospital from a local clinic on April 16, 2015, complaining of intermittent upper abdominal pain and anorexia for 10 d. Following the occurrence of these symptoms, the patient reported no weight loss. We did not know the medical history of the patience except for laparoscopic resection of an ovarian cyst, which was performed in Fuzhou People's Hospital (Jiangxi, China) 10 years previously. The patient's family number was very important, since the patient's mother and father had all died of cancer. No abnormalities were observed during physical examination. Laboratory examinations on admission revealed: white blood cell (WBC) count of 5.41 × 10^9/L (normal range 4-10 × 10^9/L), hemoglobin 75 g/L (normal range 110-150 g/L), platelet count 151 × 10^9/L (normal range 100-300 × 10^9/L), alanine aminotransferase 49 U/L (normal range 5-35 U/L), aspartate transaminase 48 U/L (normal range 5-40 U/L), γ-glutamyl transpeptidase 87 U/L (normal range ⩽ 32 U/L), and albumin 29.6 g/L (normal range 34-48 g/L). Serum tumor markers, including α-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and carbohydrate-125 (CA-125), were all within normal ranges. A coagulation function test was performed and no positive results were observed. Magnetic resonance imaging (MRI) of the abdomen has shown a well circumscribed heterogeneous mass of 7.8 cm × 5.7 cm and multiple nodules in the liver parenchyma (Figure 1). So the diagnosis of the primary splenic malignancy with hepatic metastasis was suggested based on imaging findings. A hand-assisted laparoscopic splenectomy was then performed not only for the purpose of curing the disease, but also for histopathologic diagnosis. In this process, the patient had received 2 units of fresh frozen plasma and frozen plasma, 3 units of blood cells and 6 units of cryoprecipitate. Intraoperatively, we observed a scleroid tumor mass 6 cm × 5 cm in size in the lower pole of the spleen. A cross-section of the specimen demonstrated a well-circumscribed grayish yellow lesion. In accordance with the imaging findings, laparotomy revealed micrometastases in the liver. Thus, a histopathologic biopsy was carried out. The pathologic diagnosis of the excised spleen was originating from the spleen (Figure 2) and liver metastasis, which were confirmed by pathologic examination of the biopsied specimen. Immunohistochemical analysis showed that the tumor cells were positive for CD31+++ (Figure 3A), CD34+++ (Figure 3B), FVIII (Figure 3C), Ki-67 + 30% (Figure 3D), and were negative for CD68, cytokeratin, epithelial membrane antigen, desmin, actin, smooth muscle actin, and lysozyme. The patient leave the hospital after 15 d with no complications, but succumbed to the disease 35 d after surgery.

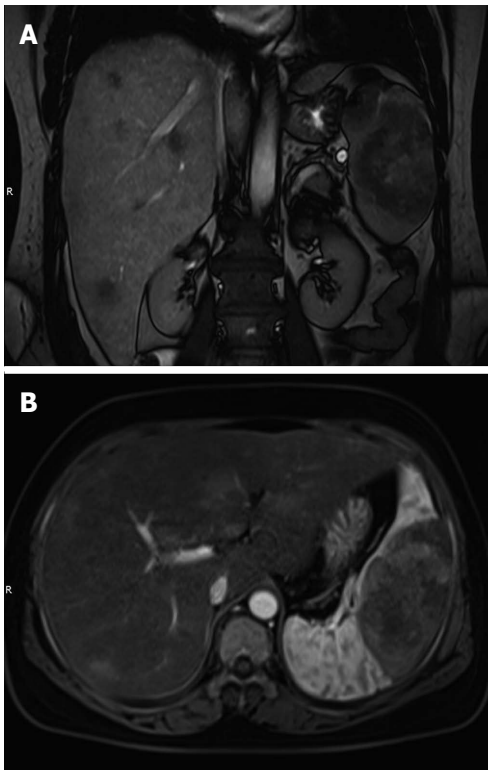

Figure 1 Magnetic resonance imaging. A: Coronal T2-weighted image revealed multiple nodular lesions in the liver parenchyma; B: Contrast-enhanced magnetic resonance imaging showed marked heterogeneous enhancement in the splenic mass, and multiple high signal intensity lesions in the liver.

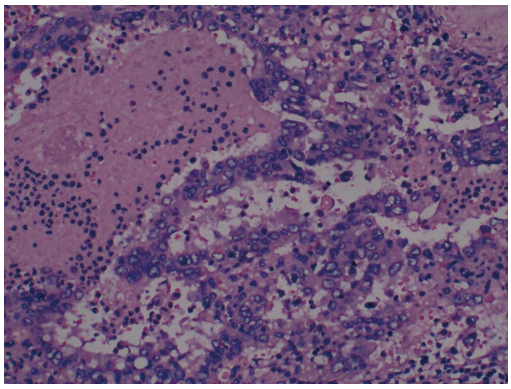

Figure 2 Histopathologic findings of the splenic specimen. Clusters of spindle tumor cells infiltrating the spleen parenchyma (HE stain; magnification × 200).

DISCUSSION

Although the spleen play an important role in our life and it is also an important immune organ, it rarely reported as the beginning of tumors. Primary malignant tumors of the spleen are extremely rare and include lymphoma, reticulum cell sarcoma, fibrosarcoma, and angiosarcoma which is also recognized as malignant hemagioendothelioma. It is reported that PSA originates from immature endothelial-type cells, the immunohistochemical techniques has confirmed it[9]. PSA is a very aggressive neoplasm, it could last 4.4-14 mo[2,3]. In the majority of patients with PSA, distant metastasis is present at the time of laparotomy, with the most common sites has occurred in lungs, bones, lymph nodes, adrenal glands and others[2,3,5]. The findings in our case were consistent with these results, as liver metastasis was identified.

The pathogenesis of this highly aggressive malignancy remains unclear. Possible causative factors include ionizing radiation, arsenic, vinyl chloride and chemotherapy for lymphoma[10,11]. However, some reports indicate that angiosarcoma of the spleen develops from previously existing benign tumors, such as hemangioma or hemangioendothelioma[4,6]. None of these factors were involved in our case. Delacruz *et al*[12] reported a 69-year-old woman who presented with splenic angiosarcoma during the follow-up for a history of liposarcoma of the right buttock. Three years prior to the patient's admission, she had received neoadjuvant chemotherapy for the liposarcoma. In addition, a remarkable family medical history was noted for a sister with angiosarcoma of the spleen and her mother with breast cancer. Our patient's family history was significant for the death of her mother due to gastric cancer and the death of her father due to lung cancer. With the information provided, we made an assumption that family history may play a significant role in the pathogenesis of PSA.

The clinical manifestations of PSA vary significantly. Upper abdominal pain is the most common manifestation[3]. Other symptoms include weakness or fatigue, shortness of breath, fever, chest pain, weight loss and anorexia[3,13]. A minority of patients are asymptomatic and PSA is discovered incidentally. In severe cases, patients may develop spontaneous splenic rupture which is one of the most severe complications of this disease with a reported incidence of up to 25%[3]. However, it has been reported that splenic rupture was not combined with the clinical outcome[3]. As we all know, a total of 17 cases of splenic rupture secondary to angiosarcoma had been reported until February 2015. All of these cases had undergone splenectomy, and postoperative survival ranged from 1 d to 7 mo. When do the physical examination, the disease could also presented with the splenomegaly identified, and quadrant abdominal mass[14].

Laboratory findings described combined with the disease include anemia, thrombocytopenia, leukocytosis, and an elevated erythrocyte sedimentation rate[2,3]. None of these abnormalities was present in our patient, with the exception of anemia. Tumor markers (AFP, CEA, CA-125 and CA19-9) are always within normal ranges or only mildly elevated.

While there is still a lack of standardization, the majority of the diagnosis of angiosarcoma is suggested at the imaging of the patients[14]. The most common ultrasonographic findings are represented by splenomegaly, ill-defined solid and others[15,16]. Computed tomography could show the enlarged spleen with solitary or multiple nodular masses of heterogeneous low attenuation. Some of these masses has shown the peripheral enhancement, and the margins of the lesions could not see clearly[13,16,17]. On MRI, areas of increased and decreased signal intensity may be seen on images obtained with both T1- and T2-weighted pulse sequences, and contrast-enhanced MRI reveals heterogeneous enhancement within the tumor[18,19]. Low-signal-intensity areas on MRI probably represent siderotic nodules[20]. In our case, only MRI was performed. Nonspecific clinical presentation and laboratory test results emphasize the essential role of imaging in the diagnosis of PSA.

The therapeutic strategies for PSA are limited as the disease is extremely rare and has highly aggressive characteristics. Splenectomy is the predominant treatment method for PSA without metastasis. Chemotherapy and radiotherapy have also been reported[21,22]. However, the role of these adjuvant therapies still remain to be defined. In addition to patients with distant metastasis, splenectomy is suggested for pediatric angiosarcoma of the spleen. Due to the immune system of infants is not developed very well, so that a complete splenectomy may have negative effects on system. A complete splenectomy may increases postoperative risks, including the possibility of overwhelming post-splenectomy infection (OPSI). OPSI occurs at an estimated incidence of 0.23%-0.42% per year, with a mortality of 38%-69%[23]. Splenic rupture may occur in PSA, thus early diagnosis of PSA

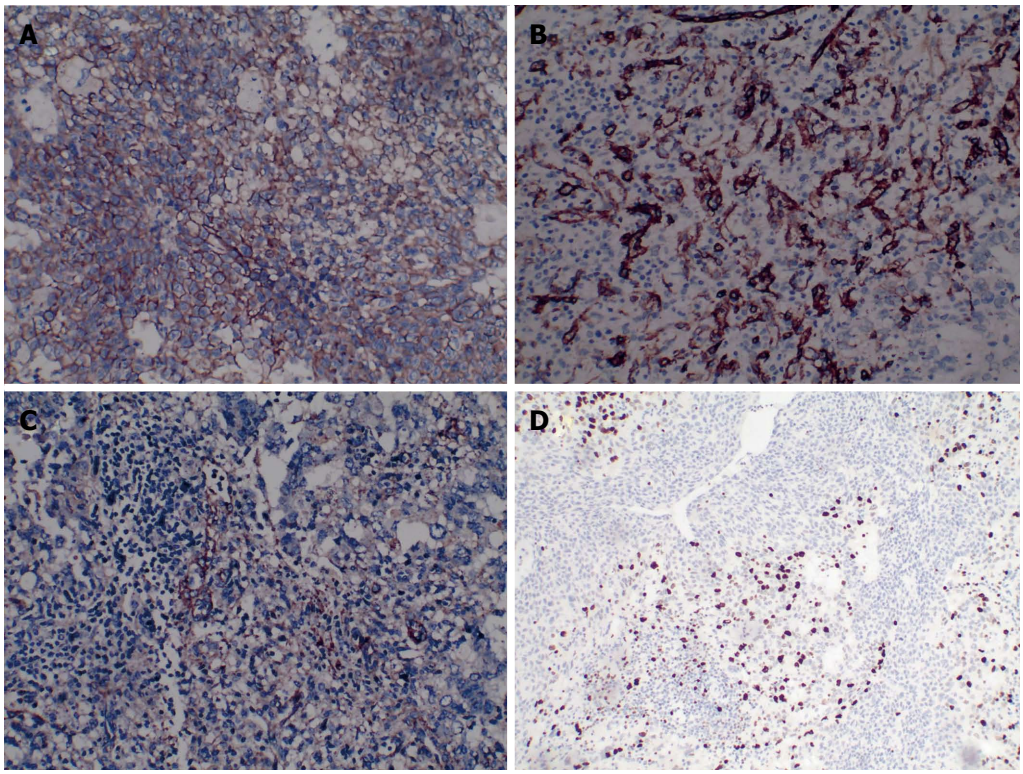

Figure 3 Immunohistochemical examination of the splenic specimen. A: Specimen stained positive for CD31 (magnification × 200); B: Specimen stained positive for CD34 (magnification × 200); C: Specimen stained positive for FVIII (magnification × 200); D: Ki-67 proliferation index less than 30% (magnification × 100).

followed by splenectomy before rupture may yield a more favorable survival rate[2,24]. Montemayor *et al*[25] found that patients with splenic angiosarcoma had a longer survival time if splenectomy was performed prior to rupture rather than after rupture (14.4 mo *vs* 4.4 mo).

Similar to other aggressive neoplasms, PSA is a malignancy with a poor prognosis. It was reported by Neuhauser *et al*[3] that PSA has a survival of 5 mo, irrespective of the type of treatment administered. We report a case of a 46-year-old woman with PSA and liver metastasis, who died 35 d after surgery. This case report together with the literature review of previous cases provide an alert for clinicians that PSA should be considered when the clinical presentation includes symptoms such as upper abdominal pain, hematology abnormalities (anemia, leukocytosis, thrombocytopenia, and/or elevated erythrocyte sedimentation rate). In addition, imaging examination is essential for early diagnosis of PSA. Considering that early diagnosis followed by splenectomy shows an advantage in terms of survival rate, early diagnosis should be a hot topic in the treatment of this disease.

COMMENTS

Case characteristics

A 46-year-old woman with a history of laparoscopic resection of an ovarian cyst presented with upper abdominal pain and anorexia for 10 d.

Clinical diagnosis

Intermittent upper abdominal pain and anorexia may be nonspecific symptoms. No abnormalities were found during physical examination.

Differential diagnosis

Differential diagnosis included hemangioma, littoral cell angioma, lymphangioma and hemangiopericytoma.

Laboratory diagnosis

White blood cell count 5.41 × 10^9/L, hemoglobin 75 g/L, platelet count 151 × 10^9/L, alanine aminotransferase 49 U/L, aspartate transaminase 48 U/L, γ-glutamyl transpeptidase 87 U/L, and albumin 29.6 g/L. Serum tumor markers were within normal ranges.

Imaging diagnosis

Magnetic resonance imaging of the abdomen revealed an enlarged spleen with a well circumscribed heterogeneous mass measuring 7.8 cm × 5.7 cm and multiple nodules in the liver parenchyma.

Pathological diagnosis

Histopathologic diagnosis was angiosarcoma originating from the spleen, and liver metastasis. Splenic tumor cells stained positive for CD31, CD34+++, FVIII, Vimentin+++, and Ki-67 + 30%.

Treatment

A hand-assisted laparoscopic splenectomy was performed not only for the purpose of curing the disease, but also for histopathologic diagnosis.

Related reports

There are few reports of liver metastasis from splenic angiosarcoma in the literature. However, the liver is the most frequently reported organ in terms of

distant metastasis with an incidence of 70%. The etiology of Primary splenic angiosarcoma (PSA) has not been clearly elucidated. Median survival has been reported to be 12 mo.

Term explanation

PSA is a rare splenic tumor of significant malignancy, with a reported incidence between 0.14 and 0.25 per million persons.

Experiences and lessons

Etiology, clinical diagnosis and treatment of the disease remain challenging. Early diagnosis followed by splenectomy result in a favorable survival rate.

Peer-review

The reviewer agrees with the authors that early diagnosis followed by splenectomy, it is beneficial for better survival of the patients because only histopathological examination is essential for diagnosis. Although primary splenic angiosarcoma is quite widely featured, there are two reasons why the article should be published: (1) the manuscript is very well prepared; and (2) it is necessary to remind us about this type of neoplasm.

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