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EDITORIAL

Impact of homeobox genes in gastrointestinal cancer

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Abstract

Homeobox genes, including *HOX* and non-*HOX* genes, have been identified to be expressed aberrantly in solid tumors. In gastrointestinal (GI) cancers, most studies have focused on the function of non-*HOX* genes including caudal-related homeobox transcription factor 1 (CDX1) and CDX2. CDX2 is a crucial factor in the development of pre-cancerous lesions such as Barrett's esophagus or intestinal metaplasia in the stomach, and its tumor suppressive role has been investigated in colorectal cancers. Recently, several *HOX* genes were reported to have specific roles in GI cancers; for example, *HOXA13* in esophageal squamous cell cancer and *HOXB7* in stomach and colorectal cancers. HOXD10 is upregulated in colorectal cancer while it is silenced epigenetically in gastric cancer. Thus, it is essential to examine the differential expression pattern of various homeobox genes in specific tumor types or cell lineages, and understand their underlying mechanisms. In this review, we summarize the available research on homeobox genes and present their potential value for the prediction of prognosis in GI cancers.

Key words: Homeobox genes; *HOX* genes; Caudal-related homeobox transcription factor 2; Gastrointestinal cancers; *HOXB7*

Core tip: Aberrant up- or downregulation of homeobox genes may play pivotal roles in the development and progression of gastrointestinal (GI) cancers. Core research in GI cancers has focused on non-HOX genes including caudal-related homeobox transcription factor 2. However, recent studies have demonstrated significant functions of specific *HOX* genes, including *HOXB7*, *HOXA13*, and *HOXD10*, in GI cancers. Here, we review the major research data concerning the deregulation of homeobox genes in GI cancers and their underlying mechanisms.

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INTRODUCTION

The homeobox genes were first discovered in *Drosophila melanogaster* where their mutation led to malformations of body parts[1]. As the name implies, homeobox genes play crucial roles in the development of the embryo along the anterior-posterior axis. The human genome contains about 235 functional homeobox genes, most of which are dispersed throughout the genome and contain a highly conserved 180 nucleotide sequence (homeobox) encoding 60 amino acids along the DNA-binding protein domain (homeodomain)[2]. A typical characteristic of the homeodomain is its DNA-binding nature; it functions as a transcription factor by binding to the promoter of various target genes. Several cofactors, such as pre-B-cell leukemia transcription factor 2 (PBX2) or myeloid ecotropic viral integration site (MEIS), interact with homeobox genes to form a protein complex and facilitate the specificity and stability of homeobox genes by binding to promoter DNA[3].

Homeobox genes are generally classified as class Ⅰ (*HOX*) and class Ⅱ (non-*HOX*). In humans, 39 *HOX* genes have been identified. They cluster into 4 groups named A, B, C, and D, located in 7p15.3, 17q21.3, 12q13.1, and 2q31, respectively[4]. Each HOX gene in a cluster is arranged from the 3′ to 5′ end and named from 1 to 13. HOX genes located at the 3′ end are expressed early in development and in anterior tissues, while HOX genes at the 5′ end are expressed later and in posterior tissues[5].

Numerous studies have revealed that various homeobox genes have either tumor-suppressive or tumor-promoting effects according to their aberrant expression patterns in certain organs. In terms of their oncogenetic properties, homeobox genes are normally expressed during the embryonic period and are reactivated in tumors, while being downregulated in normal differentiated adult tissues. In contrast, certain homeobox genes are expressed in normal differentiated adult tissues, but are downregulated in tumors[1]. This aberrant reduced or enhanced expression of homeobox genes is regulated by several mechanisms, such as loss of heterozygosity, gene amplification, CpG island promoter hypermethylation, or histone deacetylation, and consequently contributes to the development and progression of cancer.

Interestingly, a homeobox gene may have both tumor-promoting and tumor-suppressing properties depending on the specific organs or cell lineages where it is expressed. For example, HOXA9 is downregulated in lung cancer tissues compared to that in surrounding non-cancerous tissues by an epigenetic silencing mechanism, whereas it is upregulated in acute lymphocystic leukemia[6,7]. HOXB13 is another example that is upregulated in breast cancer but downregulated in prostate cancer compared to surrounding normal tissues[8,9]. Several long and short non-coding RNAs are also involved in the regulation of transcription or expression of homeobox genes. For example, *HOX* transcript antisense intergenic RNA (HOTAIR), a long non-coding RNA, is located in the *HOXC* locus near the 5′ end, and recruits polycomb repressive complex 2 to lead epigenetic silencing of the *HOXD* locus[10]. MicroRNAs (miRNAs), including miR-10a, miR-10b, miR-196a, and miR-196b, are also located within the HOX clusters and target multiple *HOX* genes to regulate their expression post-transcriptionally[4]. Therefore, it is important to understand the aberrant expression pattern of homeobox genes in specific cancer types or cell lineages, and their underlying mechanisms for carcinogenesis and invasion of certain types of cancer.

In this editorial, we summarize the outcomes of previous studies of homeobox genes that showed valid influences on solid tumors in the gastrointestinal (GI) tract, including esophageal, gastric, and colorectal cancers (CRCs). This article provides information on the underlying molecular mechanisms, aberrant expression in GI cancer tissues, and the potential value of various homeobox genes for early recognition or prediction of prognosis in GI cancers.

ESOPHAGEAL CANCER

Most studies of homeobox genes in Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) have focused on non-*HOX* genes, especially caudal-related homeobox transcription factor 2 (CDX2). Generally, acid and bile reflux at the esophagogastric junction promotes dedifferentiation of the basal layer of the esophageal squamous epithelium. This is where secretion of CDX2 is increased, and morphogenic and metaplastic changes occur, eventually leading to the development of intestinal-type squamous to columnar metaplasia[11]. Indeed, CDX2 plays a crucial role in the development of BE, a major precursor of EAC. In addition, several previous studies showed that mRNA and protein expression of CDX2 was increased significantly in BE and EAC compared to normal esophageal tissues, although no significant difference could be found between BE and EAC[12,13]. The expression of CDX2 protein was well-conserved in an EAC cell line, but was not detected in esophageal squamous cell carcinoma (ESCC) cells. Furthermore, demethylation or exposure of esophageal squamous epithelial cells to acid or bile induced CDX2 as well as other intestinal markers. These findings suggested that CDX2 is a key modulator of intestinal metaplasia of esophageal squamous cells in response to acid or

Table 1 Aberrant expression of *HOX* and non-*HOX* genes in esophageal cancer

Homeobox gene	Change	Underlying mechanism	Ref.
BE/EAC			
CDX2	↑ in BE/EAC No difference between BE and EAC	Concomitant decrease of PITX1 Association with β-catenin	[12,13]
HOXB5, B6, B7	↑ in BE/dysplasia/EAC	Induction of intestinal markers such as KRT20, Muc2 and villin	[15]
ESCC			
CDX2	↓ in a ESCC cell line and tissues	Promoter hypermethylation	[18]
MEIS	↓ in ESCC, inversely related with nodal status and high tumor stage	Concomitant increase of SOX2	[24]
HOXA7, A9, C6	↑ in ESCC	Not presented	[16]
HOXA5, A10, B13, C6, C10, C13, D3	↑ in BE/EAC, highest in T2 stage	Not presented	[17]
HOXA13	↑ in ESCC, associated with OS and DFS	Targeting annexinA2, MnSOD, ERAB	[19-21]
HOXB7	↑ in ESCC, associated with T/N stage and DFS	Not presented	[23]

BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; MnSOD: Manganese superoxide dismutase; OS: Overall survival; DFS: Disease free survival.

bile reflux[14]. In terms of *HOX* genes, a previous well-designed study showed that mid-cluster *HOXB* genes (*HOXB5*, *B6*, and *B7*) were upregulated in BE tissue as well as in dysplasia and EAC. However, no significant difference was observed between BE with dysplasia and EAC. Furthermore, these mid-cluster *HOXB* genes induced several intestinal markers including KRT20, Muc2, and villin in esophageal cells in a CDX2-independent manner[15].

A previous study using the reverse transcriptase-polymerase chain reaction (RT-PCR) showed that *HOXA7*, *A9*, and *C6* mRNAs were overexpressed significantly in ESCC tissues compared to non-cancerous surrounding tissues[16]. A microarray study showed that the mRNA expression of several *HOX* genes, including *HOXA5, A10, B13, C6, C10, C13*, and *D3*, was upregulated significantly in ESCC tissues compared to normal esophageal mucosa, and these genes were differentially expressed according to the T stage; expression was the highest in T2[17]. This study also showed that several non-*HOX* genes, including *CDX1* and *CDX2*, were expressed at higher levels in ESCC than normal esophageal mucosa. However, another crucial study demonstrated that most of the expression of CDX2 in ESCC cell lines and tissues was governed by an epigenetic silencing mechanism that was not found in EAC, CRC, or normal esophageal tissues[18]. This suggested that aberrant inactivation of CDX2 is an important step toward the development of ESCC.

Among the *HOX* genes, *HOXA13* has been most actively investigated in ESCC. A previous pivotal study very nicely showed the tumorigenic effect of HOXA13 *in vivo,* and that there was a significant association between HOXA13 and both median and disease-free survival[19]. Chen and his colleagues, using knockdown of HOXA13 in ESCC cell lines and 2-dimmensional electrophoresis, suggested that annexinA2, manganese superoxide dismutase (MnSOD) and endoplasmic reticulum-associated amyloid β-binding protein (ERAB) were crucial target genes of HOXA13[20]. These researchers also used ESCC tissues to show that co-expression of HOXA13 with annexinA2 and SOD was significantly associated with poor prognosis[21]. Other *HOX* genes, such as *HOXA9* and *B7*, were also upregulated in ESCC at advanced T or N stages, and in patients with poor prognosis[22,23]. Meanwhile, a recent study demonstrated that *MEIS1*, a non-*HOX* homeobox gene, was downregulated in ESCC patients and was associated inversely with advanced TNM stage. The mechanism was thought to be mediated by upregulation of SRY (sex determining region Y)-box 2 (SOX2) in ESCC cells[24] (Table 1).

STOMACH CANCER

The most extensively researched homeobox genes in stomach cancer are *CDX2* and *CDX1*. These genes are closely involved in the development of intestinal metaplasia of the gastric mucosa. A previous pivotal study demonstrated the causal role of CDX2 in the development of intestinal metaplasia in the stomach by using a *Cdx2*-expressing transgenic mouse model[25]. The *Cdx1* transgenic mouse also exhibited significant intestinal metaplasia, although the characteristics were somewhat different from the *Cdx2* transgenic mouse; the former replaced the gastric mucosa with intestinal metaplasia involving all four intestinal epithelial cell types (absorptive enterocytes, goblet, enteroendocrine, and Paneth cells), whereas only pseudopyloric gland metaplasia was observed in the *Cdx2* transgenic mouse[26]. This phenomenon suggested that a different mechanism and roles between CDX1 and CDX2 may exist in the differentiation of intestinal metaplasia.

In human stomach, ectopic expression of CDX1 and CDX2 was observed frequently in intestinal metaplasia tissues. However, only CDX2 was an independent factor of intestinal type gastric adenocarcinoma[27].

Another study showed that the expression of CDX2 in gastric cancer was governed mainly by promoter hypermethylation. This suggested that aberrant downregulation of CDX2 might promote gastric carcinogenesis[28]. Liu *et al*[29] suggested that CDX2 was associated mainly with the formation of intestinal metaplasia of gastric mucosa, and was less involved in dysplasia and cancer, by demonstrating that the expression of CDX2 protein was highest in complete type intestinal metaplasia, followed by incomplete intestinal metaplasia, dysplasia, and the lowest in gastric cancer tissues. The exact molecular characteristics of CDX2 in the development of intestinal metaplasia and gastric cancer should be further evaluated. Indeed, the unique characteristics of CDX2 are associated with both oncogenic and tumor-suppressive functions, and these ambivalent roles of CDX2 might be tissue- or site-specific. At present, CDX2 appears to be involved in the initiation of the process leading to intestinal type gastric neoplasia such as induction of intestinal metaplasia[30].

Several other non-*HOX* homeobox genes, including intestine-specific homeobox (*ISX*), prospero homeobox 1 (*PROX1*), paired-related homeobox 1 (*PRRX1*), iroquois-class homeodomain (*IRX1*), and pancreatic-duodenal homeobox 1 (*PDX1*), have been investigated for their relationship with intestinal metaplasia and gastric cancer. Among these genes, *ISX*, *PROX1*, and *PRRX1* were associated with the promotion of gastric cancer, suggesting their oncogenetic roles[31-33]. Specifically, ISX was upregulated in intestinal metaplasia and its levels correlated significantly with CDX2 expression in mice with chronic *Helicobacter felis* infections. However, ISX also enhanced cyclin D1 (a G1 → S cell cycle modulator) and CD44 (a stem cell marker of gastric cancer) expression, and its protein expression was increased significantly in undifferentiated-type gastric cancer, unlike CDX2[31]. PROX1 promoted cellular proliferation, angiogenesis, and epithelial-mesenchymal transition (EMT) *in vitro*. Furthermore, its tissue expression was significantly associated with advanced stage, undifferentiated type, lymph node metastasis, and poor prognosis[32]. PRRX1 also showed EMT-promoting functions *via* inducing the Wnt/β-catenin pathway, and was significantly associated with advanced-stage and distant metastasis[33]. In contrast, several *in vitro* studies showed that the expression of IRX1 and PDX1 mRNA was downregulated in gastric cancer cells by an epigenetic silencing mechanism *via* promoter hypermethylation, suggesting their tumor-suppressive functions[34-36]. Another study demonstrated that PDX1 expression was associated with the pseudopyloric gland of intestinal metaplasia tissues, and was decreased in patients with advanced stage and lymph node metastasis, compared to early stage gastric cancer[37]. However, a few studies demonstrated a significant relationship between various non-*HOX* homeobox genes and clinicopathological parameters such as TNM stage, differentiation, overall and disease-free survival rate of gastric cancer patients. The nature of this relationship requires further study.

Recently, investigations into the role of *HOX* genes in gastric carcinogenesis and progression have been performed. One notable study used microarray analysis to reveal the global expression patterns of 39 human *HOX* genes among 12 pairs of gastric cancer and non-cancerous tissues. The authors showed that the expression of *HOXA1, A4, A10, A13, B7*, and *C10* was increased significantly in cancer tissues. Among these genes, upregulation of HOXA13 was associated significantly with T stage, M stage, advanced UICC stage, histologic differentiation and relapse. Furthermore, patient with positive HOXA13 expression had a lower overall survival and disease-free survival compared with patients with negative HOXA13 expression. The contribution of HOXA13 towards tumorigenesis and aggressive biologic behavior in gastric cancer might be associated with downregulation of tumor growth factor-β (TGF-β) and its downstream target of Runt-related transcription factor 3 by antagonizing Smad3[38]. Concurrent researches on individual *HOX* genes in gastric cancer have been conducted. An *in vitro* study showed that *HOXB5* promoted migration and invasion of gastric cancer cells by binding directly to the *CTNNB1* promoter and thus activating the Wnt/β-catenin signaling pathway[39]. Another pivotal study showed that HOXD10 mRNA expression was downregulated significantly in stomach cancer tissues compared to normal surrounding tissues. This downregulation was caused by promoter hypermethylation, and the aberrant reduction of HOXD10 expression led to proliferation, migration, invasion, and tumorigenesis in gastric cancer cells[40]. We reported recently that *HOXB7*, one of the most widely investigated oncogenic *HOX* genes, was highly expressed in primary or metastatic gastric cancer tissues compared to chronic gastritis or intestinal metaplasia tissues. This suggested that HOXB7 might be involved in the progression rather than initiation process of gastric cancer[41]. This phenomenon has been validated by *in vitro* studies showing that overexpression of HOXB7 in gastric cancer cells promoted cellular invasion and migration, and inhibited apoptosis, whereas silencing HOXB7 showed the opposite effects[41,42].

The main target of HOXB7, and the mechanism involved in the upregulation of HOXB7 in cancer, are still under controversy. We suggested that HOXB7 regulates Akt/PTEN signaling to induce migration and invasion of gastric cancer cells, by using transient transfection of a HOXB7-expressing plasmid and HOXB7 siRNA. A recent well-designed study demonstrated that HOXB7 promoted the EMT and invasiveness of breast cancer

Table 2 Aberrant expression of *HOX* and non-*HOX* genes in gastric cancer

Homeobox gene	Change	Underlying mechanism	Ref.
CDX2	↑ in complete IM > incomplete IM > dysplasia > GC Associated with differentiated type GC	Promoter hypermethylation in GC Decreased intake of green tea or cruciferous vegetables	[27-29]
ISX	↑ in IM and GC Upregulated in undifferentiated type GC	Increase of cyclin D1 and CD44	[31]
PROX1	↑ in GC Associated with undifferentiated type, advanced stage and poor OS	Inhibition of apoptosis, promoting lymphangiogenesis and angiogenesis	[32]
PRRX1	↑ in GC Associated with advanced stage and distant metastasis	Induction of Wnt/β-catenin	[33]
IRX1	↓ in GC	Promoter hypermethylation	[35]
PDX1	↓ in GC ↑ in pseudopyloric gland IM Inversely related with advanced T/ N stage and undifferentiated type GC	Promoter hypermethylation, histone hypoacetylation	[34,36,37]
HOXA13	↑ in GC Associated with advanced TNM stage, undifferentiated type and poor response to chemotherapy	Not presented	[38]
HOXB5	↑ in GC	Upregulation of β-catenin	[39]
HOXB7	↑ in primary or metastatic cancer than chronic gastritis or IM Associated with advanced TNM stage and undifferentiated type GC	Modulation of PI3K/Akt/PTEN axis	[41,42]
HOXD10	↓ in GC	Promoter hypermethylation Induction of IGFBP3	[40]

Note: PROX1, PRRX1, HOXA13 and HOXB7 are associated with advanced TNM stage, while PDX1 is inversely associated; ISX, PROX1, HOXA13 and HOXB7 are associated with undifferentiated type GC. IM: Intestinal metaplasia; GC: Gastric cancer; OS: Overall survival; PI3K: Phosphatidylinositol-3 kinase; IGFBP3: Insulin like growth factor binding protein 3.

cells by regulating the TGF β2-SMAD3 axis[43]. Several receptor tyrosine kinase signaling pathways, including beta fibroblast growth factor and epidermal growth factor receptor, were also reported to be activated by HOXB7 in breast cancer cells[44-46]. Thus, HOXB7 might be simultaneously involved in various key molecular signaling pathways involving cancer progression, which supports the potential value of HOXB7 as a promising therapeutic target. Several miRNAs, including miR-196a and miR-196b, were suggested as key regulators of HOXB7 expression in other types of cancer[47,48]. Further investigations to reveal the mechanisms underlying the induction of HOXB7 and its targets in gastric cancer are needed (Table 2).

CRC

Similar to esophageal and gastric cancer, *HOX* and non-*HOX* homeobox genes have been investigated for their unique roles in the development and progression of CRC. Among these genes, CDX2 in colon cancer cells has been researched extensively and reported to regulate the expression of cell junctional proteins. These proteins include liver-intestine cadherin (LI-cadherin)[49] or protocadherin Mucdhl[50]. Loss of CDX2 in colon cancer cells downregulated Mucdhl, thereby eliminating the latter's inhibition of Wnt/β-catenin activity. Inflammatory cytokines, such as tumor necrosis factor-α, mediated this process (loss of CDX2 and induction of Wnt/β-catenin signaling) in CaCo2 colon cancer cells[51]. Furthermore, significant tumor formation was observed when heterozygous *Cdx2*$^{+/-}$, but not wild-type mice, were treated with the DNA mutagen azosymethane. This indicated that CDX2 had a tumor-suppressive function in CRC[52]. At the tissue level, reduced expression of CDX2 in colorectal adenoma or cancer was associated significantly with right side tumors, poorly differentiated or high-grade carcinomas, advanced stage, poor prognosis, CpG island methylator phenotype, and mismatch repair-deficient tumors[53-55].

Previous pivotal studies showed that CDX1 inhibited the proliferation of colon cancer cells by regulating the cyclin D1 or β-catenin/T-cell factor (TCF) pathways[56,57], and that tissue expression of CDX1 was increased significantly in adenomatous polyps but abolished in adenocarcinomas. Furthermore, a novel *in vitro* study showed that CDX1 was governed by miR-215 to promote differentiation and inhibit stemness in colon cancer cells[58]. These data suggested that CDX1 might play a crucial role in the transformation of benign adenomas to malignant tumors.

Other types of non-*HOX* homeobox genes have been investigated for their roles in CRC. The expression of the

Table 3 Aberrant expression of *HOX* and non-*HOX* genes in colorectal cancer

Homeobox gene	Change	Underlying mechanism	Ref.
CDX1	↑ in adenomatous polyp, ↓ in CRC	Regulation of cyclin D1 and β-catenin/TCF pathway Regulated by miR-215	[55,56,58]
CDX2	↓ in adenoma and CRC Inversely associated with right side tumor, poorly differentiated type, advanced stage, poor prognosis, CIMP, MMR-deficient tumor	Loss of Mucdhl Induction of Wnt/β-catenin axis	[50-55]
ALX4	↓ in dysplasia and CRC	Promoter hypermethylation	[59]
PROX1	↑ in CRC Associated with advanced stage and lymph node metastasis	Induction of β-catenin/TCF axis Inhibition of E-cadherin activity	[60,61]
HOXA4, D10	↑ in CRC	Stem cell overpopulation and crypt renewal	[63]
HOXA5, A9, A10, C6	Proximal colon tumor > distal colon tumor	Not presented	[64]
HOXB13	Distal colon tumor > proximal colon tumor	Not presented	[64]
HOXB7	↑ in CRC Associated with advanced stage, T stage, distant metastasis and poor OS	Activation of PI3K/Akt and MAPK pathways	[67]

CRC: Colorectal cancer; TCF: β-catenin/T-cell factor; CIMP: CpG island methylation phenotype; OS: Overall survival; PI3K: Phosphatidylinositol-3 kinase; MAPK: Mitogen-activated protein kinase.

airstaless-like homeobox-4 gene (ALX4) was aberrantly reduced in colorectal dysplasia or adenocarcinoma compared with normal colonic mucosa, through DNA methylation[59]. In addition, PROX1 promoted neoplastic transformation, tumorigenesis, and the EMT *via* induction of the β-catenin/TCF pathway and inhibition of E-cadherin activity[60,61].

Relatively few data concerning *HOX* genes have been presented in terms of CRC compared to other GI cancers. A previous quantitative RT-PCR study showed that the expression of several *HOX* genes, including *HOXA9, B3, B8*, and *B9*, was increased significantly in left side colon cancer tissues compared to surrounding normal tissues. In contrast, the expression of *HOXB2, B13, D1, D3, D4, D8*, and *D12* was significantly decreased[62]. A recent gene microarray and immunohistochemical study showed that the expression of HOXA4 and HOXD10 was significantly increased in CRC tissues compared to that in normal tissues. Furthermore, the expression of these genes was clustered in the crypt bottom rather than the top or middle of the crypt where the stem cell niche was overpopulated[63]. Remarkably, *HOX* genes showed a tendency to be differentially expressed in colon tumors according to their location. Specifically, several *HOX* genes, including *HOXA5, A9, A10*, and *C6*, were expressed at higher levels in the proximal colon, and gradually decreased in the distal colon and rectum. *HOXB13* was an exception that showed the opposite pattern[64]. Previous studies showed that expression pattern of HOXB13 was site-specific, which was mainly confined to prostate, rectum and distal colon[65], and HOXB13 inhibited the β-catenin/TCF signaling pathway as post-translational manner, which was downregulated in colorectal tumors[66].

A previous pivotal study demonstrated the prognostic value of HOXB7 in CRC. Patients in the high HOXB7 CRC group had a poorer prognosis than those in the low HOXB7 group. In addition, the tumorigenic and anti-apoptotic effects of HOXB7 in colon cancer cells were mediated by the phosphatidylinositol-3-kinase/Akt and mitogen-activated protein kinase pathways[67] (Table 3).

CONCLUSION

Several homeobox genes are expressed aberrantly in various types of cancers, and the GI tract is no exception. Previous studies focused mainly on the roles of non-*HOX* genes, such as *CDX1* and *CDX2*, in GI cancers. Recently, several *HOX* genes have been investigated and shown to have specific roles in the development and invasion of GI cancers (Figure 1). However, intensive understanding of the underlying mechanisms including their transcriptional target genes, and co-factors or downstream effectors of homeobox genes in GI cancers are still lacking. Moreover, current knowledge of the homeobox genes in GI cancer could not reach the clinical efficacy of therapeutic targets or biomarkers, which need to be fulfilled in the future research.

Recent studies demonstrated the significant contribution of several *HOX* genes to chemoresistance. For example, downregulation of HOXA1 under regulation of HOTAIR or miR-100 enhance chemoresistance in pancreas cancer and small cell lung cancer[68,69]. An improved understanding of the mechanism of this effect may reveal a means to create tailored, precision medicine of GI cancers. Meanwhile, the regulation of homeobox genes by several non-coding RNAs, including miRNAs, may provide a means to restore the aberrant expression of homeobox genes in GI cancers. Finally, the differential expression pattern of homeobox genes in various cancers may provide valuable information for

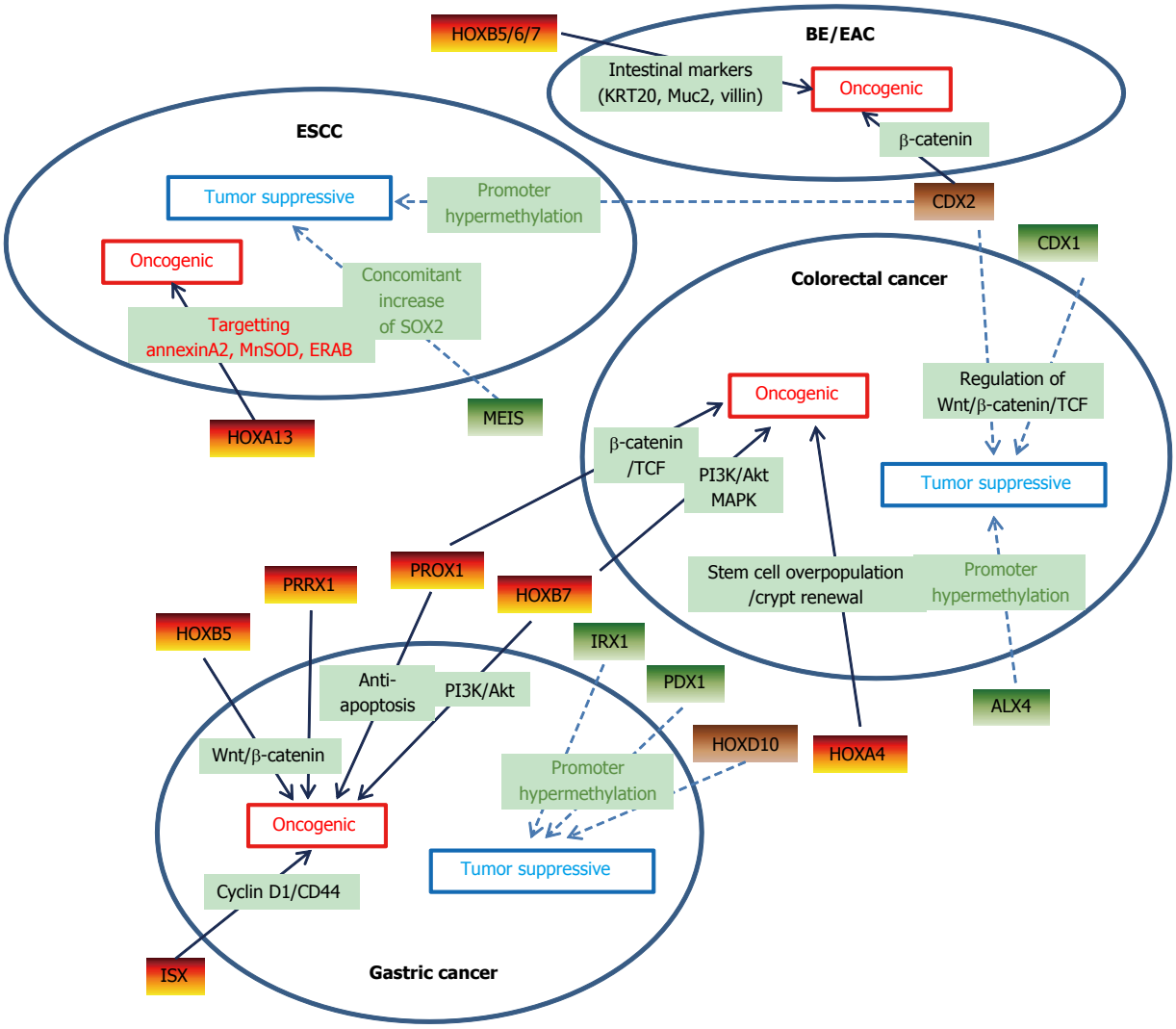

Figure 1 Schematic diagram of homeobox genes which have diverse effects on gastrointestinal cancers. Solid arrow indicates upregulated homeogox genes and dashed arrow indicates downregulated ones. Note that CDX2 shows tumor suppressive function in colorectal and esophageal squamous cell cancer whereas oncogenic effect on the formation of Barrett's esophagus and esophageal adenocarcinoma. HOXD10 also shows dual function, which has tumor suppressive effect on gastric cancer whereas oncogenic effect on colorectal cancer. BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma.

the diagnosis of challenging cases of GI tumors.

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REVIEW

Review of endoscopic radiofrequency in biliopancreatic tumours with emphasis on clinical benefits, controversies and safety

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Abstract

Most pancreatic cancers and extrahepatic cholangiocarcinomas are unresectable at the time of diagnosis, and even in case of a resectable cancer, for elderly or patients with coexistent comorbidities, surgery is not an option. Current treatment alternatives in these scenarios are very limited. Biliary stenting with self-expanding metal stents (SEMS) is the mainstay palliative treatment of biliary obstruction due to unresectable pancreatic cancer or cholangiocarcinoma. Nevertheless, more than 50% of SEMS become occluded after 6 mo due to tumour over- and ingrowth, leading to hospital readmissions and reinterventions that significantly impair quality of life. Regimes of chemotherapy or chemoradiotherapy also provide minimal survival benefits. Therefore, novel therapies are eagerly awaited. Radiofrequency (RF) energy causes coagulative necrosis leading to local destruction of the accessed malignant tissue and has an established role in the treatment of malignancies in several solid organs, especially liver cancers. However, pancreatic and extrahepatic biliary cancers are not easily accessed by a percutaneous route, making the procedure dangerous. Over the past five years, the development of dedicated devices compatible with endoscopic instruments has offered a minimally invasive option for RF energy delivery in biliopancreatic cancers. Emerging experience with endoscopic RF ablation (RFA) in this setting has been reported in the literature, but little is known about its feasibility, efficacy and safety. A literature review makes it clear that RFA in biliopancreatic tumours is feasible with high rates of technical success and acceptable safety profile. Although available data suggest a benefit of survival with RFA, there is not enough evidence to draw a firm conclusion about its efficacy. For this reason, prospective randomized trials comparing RFA with standard palliative treatments with quality-of-life and survival endpoints are required. Anecdotal reports

have also highlighted a potential curative role of RFA in small pancreatic tumours and benign conditions, such as ductal extension of ampullomas, intrahepatic adenomas or non-tumoural biliary strictures. These newest indications also deserve further examination in larger series of studies.

Key words: Radiofrequency ablation; Pancreatic tumour; Endobiliary radiofrequency; Cholangiocarcinoma; Biliary stricture

Core tip: Most pancreatic cancers and extrahepatic cholangiocarcinomas are unresectable at the time of diagnosis. Radiofrequency (RF) energy causes coagulative necrosis leading to local destruction of the accessed malignant tissue. Endoscopic RF has emerged as a novel ablative therapy. In the present study, we aim to review general principles and technical aspects and to evaluate clinical benefits and complications of endoscopy-guided RF in biliary and pancreatic indications based on recent literature.

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INTRODUCTION

Extrahepatic cholangiocarcinomas (CC), including hilar tumours, and pancreatic carcinomas (PC) are aggressive cancers often discovered at an advanced stage for curative surgical resection. Less than 20% of PC and 30% of CC are resectable at the time of diagnosis; moreover, surgery is not always an option in patients with poor functional status or coexisting comorbidities[1-4]. Chemotherapy and radiotherapy provide minimal survival benefits in patients with unresectable locally advanced pancreatic or biliary cancer, and the average survival is measured in months rather than years[5,6]. The need for novel therapies to positively impact survival has led to the development of a variety of local ablative methods, among which radiofrequency (RF) has generated wide interest after its first application for hepatic tumours in the early 1990s[7-9].

RF has been widely used percutaneously or intraoperatively in malignancies of several solid organs, such as the liver, breast, lung and kidney[10]. However, PC is not usually amenable to percutaneous RF treatment because of the difficult visualization of these deeper tumours with the risk of thermal injury of the adjacent duodenum and blood vessels. Intraoperative RF ablation (RFA) provides better visualization and the ability to manipulate nearby structures, but many patients with biliopancreatic cancers are also unfit for surgery. Percutaneous endobiliary RF of extrahepatic CC has been shown to be successful, but percutaneous transhepatic bile duct access is an invasive technique; therefore, endoscopic bile duct access by endoscopic retrograde cholangiopancreatography (ERCP) is usually favoured over the percutaneous approach[11,12]. The development of new over-the-wire and flexible RF probes that can be placed down the working channel of an endoscope or through an endoscopic ultrasonography (EUS) needle has allowed for a minimally invasive approach for delivering RF under endoscopic guidance in pancreatic and extrahepatic biliary cancers[13,14]. Nevertheless, data on safety and efficacy are scarce. In the present study, we aim to review general principles and technical aspects and to evaluate clinical benefits and complications of endoscopy-guided RF in biliary and pancreatic indications based on recent literature.

GENERAL RF PRINCIPLES, LIMITATIONS AND COMPLICATIONS

RFA creates an electrical circuit, either through the body with monopolar probes, between an electrode positioned in the tumour and a grounding pad placed on the skin, or between two interstitial electrodes with bipolar catheters, by using an alternating current with a frequency in the range of radio waves (400-500 kHz). Ions within the tissue try to follow the alternating path of the current, and thus, current flowing through the tissues leads to ion agitation and subsequent frictional heat. Friction heats the surrounding tissues to 50-100 ℃ causing protein denaturation followed by cell dehydration and coagulative necrosis[10,15,16]. Because of the poor electrical conductivity of tissues, the closest areas to the electrode experience the highest current and temperature, whereas tissues farther away are heated by thermal conduction; in these regions, the heat may not be sufficiently high to cause necrosis[15].

Therefore, a key limitation is the extent of coagulation produced by RF, which is often insufficient to cover the tumour volume[16]. This deficiency is mainly related to the physical consequences of RF. During the desiccation induced by RF, tissues become dehydrated and charred with the loss of ions. Then, current stops leading to a rise in impedance, which limits the volume of tissue successfully ablated[10,15-17]. This roll-off phenomenon may be reduced by using pulsed RF, which allows for the tissue to cool and rehydrate between pulses, with a decrease in impedance enabling larger volumes of thermal destruction. Another strategy involves the use of internally cooled electrodes by circulating water that increases the temperature at the interface tissue electrode, limiting the charring

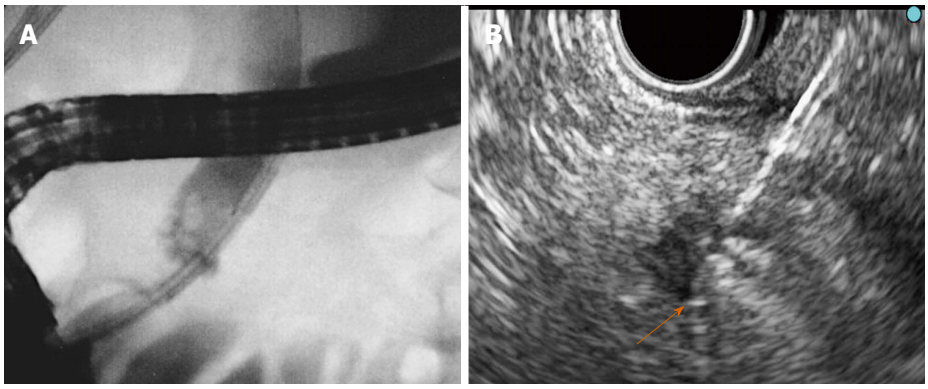

Figure 1 Endobiliary and pancreatic radiofrequency ablation. A: Fluoroscopy: The Habib TM EndoHBP inside the common bile duct; B: EUS: The EUSRA RF electrode (STARmed) inserted in a PNET. Tip of the probe (orange arrow). EUS: Endoscopic ultrasonography; RF: Radiofrequency.

process and allowing for longer lasting current flow[10,16]. However, the extent of thermal injury is also dependent on the length and gauge of the electrode, the temperature generated and the length of RF. The selection of the correct settings in the generator, the optimal application time and the development of bipolar probes and multiple hooked electrodes in an array are other approaches for optimizing the tumour volume ablated[16]. Another cause of incomplete tumour ablation is the heat-sink effect created by the proximity of large vessels to tumours. Vascular flow may dissipate heat and cool the adjacent tissues, preventing the required temperature from being attained, which is particularly important when considering PC[15,16].

In addition to thermal injury, recent studies in animal models have suggested that RF may stimulate systemic antitumor immunity, which acts synergistically in subsequent tumour eradication. RFA generates large amounts of cellular debris that results in increased dendritic cell infiltration, inducing tumour-specific T-cell responses[18]. Another plausible mechanism by which an anti-tumour immunity response can be triggered is the induction of heat shock protein (HSP) expression because hyperthermia has been reported to enhance the immunogenicity of cancer cells concomitantly with the expression of HSP[19].

There are two different categories of complications resulting from RF. The first category corresponds to complications related to thermal therapy and includes a flu-like syndrome that is usually resolved within the first 24 h, post-procedure pain, skin burns at the grounding pad site and thermal injury of surrounding structures[10,20]. During RFA with monopolar probes, a similar amount of energy is generated at the ground and at the electrode surface. The surface area, orientation and material of the pad as well as the electrode to pad distance affect the grounding pad temperature. With small grounding pad areas and high-current RF, deleterious heating effects may be observed at the ground site. Second- and third-degree skin burns are now uncommon owing to the use of large-area foil pads oriented to maximize the leading edge of the ground to safely dissipate heat. The risk of skin burns is also obviated using bipolar probes. Structures adjacent to a tumour may become irreversibly coagulated during the procedure[16,20]. The damage of vessels and the gastrointestinal wall with secondary perforation are the most feared complications. These complications may be avoided by maintaining a 1 cm separation between these structures and the targeted tumour. However, vascular damage is not as common as anticipated due to the protective effect of the heat-sink phenomenon. The second category of complications includes those related to electrode placement[20]. The complications consist mainly of bleeding, infection and tumour seeding and depend on the access route and technique applied to the targeted organ (ERCP or EUS-FNA in the case of CC and PC, respectively).

RADIOFREQUENCY DEVICES FOR ENDOSCOPY-GUIDED THERAPY IN BILIOPANCREATIC INDICATIONS

Six different RF probes have been developed that enable endoscopic RF in the pancreas and the bile duct. Two of them are designed to be used over a guide wire during ERCP for biliary strictures (Habib™ EndoHBP and ELRA™), and the other four are used under EUS guidance for pancreatic tumours (Habib™ EUS RFA, Cryotherm probe, EUSRA RF electrode and a 19-gauge EUS-FNA needle) (Figure 1). Among them, the 19-gauge EUS-FNA needle has been used only in liver procedures on animal models, the Habib™ EUS RFA and the EUSRA RF electrodes are monopolar probes and the other three are bipolar. A cooling system is available only with the cryotherm probe, the ELRA™, and the EUSRA RF electrode. Other technical characteristics are presented more extensively elsewhere[21-26].

ENDOSCOPY-GUIDED RF PROCEDURE

RF ablation of pancreatic tumours is performed by

using a convex linear-array echoendoscope. When using the cryotherm or the EUSRA RF electrodes, the probe is passed through the operative channel of the echoendoscope and directly inserted into the target mass. However, with the Habib™ EUS RFA, a 19-gauge EUS needle is first placed into the mass, positioning the needle tip at the far end of the mass. The stylet is then removed, and the RF probe is introduced through the needle. Finally, the needle is withdrawn by 3 cm to avoid direct contact between the metallic needle and the active electrode[27]. Regardless of what needle is used, real-time Doppler imaging is helpful to avoid major vessel injury. The ablation must start at the far end of the lesion, and for large lesions the probe is repositioned along the same trajectory or using a fanning technique to ablate the entire lesion[25,28].

For biliary RF, the biliary tract is cannulated by conventional ERCP, and biliary tree opacification is performed to clearly determine the location of the stricture and to delineate its length and diameter. Although not necessary, a sphincterotomy is usually performed. Depending on the stricture diameter, balloon dilation of the stricture may be required before inserting the RF catheter. The probe is then introduced over the guide until the stricture is reached[29,30]. RF energy is delivered over the selected period, and before moving the probe, a rest period of 1 min is observed to prevent tissues from adhering to the electrodes. Based on the stricture length, several RF applications are performed during the same session, from the proximal margin of the stricture to the distal one, with minimal overlap to reduce the risk of complications. In patients with Klatskin tumours, RF is also applied to more than one stricture (common bile duct, left and/or right hepatic ducts) in the same session. After withdrawing the probe, coagulated tissue debris are removed with balloon sweeps, and a plastic or metal stent is placed to ensure biliary drainage[29,30].

The selected power and time settings in endoscopy-guided biliopancreatic RF vary among the different probes and are those recommended by manufacturers based on the results of preclinical studies in animals and *ex vivo* human studies

ENDOSCOPIC BILIOPANCREATIC RF IN CLINICAL PRACTICE: REVIEW OF LITERATURE

To evaluate the feasibility, clinical efficacy and safety of endoscopy-guided biliopancreatic RF, an electronic search was performed in Pubmed and Embase. The review was restricted to English literature published up to March 2016. The search terms used were "pancreatic cancer" or "pancreatic tumour" or "cholangiocarcinoma" or "biliary cancer" or "biliary stricture" and "radiofrequency ablation". The reference list of published articles was hand-searched to select original studies reporting on endoscopic RF for biliopancreatic cancers in humans. Because of the paucity of reports on this subject, we aimed to consider all types of evidence available. For this reason, we also included case reports and relevant abstracts reporting on technical feasibility, clinical outcomes or complications of endoscopy-guided biliopancreatic RF. Studies whose patients were included in further larger series were not considered. Indications, technical details, technical success, clinical outcomes, impact on survival, complications and mortality were extracted and further discussed.

EUS-guided RF on pancreatic tumours

Results: A literature search using the terms "pancreatic cancer" or "pancreatic tumour" and "RF ablation" yielded 276 relevant articles from the Pubmed and Embase databases. Of these articles, only seven were suitable and corresponded to four prospective studies, one case series of three patients and two case reports[25,27,28,31-34]. Data extracted from these articles are summarized in Table 1.

Overall, 42 patients underwent EUS-guided RFA, and indications were advanced unresectable PC in 28 patients (2 in the uncinated process, 20 in the head, and 6 in the tail), PNET in 7, mucinous cysts in 4, IPMN in 2 and microcystic adenoma in 1. All patients with a resectable tumour were either unfit for surgery or refused surgery. Among patients with PNET, three corresponded to symptomatic insulinomas (hypoglycemia with recurrent episodes of seizures or syncope and frequent eating with significant weight gain)[27]. One patient with IPMN presented recurrent tumour bleeding through the ampulla[34]. Technical success was achieved in 36 patients (86%), but in 6 patients it was not possible to introduce the CTP inside the tumour. The proposed explanation was that the stiffness due to the duodenal infiltration and desmoplastic reaction prevented the probe insertion[32]. The required number of RF sessions was not specifically reported in the majority of studies. The selected power and application time varied widely, ranging from 5 to 50 W and from 10 to 360 s, respectively, primarily depending on the tumour size.

Following RF treatment, the four symptomatic tumours became asymptomatic. No further bleeding occurred in the patient with IPMN during 10 wk of follow-up. Biochemical improvement was observed in the first 48 h after RF in the three insulinomas, and these patients remained free of symptoms during a 12-mo follow-up[25]. A favourable response was observed in the remaining PNETs, either with complete ablation estimated at 1-month image exam in one case or with a vascularity change with central necrosis in the other two PNETs[27,31,33]. Two mucinous cysts had complete resolution, and the volumes of the other four cysts were nearly halved (48% reduction in volume)[27]. One study in patients with unresectable PC focused on the feasibility and safety of EUS-guided

Table 1 Endoscopic ultrasonography-guided radiofrequency ablation on pancreatic tumours

Ref.	*n*	Indication	Mean size mm (range)	RF device	Thermokinetics	RF sessions	Outcome	Survival (range)	Complications
Armellini *et al*[31], 2005	1	PNET	20	18 G Needle electrode (STARmed)	NA	NA	Complete ablation	-	No complication
Arcidiacono *et al*[32], 2012	22	Locally advanced PC	36 (23-54)	CTP	18 W (heating) 650 psi (cooling) 107 (10-360) s	NA	Significant volume reduction in 16 patients (P = 0.07) Technical failure in 6 patients	6 mo[1] (1-12)	Early: 3 transient abdominal pain 1 minor duodenal bleeding Late: 2 jaundice 1 duodenal stricture 1 cystic fluid collection
Rossi *et al*[33], 2014	1	PNET	9	Habib EUS RFA	10-15 W 360 s	1	Complete thermal ablation No recurrence (34 mo follow-up)	-	No complication
Weigt *et al*[34], 2014	1	IPMN (recurrent bleeding)	10	Habib EndoHBP	8 W 90 s	NA	2 cm ablation No rebleeding (10 wk follow-up)	-	Mild acute pancreatitis
Pai *et al*[27], 2015	8	Mucinous cyst (4) IPMN (1) Microcystic adenoma (1) PNET (2)	41 (24-70) 35 20 27 (15-40)	Habib EUS RFA	5-25 W 90-120 s	4.5 (2-7)	2 cyst resolution 4 cyst reduction (48 % reduction) 2 PNET with vascularity change	-	2 mild abdominal pain
Lakhtakia *et al*[25], 2015	3	Insulinoma (Hypoglycemia)	18 (14-22)	18 G Needle electrode (STARmed)	50 W 10-15 s	NA	No recurrent hypoglycemia (12 mo follow-up)	-	No complication
Song *et al*[28], 2016	6	Locally advanced PC (4) Metastatic PC (2)	38 (30-90)	18 G Needle electrode (STARmed)	20-50 W 10 s	1.3 (1-2)	Necrosis at the ablation site	NA	2 mild abdominal pain

[1]Among 13 patients; 2 patients were lost to follow-up and other patient died during hospitalization. PNET: Pancreatic neuroendocrine tumour; NA: Not available; PC: Pancreatic cancer; IPMN: Intraductal pancreatic mucinous neoplasm.

RF, and therefore, survival, the main outcome in this group, was not evaluable[28]. Two of sixteen patients were lost to follow-up, and another one died during hospitalization in another study involving patients with locally advanced PC. The median survival of the remaining 13 patients was 6 mo[32].

There was no procedure-related mortality, and no patient required surgery. Mild early complications were observed in 9 of 36 patients (25%) with successful RF treatment. The most frequent complication, observed in seven patients, was mild abdominal pain that lasted 24 h after treatment and responded to common analgesics. There was one case of mild acute pancreatitis and one case of duodenal bleeding treated endoscopically without the need for blood transfusion[32,34]. One patient had a cystic fluid collection between the pancreas and the left hepatic lobe as a late complication. The collection was asymptomatic and resolved spontaneously[32].

Discussion: Current experience, although preliminary, demonstrates that EUS-guided RF is a feasible treatment. However, technical failure occurred in six cases (14%) when using a cryotherm probe. Results of recent studies involving percutaneous and intraoperative RF suggest that pancreatic RF may be dangerous without additional cooling because of the risk of unintended thermal injury of surrounding structures[35]. The CTP for EUS-guided RF that incorporates a cooling system cannot be inserted through a EUS needle because of its larger diameter and, therefore, it is introduced directly inside the echo endoscope channel. This characteristic, along with the flexible nature of this probe, makes it difficult to enter a hard tumour with a desmoplastic reaction. Technological improvements providing these probes with cutting current, like a needle-knife, or rendering the probes thin enough to be inserted through a EUS

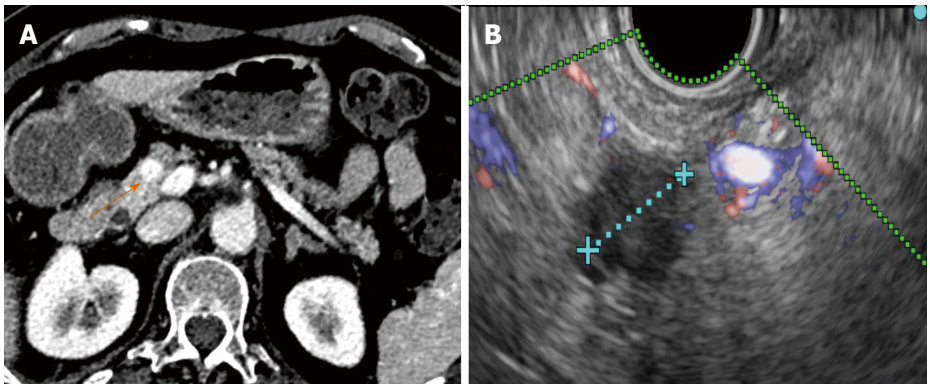

Figure 2 Ten millimeter pancreatic neuroendocrine tumour before radiofrequency ablation. Chromogranin at diagnosis: 239 ng/mL. A: The corresponding CT image (orange arrow); B: The corresponding Doppler endoscopic ultrasonography image.

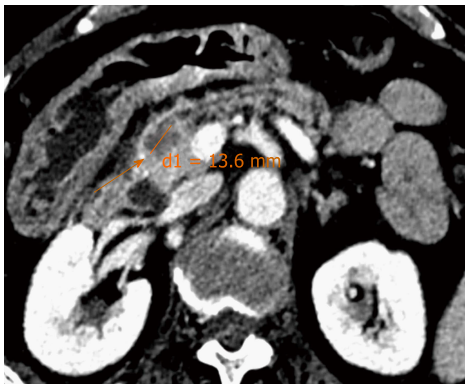

Figure 3 Same pancreatic neuroendocrine tumour showing necrosis (orange arrow) two days after Radiofrequency ablation. Chromogranin level decreased to 36 ng/mL.

needle, may increase the success rate.

Although the technical feasibility of EUS-guided RF may be accepted, its clinical efficacy is more difficult to affirm because of the scarcity of available experience. The intended effect is primarily palliative, and possible recurrence must be expected, although some authors have suggested that it may be curative for small tumours (Figures 2 and 3). Beneficial effects with immediate relief of symptoms were observed in the four symptomatic tumours, but the follow-up period was very limited. Therefore, it is impossible to predict the recurrence rate, time until symptom reappearance and how many frequent RF sessions would be required. In addition, for locally advanced PC, EUS-guided RF aims to improve quality of life and to prolong survival by means of a cytoreductive effect. However, no study has had survival as a primary endpoint, a quality-of-life assessment has not been performed and there are no randomized studies comparing EUS-guided RF with the standard palliative treatment.

The successful results of RF in hepatic tumours and the need for less invasive alternatives to surgery for pancreatic tumours have prompted to attempt RF in the pancreas. Nevertheless, important biological and anatomical differences between the liver and the pancreas may determine very different safety profiles of RF in the two contexts. First, the pancreas is a highly thermosensitive organ, and thermal injury may lead to serious inflammatory consequences[27]. Second, hepatic tumours are usually surrounded by normal parenchyma, and thermal injury beyond the hepatic tumours does not usually affect important structures, whereas pancreatic tumours often encase vessels and the distal bile duct or are in contact with the gastric or duodenal wall. For this reason, the safety of intraoperative and percutaneous pancreatic RF is still under debate, and frequent and severe complications have been reported[36-38]. In contrast, only mild complications have been described for EUS-guided RF in pancreas, even when RF without additional cooling was applied. No case of severe acute pancreatitis, duodenal perforation, severe gastrointestinal haemorrhage or bile leak have occurred after RF under EUS guidance. Although all patients with a locally advanced cancer in one series had major vessel involvement, no case of portal or splenic thrombosis was reported[32]. One explanation for this low morbidity may be that EUS is the best modality for real-time imaging of the pancreas, minimizing the risk of inadvertent damage of adjacent anatomical structures. Nevertheless, mostly large and advanced tumours have been included in these series, and adjacent normal structures were likely far from the probe. In small benign lesions, other injuries may be observed (Figures 4 and 5). Further prospective series are needed to confirm the low morbidity.

Endoscopy-guided RF of biliary strictures

Results: The primary search identified 227 publications. Titles and abstracts were screened for relevance, and 174 records were excluded. After a full-text review of the 53 remaining studies, 25 papers were determined to be eligible for inclusion (Tables 2 and 3)[39-64]. The selected studies comprised eight retrospective series, two prospective trials, seven case reports and eight abstracts. Review of article references yielded one more abstract[39].

Table 2 Endoscopic radiofrequency ablation for biliary strictures

Ref.	*n*	Indication	Stricture length (mm)	Thermokinetics power - time	RF sessions	Technical success	Stricture diameter before RF (mm)	Stricture diameter after RF (mm)	Stent patency (d)	Median survival (mo)	Complications
Pozsár *et al*[39], 2011	5	Occluded SEMS (malignant strictures)	15	10 W - 120 s	2 (1-3)	100%	2	4.7	62 (9-236)	-	No complication
Monga *et al*[40], 2011	1	CC	15	5 W - 120 s	1	100%	-	-	-	-	No complication
Steel *et al*[41], 2011	21	16 PC 6 Klatskin/ intrahepatic CC	-	7-10 W 120 s	2 (1- 4)	100%	0 (0-1)	4 (3-6)	76% at 90-d FU	-	1 hyperamylasemia 2 cholecystitis 1 rigors
Yoon *et al*[42], 2012	1	CHD CC	-	7 W - 90 s	2	100%	-	-	-	-	No complication
Mavrogenis *et al*[43], 2012	1	Intrahepatic adenoma	-	-	-	100%	-	-	-	-	-
Dzeletovic *et al*[44], 2012	1	Ampullary adenoma with CBD invasion	10	1	-	100% (Complete ablation)	-	-	-	-	CBD stenosis
Sonpal *et al*[45], 2012	1	Occluded SEMS (Klatskin CC)	-	8 - 10 W 90 s	2	100%	-	-	90	-	-
Lewis *et al*[46], 2012	5	4 CC 1 colon met.	-	7-10 W 90 s	1 (1-2)	100%	-	-	-	-	No complication
Watson *et al*[47], 2012	3	3 Klatskin CC	2	7-10 W 90 s			3	6			No complication
Kallis *et al*[48], 2015	11	Occluded SEMS: 6 PC/3 CC 2 liver met	-	-	1 (1-2)	100%	-	-	146	-	No complication
Topazian *et al*[49], 2013	1	Intrahepatic adenoma		10 W - 90 s	1	100% (Complete ablation)	-	-	-	-	Hepatic artery pseudoaneurysm
Figueroa-Barojas *et al*[50], 2013	20	11 CC/7 PC 1 IPMN/1 Met.	15.2 (3.5-33)	7-10 W 120 s	-	100%	1.7 (0.5-3.4)	5.2 (2.6-9)	100% at 30-d FU		5 pain 1 mild pancreatitis and cholecystitis
Alis *et al*[51], 2013	10	CC	20 (20-35)	10 W - 120 s	3 (3-4)	100%	1.5 (1.5-2)	5 (4-7)	270 (180-450)	-	2 mild pancreatitis
Lui *et al*[52], 2013	1	Occluded SEMS (Klatskin CC)		10 W - 150 s	1	100%	-	-	60	-	No complication
Law *et al*[53], 2013	2	PC	-	10 W - 120 s	1	100%	-	-	-	-	-
Tal *et al*[54], 2014	12	2 intrahepatic CC 8 Klatskin IV CC 2 GB can. 1 gastric Met	-	8-10 W 60-90 s	1 (1-5)	100%	-	-	-	8.5	3 hemobilia (2 deaths) 3 cholangitis

CHD: Common hepatic duct; CBD: Common bile duct; CC: Cholangiocarcinoma; PC: Pancreatic cancer; GB: Gallbladder; SEMS: Self-expanding metal stent; Met: Metastasis; FU: Follow-up.

Over the last 5 years, a total of 293 patients were reported in the literature to have undergone biliary RF under endoscopic guidance. The indications in the literature were malignant strictures in 232 patients (79%), occluded self-expanding metal stents in 48 (16%), benign non-tumoural strictures in 9 (3%), intrahepatic adenoma in 2 (0.6%) and bile duct ingrowth of ampulloma in the remaining 2 patients (0.6%). In some studies, all malignant strictures corresponded to cholangiocarcinoma, but in others malignant strictures encompassed pancreatic cancer, gallbladder cancer, hepatic carcinoma and metastatic cancers as well. Only one study reported patients with benign non-tumoural strictures and included four postsurgical strictures, three after liver transplant and two chronic inflammatory strictures[55]. The RF

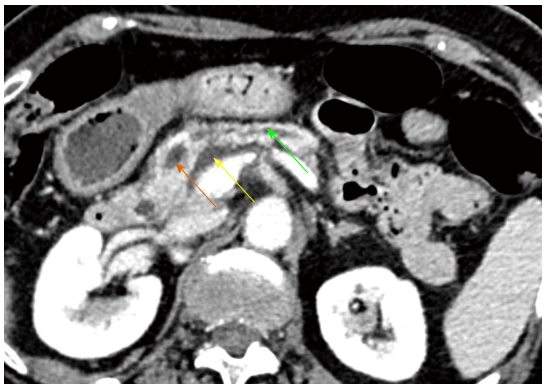

Figure 4 Patient suffered a mild acute pancreatitis 3 wk after radiofrequency ablation. CT revealed a peripancreatic fluid collection (yellow arrow) and tumour necrosis (orange arrow) with slight dilation of the upstream pancreatic duct (green arrow).

probe used in all the reported cases was the Habib™ EndoHBP. The ELRA™ electrode has been launched to the market recently, and to date, only one experimental study on animals has been reported[65]. Power and time settings ranged from 5 to 10 W and from 60 to 180 s, respectively; nevertheless, most studies applied RF at 10 W over a period of 90 s. In most studies, patients underwent RFA only once. However, some operators performed RF either twice during the same session with a rest period of 1-2 min or at every ERCP for stent exchange during follow-up.

Technical success with satisfactory placement and deployment of the RF catheter was achieved in all patients. Only in one study was RF not applied in one included patient due to an irretrievable plastic stent with proximal migration. Because RFA was not attempted, we did not consider this case a technical failure[41]. Regarding efficacy, three main outcome measures may be considered: biliary decompression, stent patency and survival. Biliary decompression was possible in all cases but two (99%). In one patient, extensive intrahepatic biliary malignancy prevented successful biliary drainage[41], and in one other, biliary decompression was not achieved, despite successful RFA and endoscopic stenting requiring percutaneous drainage[51]. However, stent patency and survival have not been uniformly described. Only five studies have detailed data about stent patency in patients with malignant strictures treated with RF before placing a self-expanding metal stent (SEMS)[40,50,51,58,62]. In two studies, the mean lengths of stent patency in 10 and 58 patients with malignant strictures were 270 and 170 d (range 180-450 and 63-277)[51,58]. The three other studies reported 96 to 100 %[41,50,62] and 76 % of stent patency at 30 and 90 d[41] of follow-up, respectively. Moreover, RFA of occluded stents achieved 60, 62, 90, 114, 146 and 180 d of mean patency in six studies, and 62% of stents were still patent at 90 d of follow-up after RF for stent occlusion in one abstract report[64]. Six authors evaluated the

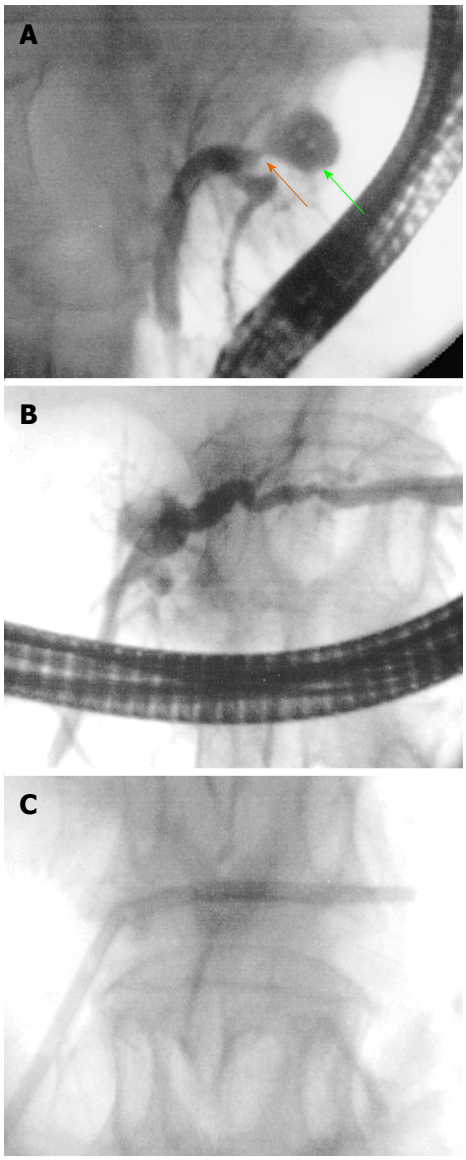

Figure 5 Pancreatic duct stenosis after pancreatic radiofrequency ablation. A and B: ERCP revealed a necrotic cavity (green arrow) and a pancreatic duct stenosis (orange arrow); C: A plastic stent was inserted.

survival of patients treated with RF before biliary stenting, and it was always longer than 8 mo, ranging from 8.5 to 18 mo[54,57,58,62,63]. As secondary outcomes, differences between luminal diameter before and after RFA, as determined by repeated cholangiography, were recorded in some studies, and a significant increase in diameter was observed immediately after RF treatment in all cases[39,41,50,62].

Two studies compared the stent patency and survival of patients treated with SEMS following RFA with those of patients undergoing biliary stenting with SEMS alone, which represents the conventional practice. These studies were not considered in the present analysis because patients in both cases were included in larger series. In the first study by Sharaiha *et al*[66] 26 patients, who underwent RF, were matched with 40 patients receiving a SEMS alone. There was no

Table 3 Endoscopic radiofrequency ablation for biliary strictures (continuation)

Ref.	*n*	Indication	Stricture length (mm)	Thermokinetics power - time	RF sessions	Technical success	Stricture diameter before RF (mm)	Stricture diameter after RF (mm)	Stent patency (d)	Survival (mo)	Complications
Hu *et al*[55], 2014	9	4 postsurgical 3 liver transplant 2 chronic inflam	-	10 W - 90 s	1	100% (5 complete resolution 4 improvement)	-	-	-	-	2 abdominal pain 2 transient leucocytosis 1 mild pancreatitis
Uppal *et al*[56], 2014	2	Prehepatic transplant 1 LHD-CHD CC 1 RHD-CHD CC	-	-	-	100% (No malignancy in explant)	-	-	-	19-35 mo FU	1 hemobilia
Strand *et al*[57], 2014	16	13 Klatskin CC 1 intrahepatic CC 2 extrahepatic CC	-	7 W - 90 s	-	100%	-	-	-	9.6	Occurrence/ month: Stent occlusion 0.06 Stent migration 0.02 Cholangitis 013 Hepatic abscess 0.02
Dolak *et al*[58], 2014	58	50 Klatskin CC 4 PC 1GB can 1 met 1 HCC 1 HCC and CC	-	10 W - 180 s	1 (1-5)	100%	-	-	170	10.6	1 partial liver infarction 5 cholangitis 2 cholangiosepsis 3 hemobilia 1 GB empyema 1 hepatic coma (1 death) 1 left bundle branch block
Mukund *et al*[59], 2014	8	Occluded SEMS: 4 GB cancer 2 CC/2 PC	-	-	1 (1-2)	100%	-	-	114		No complication
Mehendiratta *et al*[60], 2015	1	Ampullary adenoma with CBD invasion	-	7 W - 90 s	-	100% (Complete ablation)	-	-	-	-	No complication
Musquer *et al*[61], 2015	1	Occluded SEMS (CC)	-	10 W - 90 s	-	100%	-	-	180	-	-
Sharaiha *et al*[62], 2015	69	45 CC 19 PC 1 GB cancer 1 gastric cancer 3 liver met	14.5 (3.5-60)	8 W - 90 s	1 (1-4)	100%	2	4.9	96% at 30-d FU	15 for PC 18 for CC	1 pancreatitis 2 cholecystitis 1 hemobilia 3 abdominal pain
Laquière *et al*[63], 2015	12	CC 4 Bismuth Ⅰ 3 Bismuth Ⅱ 2 Bismuth Ⅲ 3 Bismuth Ⅳ	19.5 (10-35)	10 W - 90 s	1 (1-3)	100%	-	-	-	12	1 sepsis 1 cholangitis
Atar *et al*[64], 2015	21	Occluded SEMS: 11 PC/7 CC 1 GB can/2 liver met	-	10 W - 90 s	1 (1-5)	100%	-	-	62% at 90-d FU	-	-

LHD: Left hepatic duct; RHD: Right hepatic duct; CHD: Common hepatic duct; CBD: Common bile duct; CC: Cholangiocarcinoma; PC: Pancreatic cancer; HCC: Hepatocellular carcinoma; GB: Gallbladder; SEMS: Self-expanding metal stent; Met: Metastasis; FU: Follow-up.

difference in pre- and post-RF stricture diameter (20.4 ± 7.33 *vs* 23.17 ± 8.07, *P* = 0.1 and 1.6 ± 0.75 *vs* 1.38 ± 0.18, *P* = 0 respectively), mean number of ERCP (2.26 ± 1 *vs* 1.94 ± 1.27, *P* = 0.84) or survival (median survival of the groups was 5.9 mo *P* = 0.87) between the two groups. However, multivariate analysis revealed RF as an independent predictor of survival (HR = 0.9 (0.1-0.76), *P* = 0.012). The second series by Kallis *et al*[67] consisted of 23 patients undergoing RF followed by biliary stenting and 46 receiving a SEMS alone. SEMS patency rates between the RF treated group and control group were equivalent (472 d *vs* 324 d, HR = 1.186, 95%CI: 0.536-2.656, *P* = 0.669). Median survival in the RFA group was 226 *vs* 123.5 d in the control group (*P* = 0.010), and RF was observed to be an independent predictive factor of survival at 90 and 180 d (OR = 21.07, 95%CI: 1.45-306.64, *P* = 0.026; OR = 4.48, 95%CI: 1.04-19.30, *P* = 0.044, respectively). Because photodynamic therapy (PDT) has been shown to confer a significant survival advantage compared with biliary stenting, Strand *et al*[57] aimed to compare RFA with PDT in patients with unresectable CC. Overall survival was similar (9.6 mo *vs* 7.5 mo respectively, *P* = 0.799) in patients who underwent RF (*n* = 16) and in patients receiving PDT (*n* = 32).

RF was anecdotally used to treat intraductal extension of an ampullary adenoma in two cases and an intrahepatic adenoma in two other patients; all of them were successfully ablated[43,44,49,60]. In addition, nine patients with benign strictures (four post-surgery, three after the liver transplant and two with chronic inflammation) and prior unsuccessful endoscopic treatment underwent RF[55]. All the strictures improved, and complete resolution was observed in more than half.

In some studies, data on complications were not available[43,45,53,57,61,64]. Therefore, among 252 patients, complications occurred in 49 (19 %), and overall mortality was less than 2% (*n* = 3). Infectious complications, the most frequent adverse event, were reported in 8% (nine cholangitis, five cholecystitis, three cholangiosepsis, two transient leucocytosis, one gallbladder empyema and one patient with rigors). At least two cases of cholecystitis may be explained by tumour encasement of the cystic duct, as shown by CT scan and sepsis before ERCP. Four percent of patients (*n* = 10) complained of postprocedure abdominal pain well controlled with analgesics, and 2 % suffered mild acute pancreatitis. Haemobilia occurred in 4% (*n* = 9) and in two cases was fatal[54]. Another case was due to a pseudoaneurysm of the hepatic artery, which was percutaneously thrombosed with thrombin[49]. The pseudoaneurysm was related to RF due to the close temporal relationship with the RF session. Liver infarction was also described in one patient and successfully recovered with conservative treatment[58]. Thermal injury of surrounding vessels was proposed as the hypothetical cause. Finally, hepatic coma with fatal outcome was recorded once[58].

Discussion: SEMS placement is the mainstay palliative treatment of malignant biliary strictures, but more than 50% of SEMS become occluded after 6 mo. It was hypothesized that RF might lengthen stent patency. Therefore, RF has been primarily used as neoadjuvant therapy for malignant strictures prior to inserting a SEMS. Afterwards and in the same line, RF has been applied to treat SEMS occlusion by tumour ingrowth and overgrowth. Anecdotal applications in benign tumoural and non-tumoural strictures have also been reported. Current experience demonstrates that biliary RF under endoscopic guidance is feasible and easy to perform with high technical success rates. Although results seem promising, two studies comparing RF plus SEMS and the conventional palliative treatment with SEMS alone failed to show longer patency of stents after RF. However, the results of these studies suggest a benefit of survival with RF. RF was also compared with PDT, which had been shown to increase stent patency, quality of life and survival, and RF was found to provide a similar survival. Moreover, potential advantages of RF over PDT are the unnecessary limitation of sunlight exposure and its significantly lower cost. Nevertheless, it is still early to draw conclusions about the efficacy of endoscopic biliary RF because most of the available data consist of small retrospective series with high heterogeneity regarding the aetiology of malignant strictures, the power settings selected, the number of sessions, the disease stage and other concomitant therapies, such as chemotherapy. Prospective randomized trials are required to obtain more reliable results about the efficacy of biliary RF and to explore novel indications. The possibility to ablate intraductal extension of ampullary adenomas might reduce the risk of recurrence rate and the need for radical surgery after endoscopic papillectomy (Figures 6 and 7), but only two cases have been reported so far. An ongoing French prospective trial may help to draw conclusions about the interest in RF in this indication. RF may also improve the endoscopic treatment of benign strictures and may even be a rescue treatment for benign strictures refractory to conventional endoscopic treatment.

Although RF has an acceptable safety profile, complications are not uncommon and mortality is not zero. The most frequent adverse outcomes are infectious complications and postprocedure abdominal pain. Abdominal pain is usually self-limited, and the prophylactic pre- and post-procedural use of antibiotics may decrease the risk of infections. Infectious complications, as well as acute pancreatitis, may be primarily attributed to ERCP, but we cannot dismiss the possibility of an increased risk of bacterial translocation after RF and thermal injury of the neighbouring

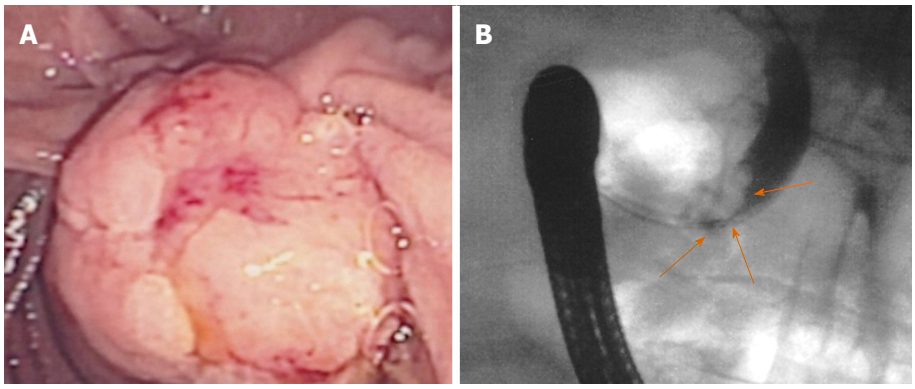

Figure 6 Ampullary adenoma with bile duct extension. A: Endoscopic image of a large lesion involving the ampulla and the adjacent duodenum; B: Bile duct extension at endoscopic retrograde cholangiopancreatography (orange arrows).

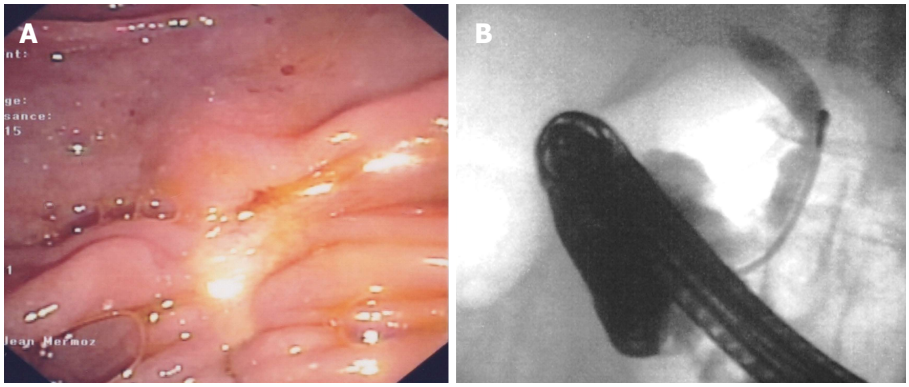

Figure 7 Results after endoscopic papillectomy and biliary radiofrequency ablation at two-year follow-up. A: Endoscopy showed complete resection without tumour recurrence; B: No longer bile duct ingrowth at endoscopic retrograde cholangiopancreatography. Biopsies were negative repeatedly.

pancreas. The most feared complications at first, such as bile duct or duodenal perforation, were not observed. Preventing biliary fistula was always pursued by inserting a plastic or SEMS after RFA. However, other serious events have been reported, such as liver infarction and fatal haemobilia. Both are believed to be secondary to thermal injury of the hepatic artery. The use of intraductal ultrasonography may help to evaluate the proximity of the hepatic artery to adjust the power settings for more limited energy delivery. This measure is especially relevant for hilar lesions located near liver parenchyma and for strictures without an associated mass.

CONCLUSION

RF is an ablative modality of treatment that has been recently added to the therapeutic armamentarium of endoscopy. In the setting of unresectable biliopancreatic cancers, in which treatment options are very limited, high expectations are held for this modality. Available experience suggests a beneficial effect on survival with RFA, but current evidence is scarce because most studies have been performed on small and heterogeneous groups of patients using a retrospective design. The safety profile appears to be acceptable, though serious complications have been reported. This finding may be explained in part because the energy settings are not clearly standardized and have been extrapolated from either *in vivo* animal models with non-tumoural tissues or *ex vivo* human studies without considering the delayed necrosis and the heat-sink effects *in vivo*. Prospective randomized controlled trials are awaited to accurately evaluate its efficacy in terms of survival and quality-of-life and to optimize energy parameters in order to reduce the risk of complications. Newest indications such as refractory benign strictures, biliary extension of ampullomas or branch-duct intraductal papillary mucinous neoplasms deserve also further assessment.

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REVIEW

Circulating predictive and diagnostic biomarkers for hepatitis B virus-associated hepatocellular carcinoma

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Abstract

Chronic hepatitis B virus (HBV) infected patients have an almost 100-fold increased risk to develop hepatocellular carcinoma (HCC). HCC is the fifth most common and third most deadly cancer worldwide. Up to 50% of newly diagnosed HCC cases are attributed to HBV infection. Early detection improves survival and can be achieved through regular screening. Six-monthly abdominal ultrasound, either alone or in combination with alpha-fetoprotein serum levels, has been widely endorsed for this purpose. Both techniques however yield limited diagnostic accuracy, which is not improved when they are combined. Alternative circulating or histological markers to predict or diagnose HCC are therefore urgently needed. Recent advances in systems biology technologies have enabled the identification of several new putative circulating biomarkers. Although results from studies assessing combinations of these biomarkers are promising, evidence for their clinical utility remains low. In addition, most of the studies conducted so far show limitations in design. Attention must be paid for instance to different ethnicities and different etiologies when studying biomarkers for hepatocellular carcinoma. This review provides an overview on the current understandings and recent progress in the field of diagnostic and predictive circulating biomarkers for hepatocellular carcinoma in chronically infected HBV patients and discusses the future prospects.

Key words: Hepatocellular carcinoma; Hepatitis B virus infection; Biomarkers; Predictive; Diagnostic; Alpha-fetoprotein; Validation; Limitations; MicroRNA

Core tip: Regular screening for hepatocellular carcinoma (HCC) in patients at risk improves their survival rates. Currently available screening methods include abdominal ultrasound and alpha-fetoprotein serum levels, but both methods lack diagnostic accuracy. Recent technological advances have enabled the identification of new predictive and diagnostic hepatitis B virus (HBV)-associated HCC biomarkers. Nevertheless, most of the studies conducted so far show design limitations. This review provides an overview on the current understanding and future prospects of circulating predictive and diagnostic biomarkers for HBV-associated HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and ranks third as cancer-related death cause due to a 5-year survival of only 15%[1]. Moreover, at a time of decreasing overall cancer-related deaths due to an immense progress in cancer diagnostics and treatment options, mortality from hepatocellular carcinoma is increasing[1,2].

Chronic hepatitis B virus (HBV) infection is a major risk factor for HCC development. Prospective cohort studies have revealed an up to 100-fold increased risk for HCC in chronically infected HBV patients[3]. Up to 50% of newly diagnosed HCC cases are attributed to HBV infection, due to both direct and indirect oncogenic effects of the virus[2,4-7]. Integration of HBV DNA in the human genome may result in genomic instability, while inflammation-related oxidative stress, caused by immunological responses, may contribute indirectly to HCC development[6-9].

Four clinical phases can be distinguished during the natural course of a HBV infection: an immune-tolerance phase, an immune active phase, an inactive carrier phase and a hepatitis B e antigen (HBeAg) negative phase[2,10]. Patients in the immune active phase and the HBeAg negative phase are at increased risk for progression towards fibrosis and ultimately the development of cirrhosis, which is a major risk factor for HCC[11,12].

Several years to decades are needed for HCC to develop in a HBV infected liver[13]. Early diagnosis of HCC in HBV patients is challenging but is proven to result in an improved long-term survival due to an increased chance to detect tumors at a resectable stage[14-19].

Importantly, also a significant number of HBV patients develop HCC in a non-cirrhotic liver[20]. Current guidelines therefore advise 6-monthly abdominal ultrasound (US) surveillance for HCC in advanced fibrosis or cirrhotic HBV patients and in non-cirrhotic patients depending on ethnic background and age[21-25]. The technique, however, faces a disappointing 63% sensitivity to detect HCC and is hampered by inter- and intra-observer variability[20]. Finding biomarkers to better predict or diagnose HCC therefore remains an important clinical and research priority.

Serum alpha-fetoprotein (AFP) levels are widely used for HCC screening and diagnostics, but the clinical utility to rule out or detect HCC is still a matter of debate. The protein lacks sensitivity and specificity to detect HCC. The recent improvement of systems biology techniques, such as proteomics and genomics, has enabled the identification of several new putative biomarkers[26,27]. This review provides an overview of diagnostic and predictive serum biomarkers for HBV-associated hepatocellular carcinoma and discusses future prospects.

DIAGNOSTIC BIOMARKERS FOR HCC: AN OVERVIEW

An overview of the discussed diagnostic circulating biomarkers with their respective sensitivities and specificities to detect HCC is displayed in Table 1. An overview of their cellular origin is displayed in Figure 1.

Alpha-fetoprotein goes off stage, its glycoforms come on stage

Alpha-fetoprotein is an oncofetal protein produced by the fetal yolk sac and liver[28]. The protein, like albumin, binds exogenous as well as endogenous substances in blood[29]. Physiologically elevated AFP levels are found in pregnant women and newborns, but decrease quickly after birth. Upregulation of AFP later on in life has been associated with various pathological conditions such as acute hepatitis, endodermal sinus tumors and HCC[30-32].

Alpha-fetoprotein was discovered in the late 1950s and has been of interest for the monitoring of HCC development in viral hepatitis patients since the early 1970s[33-35]. For a long time, AFP has been widely used together with abdominal US in routine HCC screening. Nevertheless, the most recent European and American guidelines do not endorse this practice anymore since its diagnostic accuracy is low[21-23]. The protein, most often detected by enzyme-like immunosorbent assays (ELISA), indeed faces a lack of sensitivity and specificity to detect early stage HCC in HBV patients[36]. Only 70% of all HCC's are characterized by markedly elevated AFP levels at the time of diagnosis[37-41]. Large HBV cohort studies showed a maximal sensitivity for AFP of about 75% to detect HCC at optimal cut-off levels[32,41-45].

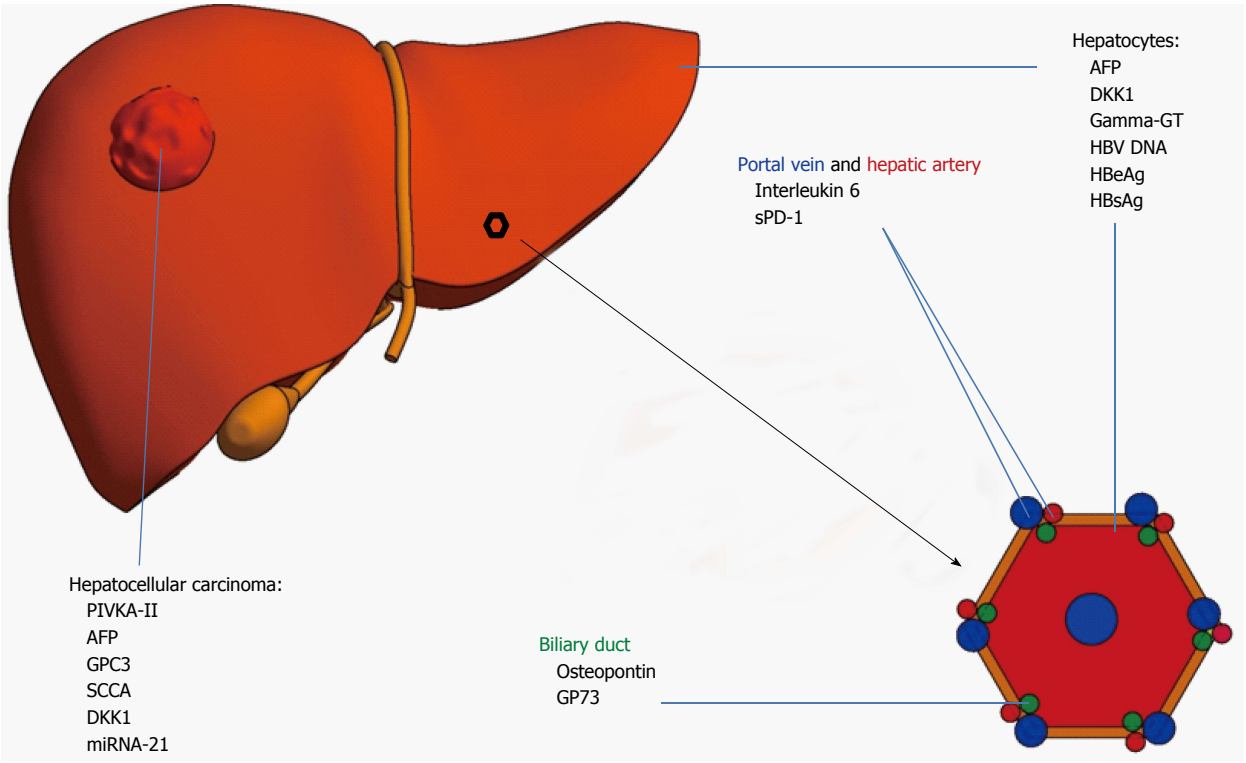

Figure 1 Cellular origin of the discussed predictive and diagnostic biomarkers in a physiological and oncological setting. Predictive biomarkers are displayed in italics. PIVKA-II: Protein induced by vitamin K absence; AFP: Alpha-fetoprotein; GPC3: Glypican-3; SCCA: Squamous cell carcinoma antigen; DKK1: Dickkopf-1 protein; miRNA: MicroRNA; COMP: Cartilage oligomeric matrix protein; GP73: Glycoprotein-73; sPD-1: Soluble programmed death-1; Gamma-GT: Gamma-glutamyltransferase.

Table 1 Diagnostic serum biomarkers for hepatitis B virus-associated hepatocellular carcinoma

Marker	Cut-off[1]	Sensitivity	Specificity	Detection method (most reliable)	Ref.
AFP	7.7-112.0 ng/mL	25%-90%	87%-97%	ELISA	[36,73]
AFP-l3	3%-20%	36%-96%	89%-94%	Liquid-Phase Binding Assay	[46,47,52]
DCP	40-150 mAU/mL	44%-91%	68%-99%	Electrochemiluminescence immunoassay	[36,53]
Osteopontin	9.3-642.5 ng/mL	73%-97%	55%-100%	ELISA	[65]
GP73	78-150 ng/mL	68%-95%	9%-97%	Immunoblotting, Western Blotting or ELISA[2]	[36,71,72]
GPC-3	2-300 ng/mL	36%-100%	40%-100%	ELISA	[36,78]
SCCA	0.12-3.80 ng/mL	42%-80%	50%-88%	ELISA	[36,81]
DKK1	1.01-2.15 ng/mL	69%-91%	62%-91%	ELISA	[86-88]
miRNA-21	NA	84%-90%	71%-92%	qRT-PCR	[83]
miRNA-122	NA	70%-82%	69%-84%	qRT-PCR	[83]

[1]Range of cut-off values used in different studies included in the discussed meta-analyses and systematic reviews; [2]Equally reliable. Relative levels were used with different internal standards to measure miRNA-concentrations. The choice of the most reliable detection method was based on recent studies involving at least 100 patients. Only biomarkers of which at least 4 different studies discussing their diagnostic potential exist, were included. AFP: Alpha-fetoprotein; DCP: Des-gamma carboxy prothrombin; GP73: Golgi-protein 73; GPC-3: Glypican-3; SCCA: Squamous cell carcinoma antigen; DKK1: Dickkopf-1; miRNA: MicroRNA; ELISA: Enzyme-linked immunosorbent assay; qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction; NA: Not applicable.

AFP-l3: The rising star under the AFP glycoforms

AFP is a glycoprotein of which three glycoforms exist: AFP-l1, AFP-l2 and AFP-l3. They are all characterized by an increased binding affinity for *Lens culinaris* agglutinin. AFP-l3, which shows the highest binding affinity is of particular interest as a biomarker for hepatocellular carcinoma. This glycoform is secreted by malignant HCC cells even at early tumor stages and in the absence of elevated AFP levels and can be detected using liquid-phase binding assays[46,47]. In addition, the fraction of AFP-l3 to total AFP in the serum correlates with the degree of malignancy[48].

Over 15 studies have addressed the clinical potential of AFP-l3 so far with sensitivity and specificity ranging from 21% to 84% and from 89% to 94% respectively[48-52]. However these studies assessing the clinical potential of AFP-l3 use different cut-off levels, test methods and patient numbers, resulting in a wide range of detected sensitivity. A study from 2009 measuring the fraction of AFP-l3 to total AFP using an automated immunologic analyzer and a cutoff of 10% AFP-l3 in 419 HCC patients and 417 cirrhotic controls, found a sensitivity of 42% to detect HCC[53]. AFP-l3 fractions were measured using Western blotting in another study, involving 388 HCC patients and 212 controls with a cutoff of 15% AFP-l3 to total AFP, resulting in a sensitivity of 21%[54]. In order to unequivocally demonstrate the superiority of AFP-l3 to AFP, large cohort studies using the same cutoff and detection method are needed. Recently AFP-l3 was suggested to be especially useful in the diagnosis of HCC in absence of elevated AFP levels, but further validation is needed[55].

Des-gamma carboxy prothrombin

Des-gamma carboxy prothrombin (DCP) is a non-carboxylated form of prothrombin, also known as protein induced by vitamin K absence (PIVKA-II). Carboxylation takes place in the hepatocytes before the protein is released into the circulation. Release of the non-carboxylated form has been associated with vitamin K deficiency and presence of HCC[56]. Elevated DCP levels, preferably measured using electrochemoluminiscence assays, were found in sera of HCC patients, suggesting proper DCP synthesis in hepatoma cells[36,57,58]. DCP has been investigated as a potential HCC diagnostic biomarker in several studies, showing a comparable to slightly higher diagnostic performance compared to AFP[59-63].

Osteopontin

Osteopontin (OPN) is a glycoprotein that constitutes a major part of the extracellular matrix of bones and teeth. In addition, low levels of the protein are being secreted by biliary epithelial cells. OPN is involved in developmental as well as immunological, tumorigenic and bone homeostatic processes[64]. Overexpression of the protein, detected using ELISA assays, was found in a wide range of tumor types including pancreas cancer, multiple myeloma and HCC[64-66]. Seven retrospective cohort studies have investigated the diagnostic potential of OPN for HCC. So far, OPN does not outperform AFP as a diagnostic marker[65].

Golgi protein-73

Golgi protein-73 (GP73) is a transmembrane protein physiologically located on the Golgi membrane of epithelial cells in different tissues, including the biliary tract[67]. Its function remains largely unknown. Liver damage, caused by viral as well as non-viral agents leads to GP73 upregulation[68,69]. Increasing GP73 serum levels are associated with advanced fibrosis stages in HBV patients[70,71]. A recent meta-analysis showed that GP73's diagnostic accuracy for HCC outperforms that of AFP[72,73]. The protein can be detected using either ELISA assays, immunoblotting or Western blot. Previous studies have shown a comparable efficacy for all three methods[36,71].

Glypican-3

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycans. It is an oncofetal antigen involved in embryonal morphogenesis[74]. Significant expression in human adults can occur in different tissues including breast and liver and indicates ongoing pathological, mostly carcinogenic processes[75,76]. GPC3 has been proposed as a novel serum marker for HCC[75]. The protein promotes HCC tumor growth through stimulation of the Wnt signaling pathway[77]. A recent meta-analysis showed an acceptable accuracy of the protein to detect HCC with a mean pooled sensitivity and specificity of 56% and 89% respectively[78]. The protein is preferably detected using ELISA assays[36].

Squamous cell carcinoma antigen

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor, physiologically located in squamous epithelial cells. It is also expressed by neoplastic epithelial cells, *e.g.,* neoplastic liver cells in which it promotes tumor growth through inhibition of apoptosis[79,80]. Increased serum levels have been detected using ELISA assays in HCC patients[36]. The protein's diagnostic accuracy for HCC has been investigated in over 12 studies and turned out to be moderate with a pooled sensitivity and specificity of 59.0% and 76.0% respectively. Nevertheless some design limitations of these studies such as a small sample size need to be taken into account[81].

Dickkopf-1 protein

Dickkopf-1 protein (DKK1) is a glycoprotein secreted by human hepatocytes. Upregulation of DKK1 expression takes place in a wide variety of cancers including prostate cancer, multiple myeloma and hepatocellular carcinoma[82-84]. Overexpression of the protein is detected in tissue as well as serum from hepatocellular carcinoma patients. Although the protein is suggested to be an inhibitor of the Wnt/β-catenin signaling pathway, its exact functions have not been fully elucidated[82,85]. A meta-analysis showed an acceptable diagnostic accuracy of DKK1, comparable to AFP, to detect HCC with a pooled sensitivity and specificity of 65% and 94% respectively[86-88]. Detection of the protein in serum is performed using ELISA assays[87].

miRNA's: Promising biomarkers for HCC detection

MicroRNA's (miRNA) are small non-coding RNA's regulating gene expression by binding to messenger-

RNA (mRNA)[89]. During recent years, circulating miRNAs have gained increasing attention for the early diagnosis and screening of hepatocellular carcinoma[90]. So far, two miRNAs, miRNA-21 and miRNA-122 show particularly high potential in HCC diagnostics[91]. miRNA-122 is a liver specific miRNA, whereas miRNA-21 is produced by different tissues including the colon, liver and heart in which it is involved in respectively tumor growth and cardiac disease development[92-94]. miRNA-21 inhibits tumor suppression by inhibiting tumor suppressor pathway activating phosphatases (*e.g.,* ATK and MAPK), whereas miRNA-122 inhibits tumor growth by acting as a tumor suppressor gene[93,95-97]. A direct correlation was observed between increasing miRNA-21 levels and increased cell proliferation[98]. In addition, high circulating miRNA-21 levels were found to be correlated with more differentiated and progressive hepatocellular carcinoma thus indicating a bad prognosis[94]. Serum miRNA-122 levels correlate inversely with the severity of liver fibrosis[99]. The antitumor properties of miRNA-122 have been successfully applied in a preclinical model to prevent HCC development[96]. The diagnostic accuracy of miRNA-21 slightly outperforms that of miRNA-122 with a pooled sensitivity and specificity of 87% and 80% respectively for miRNA-21 *vs* 68% and 73% for miRNA-122[91].

Other diagnostic biomarkers

Based on systems biology approaches, more markers with diagnostic potential in HCC screening settings have recently been identified, including fucosylated fetuin A, inter-alpha-trypsin inhibitor H4, clusterin, endoglin, soluble Axl, latent TGF-bèta binding-protein 2 as well as peroxireduxin 1, 2 and 3. The evidence for clinical utility of these markers remains low due to a lack of sufficiently large cohort studies[100-108].

In addition, several studies have been published on circulating tumor cells for HCC. However, most of published studies focus on prognosis after HCC diagnosis and prediction of disease progression rather than on the diagnosis of HCC[109-111].

PREDICTION OF THE RISK TO DEVELOP HCC

Three strategies can be applied when assessing the long-term risk for HCC. Firstly, clinical risk scores, *e.g.,* REACH-B and PAGE-B, can be calculated based on viral and host-related (*e.g.,* age and gender) clinical parameters. Most of these models have however been developed in Asian populations and lack validation in non-Asian populations[112,113]. HBeAg positivity and HBV DNA levels above 1 million copies/mL are associated with a 4- and 11-fold increased HCC-risk during 8 and 11 years of follow-up respectively[114-116]. Hepatitis B surface antigen (HBsAg) levels above 1000 IU/mL are accompanied with an up to 6.5-fold increased HCC risk in men and an up to 11-fold increased risk in women within 15 years[117]. HBsAg levels are suggested to be especially useful in case of low HBV DNA levels[118,119].

Secondly, genome-wide association studies have enabled the linkage of genetic variants to specific disease outcomes. Single nucleotide polymorphisms (SNPs) in a wide range of genes, including the Interleukin-21 and the CRP-gene, have been associated with an increased susceptibility for HCC over a variable time course[120-124]. Increasing evidence indicates that SNP's in the STAT4, MDM2 and HFE gene, determined on whole blood, are germline risk factors for HCC[125,126]. On the other hand, also somatically acquired mutations, *e.g.,* in the TP53 gene, have been associated with an increased risk for HCC[127]. All together these findings are strongly suggestive for interindividual differences in the genetic predisposition for HCC development, a predisposition that can be boosted by additional somatic mutations.

Thirdly, circulating biomolecules would be ideal as a non-invasive, predictive biomarker for HCC. An overview on the discussed predictive biomarkers including their respective increase in HCC risk is displayed in Table 2. The clinical utility of Gamma-Glutamyl Transferase (Gamma-GT) Iso-enzyme II was first evaluated as a predictive HCC marker in 1992. Patients showing persistently elevated levels of Gamma-GT Iso-enzyme II at presentation had a 86.7% risk to develop HCC within 10 years' time[128]. Gamma-GT levels above 41 U/L and AFP-levels > 5 ng/mL have later been associated with an 8-fold increased risk for HCC in a large HBV cohort followed up for 6 years[129]. The usefulness of AFP-levels for HCC prediction has, however, been assessed in several other studies with contradictory results[128-130].

Cartilage oligomeric matrix protein (COMP) is an extracellular matrix protein involved in tissue genesis and remodeling[131]. The protein is released into the circulation upon cartilage damage[132]. Overexpression of the protein in serum from HCC patients suggested that serum COMP levels reflected an individual's fibrosis stage and subsequent risk for HCC[133]. A recent study in Greece supports this hypothesis: COMP positivity (> 15 U/L) was associated with a 3-fold increased HCC risk during a median follow-up of 8 years[132,134].

In a study of 27 serum cytokines and growth factors, interleukin-6 (IL-6) levels were found to predict cancer development within a timeframe of 8 to 11 years with moderate accuracy[135]. Levels above 7 pg/mL were associated with a 3-fold increased HCC risk. As IL-6 induces C-reactive protein (CRP), the potential value of CRP in HCC risk prediction was assessed, but turned out to be disappointing[136,137]. Recently, soluble programmed death-1 (sPD-1), a soluble form of the membrane-bound programmed death 1 on T cells with a largely unknown function was put forward as a marker[138,139]. sPD-1 levels above 637.6 pg/mL at baseline reflected a 2-fold increased risk to develop HCC during a median follow-up time of 20 years, when

Table 2 Predictive serum biomarkers for hepatitis B virus-associated hepatocellular carcinoma

	Marker	Cut-off	Increased risk for HCC	Control group[1]	Follow-up time	Ref.
Viral	HBeAg	Positive	4-fold	HBeAg negative HBV patients	8 yr	[114]
	HBV DNA	> 1 million copies/mL	11-fold	HBV patients with HBV DNA < 300 copies/mL	11 yr	[115]
	HBsAg	> 1000 IU/mL	3-fold	HBV patients with HBsAg 5-9 IU/mL	14.7 yr	[117]
Host	Gamma-GT Iso-enzyme II	Positive	86-fold	GGT Iso-enzyme II negative HBV patients	10 yr	[128]
	Gamma-GT	> 41 U/L	8-fold	HBV patients with Gamma-GT ⩾ 41 U/L	5.9 yr	[129]
	AFP	> 5 ng/mL	8-fold	HBV patients with AFP ⩽ 5 ng/mL	5.9 yr	[129]
	COMP	Positive	3-fold	COMP negative HBV and HCV patients	8 yr	[134]
	IL-6	> 7 pg/mL	3-fold	HBV patients with IL-6 < 7 pg/mL	7.25 yr	[135]
	sPD-1	> 637.6 pg/mL	2-fold	HBV patients with sPD-1 < 117.3 pg/mL	20 yr	[138]

[1]Control group: group included in the study, to which the increased HCC risk was calculated. HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; Gamma-GT: Gamma glutamyltransferase; AFP: Alpha-fetoprotein; COMP: Cartilage oligomeric matrix protein; IL-6: Interleukin 6; sPD-1: Soluble programmed death 1.

compared to sPD-1 levels below 117.3 pg/mL[138].

DISCUSSION

Despite its disappointing sensitivity and specificity, AFP still remains the most widely used serum HCC biomarker. Some newly discovered circulating biomarkers, *e.g.,* AFP-l3, DCP and microRNA's show promising potential for implementation in clinical practice. However, only GP73 strongly outperforms AFP in terms of diagnostic accuracy. Large variations in sensitivity and specificity are noticed between different studies assessing the same biomarker (Table 1).

Due to the heterogeneity of HCC, one single biomarker with 100% sensitivity and specificity in all HCC cases will be hard to find. A more rational approach to increase the diagnostic accuracy might be the combination of different biomarkers[41,52,55,62,65,72,140-142]. The most recent APASL guidelines indeed recommend the combined use of AFP, AFP-l3 and DCP in HCC screening[22,142]. In favor of this approach, a meta-analysis showed that combined testing of GP73 and AFP increased the pooled sensitivity without decreasing the specificity to detect hepatocellular carcinoma. The pooled sensitivity and specificity were 87% and 85% respectively when biomarkers were combined, compared to 77% and 91% for GP73 and 62% and 84% for AFP when used alone[72].

All currently identified circulating biomarkers and their combinations definitely need more validation studies. Most of the biomarker discovery studies have been performed in cohorts of a few 100 patients. The majority of identified markers has so far not been subject of large, external validation studies[100-103,108]. Five subsequent steps are to be followed in cancer biomarker discovery. The first step is the implementation of preclinical exploratory studies. Step 2 is the development of a clinical assay. Step 3 involves retrospective studies, step 4 prospective studies and step 5 large, randomized controlled trials. Biomarkers identified in step 1 must pass all other steps before they can be termed validated biomarkers[143]. So far, only AFP has reached step 5[15].

In addition, the studies that have been conducted over the last decades are hampered by limitations in their study design. As an example the patient cohorts for HCC biomarker discovery studies are often heterogeneous regarding liver disease etiology and ethnicity. In their paper, da Costa *et al*[144] proved the need to validate biomarkers in different ethnic populations. They investigated the potential of osteopontin and latent Transforming Growth Factor beta binding-protein in HCC diagnosis in separate cohorts in Gambia, Korea, Thailand and France. The sensitivity and specificity of both markers differed (> 10%) among ethnicities. The onset of HCC occurs at a median age of 45 in sub-Saharan African people, whereas a mean age of 52 to 65 has been observed in the rest of the world[145,146]. In addition, HCC incidence varies among HBV and hepatitis C virus (HCV) patients, highlighting the importance of homogeneous patient cohorts. Future biomarker discovery and validation studies should therefore distinguish between different ethnicities and etiologies as this most probably explains the variation in sensitivities and specificities noticed between studies assessing the same biomarker (Table 1).

The inclusion of clinical parameters into biomarker scores could increase their performance. One study demonstrated that incorporation of age into combined models of biomarker testing significantly improved the diagnostic performance for HCC[140].

From a clinical point of view, however, predictive serum biomarkers would be preferred over diagnostic biomarkers to tailor HCC surveillance according to the individual needs. Proteomic approaches are encouraging, but also need a validation in larger cohorts[147,148]. Expression of Heat-shock Protein 27 was *e.g.,* detected in 90% of sera from HCC patients and in 0% of sera from non-HCC patients, which seems promising[148]. Other groups have focused on genomics and have identified a gene signature in

liver tissue of HCV infected patients predictive of HCC development[149,150]. It could be of interest to identify corresponding secretory biomarkers in blood.

CONCLUSION

Monitoring HCC development in chronic hepatitis B patients based on serum biomarkers remains challenging. During recent years, new predictive and diagnostic circulating biomarkers have been proposed. Combinations of these biomarkers show a higher potential for implementation in clinical practice, but large validation studies in homogeneous ethnic and etiological populations are urgently needed to unequivocally demonstrate their clinical utility.

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REVIEW

Recent advances in mass spectrometry-based proteomics of gastric cancer

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Abstract

The last decade has witnessed remarkable technological advances in mass spectrometry-based proteomics. The development of proteomics techniques has enabled the reliable analysis of complex proteomes, leading to the identification and quantification of thousands of proteins in gastric cancer cells, tissues, and sera. This quantitative information has been used to profile the anomalies in gastric cancer and provide insights into the pathogenic mechanism of the disease. In this review, we mainly focus on the advances in mass spectrometry and quantitative proteomics that were achieved in the last five years and how these up-and-coming technologies are employed to track biochemical changes in gastric cancer cells. We conclude by presenting a perspective on quantitative proteomics and its future applications in the clinic and translational gastric cancer research.

Key words: Gastric cancer; Mass spectrometry; Protein identification; Proteomics; Protein quantification

Core tip: Protein identification and quantification by mass spectrometry represent powerful techniques for deciphering the mechanisms underlying the biochemical anomalies that cause human diseases. Due to innovations in mass spectrometry and labeling techniques, cellular protein levels can be monitored routinely with great accuracy. This review provides a brief overview of these technological advances and their applications in gastric cancer biology.

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INTRODUCTION

Functional interpretations of the genes that are associated or linked with cancer by various genomics approaches often require other orthogonal approaches that could provide information beyond the data obtained from sequence analysis of those genes. Since Wilkins and Williams[1] first proposed the concept of the proteome in 1994, the field of proteomics has recently experienced a dramatic development that has largely been driven by technological advances in the field of mass spectrometry.

The introduction of advanced mass spectrometric instruments and bioinformatics tools has enabled the high-resolution and high-specificity analyses of thousands of proteins and their post-translational modification states in cultured cells, primary tissues, and body fluids[2]. Despite the inherent low sensitivity and undersampling suffered by mass spectrometry[3,4], researchers have started to design strategies that capitalize on this emerging technology for the elucidation of disease pathobiology[5-7].

Gastric cancer is one of the leading causes of cancer-related deaths worldwide[8,9]. With approximately one million cases diagnosed each year, gastric cancer is also one of the most common cancers, particularly in East Asia[8]. Despite a modest decline in newly diagnosed cases worldwide, the mortality rate of gastric cancer remains higher than other malignancies, mainly due to the lack of noninvasive handy diagnostics of early gastric cancer[8]. Moreover, the pathogenic mechanism underlying gastric tumorigenesis is still unknown, and the only curative treatment for gastric cancer remains surgery.

Aimed at obtaining a full understanding of the molecular determinants that drive gastric cancer, many studies have increasingly adopted advanced proteomic technologies that can help identify protein biomarkers and elucidate the molecular mechanisms of gastric cancer. This review discusses the technological breakthroughs in mass spectrometry-based quantitative proteomics and their applications in gastric cancer research.

MASS SPECTROMETRY

Basics and instrumentation

Table 1 summarizes the mass spectrometric technologies that have been adopted to study gastric cancer. Mass spectrometric measurements detect and identify the chemical composition of ionized analytes based on the mass-to-charge ratio, *m/z*. A typical mass spectrometer is composed of an ion source that ionizes the analytes, mass analyzer(s) for the detection of *m/z,* and detector(s) for counting the intensities of the ions[10]. Ionization is commonly achieved by soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI)[11] and electrospray ionization (ESI)[12], to measure the masses of proteins and peptides. In MALDI, the analytes are ionized with a crystalline matrix *via* laser pulses, whereas in ESI, the analytes are directly ionized from a solution that is typically eluted from liquid chromatography (LC) columns. MALDI is usually adopted to analyze simple samples, whereas LC-ESI is used to analyze complex mixtures.

Two major types of mass analyzers are used in current proteomics technology. In time-of-flight (TOF) mass spectrometers, the flight times of ions are measured over a fixed distance to match a specific *m/z,* and the intensity of a measurement is correlated with the amount of the ion. MALDI ionization coupled with TOF technology allows the MALDI-TOF mass analyzer to analyze proteins and peptides with a wide range of molecular weights[10]. Due to its simplicity, excellent mass accuracy, high resolution, and great sensitivity, MALDI-TOF has been widely adopted to identify proteins associated with diseases, including gastric cancer.

Using MALDI-TOF, Hu *et al*[13] have shown that overproduction of the C-X-C chemokine receptor type 1 (CXCR1) was linked with late-stage gastric cancer. The authors compared the protein abundance profiles of the MKN45 gastric cancer cell line in the presence and absence of *CXCR1* overexpression and found that the cellular levels of 29 proteins differed. As these proteins were known to participate in cell adhesion, cellular metabolism, and the cell cycle, CXCR1 was inferred to play a role in the proliferation, metastasis, and invasion of gastric cancer.

In an attempt to understand the inhibitory function of curcumin, curcumin-treated samples were analyzed with MALDI-TOF, and 75 proteins displayed significant changes in abundance. In this study, Singhal *et al*[14] identified putative biomarkers of gastrointestinal tract cancers by analyzing biopsy samples obtained from patients with gastroesophageal malignancies using MALDI-TOF.

Recently, the application of MALDI has been expanded to obtaining mass images of tissues[15]. In MALDI imaging mass spectrometry, the masses of biomolecules are probed two-dimensionally in a thin tissue section, providing valuable spatial information about the analytes that is lost in typical LC-mass spectrometry experiments. Balluff *et al*[16] have utilized MALDI imaging mass spectrometry to identify prognostic biomarkers that can be used to predict disease outcomes after surgical resection. The prognostic value of the three identified proteins (CRIP1, HNP-1, and S100-A6) was validated immunohistochemically with tissue microarrays using an independent validation cohort.

Surface-enhanced laser desorption/ionization

Table 1 Summary of recent proteomic analyses of gastric cancer

Sample	Measurand	Mass spectrometry	Identification/quantification	Ref.
Tissue samples				
GC tissue	Global proteome	MALDI TOF/TOF, LTQ Orbitrap XL	Label-free, Mascot	Balluff *et al*[16], 2011
GC tissue	Global proteome	Q-TOF	O18/O16, MassLynx (v4.0)	Zhang *et al*[73], 2013
GC tissue	Global proteome	LTQ Orbitrap	Label-free, ProLuCID (v1.3)	Aquino *et al*[74], 2014
GC tissue	Global proteome	MALDI TOF/TOF	Label-free, Mascot (v2.2)	Wu *et al*[75], 2014
GC tissue	Global proteome	LTQ Orbitrap XL	Label-free, Mascot (v2.2)	Ichikawa *et al*[38], 2015
GC tissue	Global proteome	LTQ Orbitrap XL	Label-free, Bioworks Browser (v3.3.1), Trans-Proteomic Pipeline (v4.0)	Shen *et al*[76], 2015
GC tissue	Membrane proteome	LTQ Orbitrap Velos	TMT, MaxQuant (v1.2.2.5)	Gao *et al*[31], 2015
Gastroesophageal malignancy	Global proteome	MALDI TOF/TOF	iTRAQ, Mascot	Singhal *et al*[14], 2013
Serum samples				
Sera from GC patients	Serum proteome	MALDI-TOF LTQ Orbitrap XL MS/MS	Label-free, Autoflex, Peptide mass fingerprinting	Fan *et al*[37], 2013
Sera from GC patients	Serum proteome	Triple TOF 5600	Multiple reaction monitoring	Humphries *et al*[59], 2014
Sera from GC patients	Serum proteome	MALDI-TOF Orbitrap Q-Exactive	Label-free, MaxQuant (v1.4.1.1)	Abramowicz *et al*[50], 2015
Sera from GC patients	Serum proteome	LTQ Orbitrap Velos	iTRAQ, SEQUSET HT, Mascot (v2.2)	Subbannayya *et al*[29], 2015
Sera from GC patients	Serum proteome	SELDI TOF MS MALDI TOF/TOF	Label-free, Mascot	Song *et al*[17], 2016
Cell lines				
BGC823, MKN45, SCG7901	Global proteome	MALDI TOF/TOF	Label-free, Mascot	Cai *et al*[71], 2013
AGS, AZ521, FU97, MKN7, MKN74, NCI-N87, SNU16, YCC1, YCC2, YCC3, YCC9	Global proteome	MALDI TOF/TOF	iTRAQ, ProteinPilot	Hou *et al*[69], 2013
AGS	Global proteome	Q-TOF	iTRAQ, Mascot (v2.1.1)	Hu *et al*[72], 2013
MKN45	Global proteome	MALDI TOF/TOF	Label-free, Mascot	Hu *et al*[13], 2013
OCUM-2MD3, OCUM-12	Global proteome	Q-TOF	iTRAQ, ProteinPilot	Morisaki *et al*[28], 2014
AGS, BGC823, MKN45, SGC7901	Global proteome	MALDI TOF/TOF	Label-free, Peptide mass fingerprinting	Yang *et al*[67], 2014
HGC27, MGC803, SGC7901	Global proteome	LTQ Orbitrap	Label-free, Mascot (v2.3.2), Scaffold (v4.0.5), X! Tandem CYCLONE (v2010.12.01.1)	Qiao *et al*[62], 2015
AGS	Global proteome	Q-TOF	iTRAQ, Mascot (v2.3.2)	Lin *et al*[61], 2015
HGC27	Global proteome	Triple TOF 5600	iTRAQ, Mascot (v2.3.2)	Chen *et al*[77], 2016
AGS, MKN7	Secretome	MALDI TOF/TOF	iTRAQ, ProteinPilot	Loei *et al*[51], 2011
AGS, KATO III, NCI-N87, SNU1, SNU5, SNU16	Secretome	LTQ Orbitrap Velos	SILAC, Proteome Discoverer (v1.3.0.339), Mascot, SEQUEST	Marimuthu *et al*[33], 2013
AGS, KATO III, SNU1, SNU5, MKN7, IM95	Membrane proteome	LTQ-FT Ultra	Label-free, Trans-Proteomic Pipeline, Mascot (v2.2.07)	Guo *et al*[68], 2012
AGS, IM95, KATO3, MKN7, MKN28, MKN45, NUGC3, NUGC4, SCH, SNU1, SNU5, SNU16	Membrane proteome	Q-TOF	iTRAQ, ProteinPilot	Yang *et al*[70], 2012
AGS, HGC27, KATO III, MKN45, NUGC3, SCH, SGC7901, SNU5, SNU484, TSK1	Membrane proteome	Q-TOF	iTRAQ, ProteinPilot	Goh *et al*[60], 2015
Multidrug-resistant GC cell lines: GC7901/VCR, SGC7902/ADR	Surface glycoproteome	LTQ Orbitrap XL	Triplex stable isotope dimethyl labeling, Mascot, MSQuant (v2.0a81)	Li *et al*[47], 2013
AGS	Interactome	LTQ Orbitrap Velos	Label-free, Mascot (v2.2.2)	Yu *et al*[57], 2013
AGS	Phosphoproteome	MALDI TOF/TOF	SILAC, Mascot (v2.1)	Holland *et al*[43], 2011
AGS	Phosphoproteome	LTQ Orbitrap XL	SILAC, MaxQuant (v1.3.0.5)	Glowinski *et al*[42], 2014

The studies are listed according to sample types (tissues, sera and cell lines) and measurands in the order of publication year and then in alphabetical order by the first author. GC: Gastric cancer; MALDI: Matrix-assisted laser desorption ionization; TOF: Time-of-flight.

(SELDI) is a variation of MALDI, in which the analytes are bound to a surface before the mass analysis. The surface can be modified to allow for specific binding of the analytes of interest. Like MALDI, SELDI is usually coupled with TOF for protein identification. Using SELDI-TOF, Song *et al*[17] identified 15 proteins that were differentially regulated in the serum samples from 296 gastric cancer patients.

The second type of mass analyzer is the ion trap, in which the ionized analytes are first trapped and then subjected to mass spectrometry. The ion trap is less expensive than the MALDI-TOF analyzer but is still sensitive enough to measure non-abundant analytes. Therefore, until recently, ion traps have been commonly used to obtain a majority of proteomics data, despite their relatively low mass accuracy[10].

The Fourier transform (FT) mass spectrometer is an advanced ion trap mass analyzer that exploits strong magnetic field to measure the *m/z* of ions. FT mass spectrometry boasts sensitivity, accuracy, resolution, and dynamic range[18]. These advantages make FT mass spectrometry suitable for analyzing proteins in a complex mixture. However, its application in proteomics has largely been hindered by its cost and difficulties in operation and maintenance.

The Orbitrap analyzer, a variant of the FT mass spectrometer, made its debut in 2005. Like the FT mass spectrometer, the Orbitrap mass analyzer converts the image currents produced in a trap to mass spectra by Fourier transform[19]. Orbitrap uses electrostatic forces rather than a magnetic field and, thus, does not require the expensive superconducting magnets used in FT mass spectrometers. Orbitrap is currently widely used in proteomics research and is chosen for complex proteome analysis.

Fragmentation

"Shotgun proteomics" resulted from the coupling of high-performance liquid chromatography with ESI technology[20]. In this approach, the proteome subject to mass analysis is first digested with a specific protease(s), such as trypsin. The resulting digest composed of proteolytic peptides from the entire proteins in the sample is separated by liquid chromatography before the first mass analysis of the intact peptides (MS1). Additional information about the parent peptide ions is obtained by fragmenting the parent peptides with non-proteolytic methods and measuring the *m/z* of product ions in the second mass analysis (MS2)[21].

This non-proteolytic fragmentation step is a key to peptide identification, as the amino acid sequence information is inferred from the mass spectra of the fragmented peptides[21]. The most common method used to generate fragment ion spectra of the selected precursor ions is collision-induced dissociation (CID)[22]. Electron transfer dissociation (ETD), an alternative fragmentation technique, has some advantages over CID in accurately assessing post-translational modifications such as glycosylation and phosphorylation, because ETD tends to preserve these modifications when the modified peptide is fragmented[23].

QUANTIFICATION METHODS

Instead of providing mere lists of proteins, quantitative proteomics can deliver information about the differences in proteomes between two samples, and this information may be more useful for studying biological and biochemical processes. The development of quantitative proteomics owes much to the unique labeling strategies that enables a mass spectrometer to distinguish the same proteins or peptides from different samples. These labeling strategies are designed such that labeling causes a known mass shift in the labeled protein or peptide in the mass spectrum.

In general, differentially labeled samples are mixed and analyzed in the same mass spectrometric run, where the differences in the peak intensities of the labeled peptide pairs are assumed to reflect the differences in the abundance of the corresponding proteins. For instance, it is possible to compare the proteomes from normal and cancerous tissues using this approach. Quantitative mass spectrometry-based proteomics approaches include stable isotope labeling techniques and label-free strategies.

Stable isotope labeling approaches

Stable isotope labeling entails the incorporation of stable heavy atoms such as ^{13}C and ^{15}N into specific biomolecular entities, as previously reviewed[24]. In most cases, these labels are chemically or metabolically introduced into peptides or proteins. One of the first chemical labeling strategies adopted for protein quantification by mass spectrometry was the isotope-coded affinity tag (ICAT) technique[25]. In this approach, the sulfhydryl groups in cysteine residues are covalently modified by the ICAT reagents containing "light" or "heavy" isotopes. The presence of the light or heavy ICAT tags leads to the separation and concomitant quantification of modified peptides during the precursor ion measurements in the first mass spectrometry process (MS1).

Recently, chemical labeling strategies utilizing isobaric tags, *i.e*., tags with the same molecular weight, developed for relative and absolute quantification (iTRAQ)[26] and tandem mass tags (TMT)[27] have gained popularity in proteomics. iTRAQ and TMT reagents differ from ICAT reagents in that the ε-amino group of lysine and α-amino group of the N-terminal residue in peptides are modified. The labeled peptides are quantified during the second mass spectrometry process (MS2) when the tags are released upon fragmentation of the peptides (Figure 1). Advantages of the isobaric tags include a multiplexing capacity of up to eight separate samples in a single mass spectrometric run. Additionally, because isobaric tags modify amino groups, which are more abundant than sulfhydryl groups in most proteins, the coverage of quantification by iTRAQ and TMT is also greater than by ICAT.

A large number of studies quantifying the proteomes of gastric cancer using the iTRAQ approach have been reported. Morisaki *et al*[28] applied iTRAQ to identify potential biomarkers in gastric cancer stem cells and identified nine proteins that were overproduced in gastric cancer stem cells. Using iTRAQ, Subbannayya *et al*[29] defined a set of potential biomarkers in sera from gastric cancer patients. In their study, more than 50 proteins were found to exhibit altered levels in samples from gastric cancer patients.

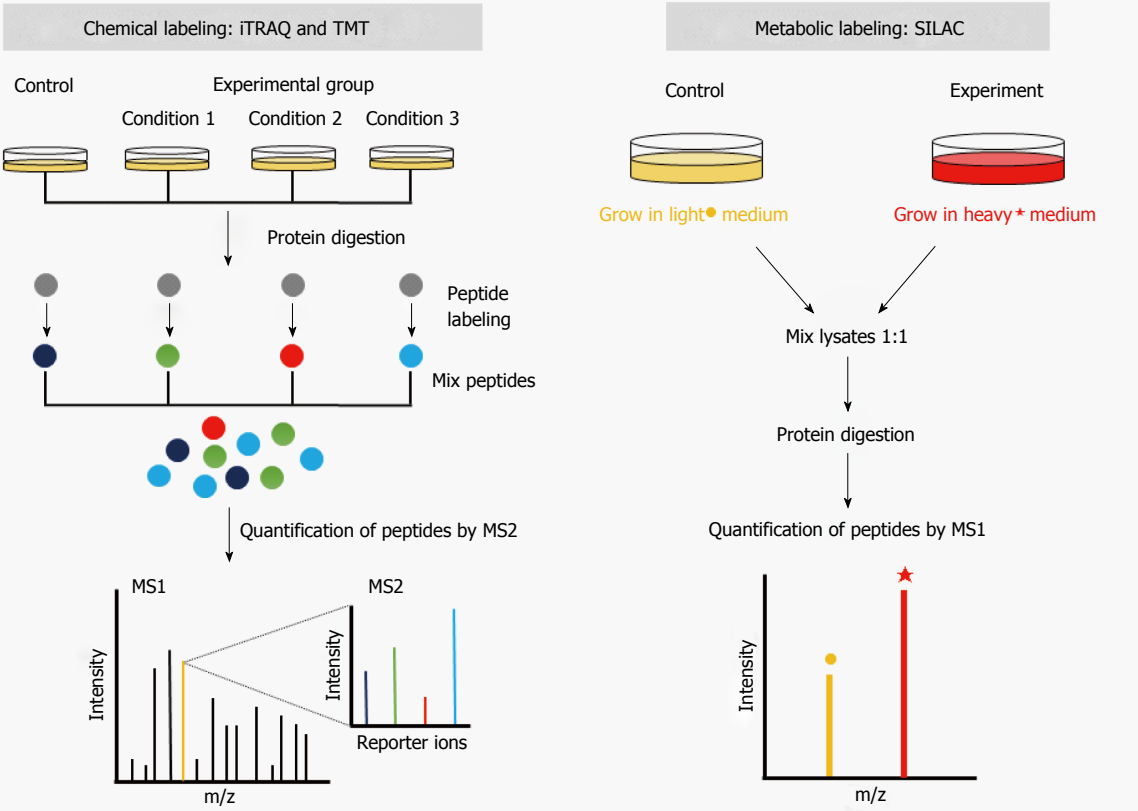

Figure 1 Schematic overview of labeling strategies used in quantitative proteomics. Chemical labeling utilizes the isobaric tags for relative and absolute quantification (iTRAQ) and the tandem mass tags (TMT). In this approach, proteolytic peptides from separate samples are labeled with discrete isobaric tags and pooled. Precursor peptide ions are fragmented (MS2) to generate reporter ions with distinct m/z, whose relative intensities represent the relative abundances of the peptides producing the corresponding reporter ion. Metabolic labeling represented by the stable isotope labeling with amino acids in cell culture (SILAC) strategy takes advantage of the metabolic incorporation of heavy amino acids into mature proteins. In this strategy, the relative peak intensities (MS1) represent the abundances of the precursor peptide ions.

TMT has also been used to quantify gastric cancer proteomes. Gao *et al*[30] found that 234 mitochondrial protein genes were differentially expressed in gastric cancer using TMT. In another study employing TMT, Gao *et al*[31] revealed that 82 plasma membrane proteins were dysregulated in gastric cancer.

An alternative to stable isotope labeling technique is metabolic labeling. This approach takes advantage of the metabolic incorporation of heavy isotopes in live cells under culture conditions. Quantification by metabolic labeling is less error-prone than chemical labeling because the labels are introduced before the samples are prepared. Stable isotope labeling with amino acids in cell culture (SILAC)[32] is one of the most popular metabolic labeling techniques (Figure 1). Developed by Ong *et al*[32], SILAC labels proteins in the cells by growing them in the medium containing heavy amino acids. The most common heavy amino acids used in SILAC are lysine-4, lysine-8, arginine-6, and arginine-10. Different combinations of heavy lysines and arginines can be used such that up to three simultaneous quantifications are possible as follows: a light sample (Lys-0 and Arg-0), medium sample (Lys-4 and Arg-6), and heavy sample (Lys-8 and Arg-10).

As trypsin is the most popular protease used for the preparation of peptide mixtures, which cleaves the carboxyl side of lysine or arginine, the use of heavy lysine and arginine in SILAC helps increase the coverage of quantification by ensuring that every peptide analyzed by the mass spectrometer contains at least one heavy amino acid. Like labeling with ICAT, the quantification of proteins labeled with the SILAC approach is carried out by comparing the intensities of precursor peptide ions in the MS1 process. Quantification employing the SILAC method has been applied in gastric cancer proteomics. Marimuthu *et al*[33] studied the secretomes from neoplastic and non-neoplastic gastric epithelial cells using SILAC. The authors identified 263 proteins that were upregulated in gastric cancer-derived cells compared to non-neoplastic gastric epithelial cells.

Label-free approaches

Another quantification strategy used in proteomics, the label-free approach, inherently suffers from low reproducibility caused by experimental errors and requires precise optimization of mass spectrometric instrumentations[34]. Nevertheless, label-free quantification can overcome some of the limitations of stable isotope labeling strategies. For example, the

time-consuming and costly labeling steps can be eliminated, and the sample numbers are not limited by the multiplexing capacity (three for SILAC and eight for iTRAQ or TMT).

Two types of label-free quantification approaches are routinely used. In spectral counting, the relative abundance of a specific protein among the samples is evaluated by the number of tandem mass (MS2) spectra that can be matched to the protein[35]. Employing this approach, Uen *et al*[36] identified biomarker candidates in plasma samples obtained from gastric cancer patients. The authors found 17 proteins with differential expression patterns in gastric cancer.

The second label-free quantitative method requires high-resolution mass spectrometers and quantifies the intensities of the precursor peptide ion, in which the number of possible tryptic peptides from a given protein are often used for normalization[24]. Using this method, Fan *et al*[37] defined novel diagnostic biomarkers for gastric cancer. The authors analyzed serum samples from gastric cancer patients and discovered four deregulated proteins that were differentially expressed in the patients' sera. Another recent label-free quantitative proteomics by Ichikawa *et al*[38] showed the prognostic importance of a tumor suppressor *PML* (promyelocytic leukemia) in treating gastrointestinal stromal tumors.

MEASURANDS OF MASS SPECTROMETRY

Post-translational modifications

In addition to quantifying the global proteome, the analysis and quantitative assessment of post-translational modifications (PTMs) of a proteome is a powerful approach to understanding the signal flux in disease samples, including gastric cancer cell lines and tissues. Mann *et al*[39] have previously reviewed PTM analysis by mass spectrometry. Of the many PTMs, phosphorylation and glycosylation associated with gastric cancer have been studied using quantitative proteomic methodologies. When only a minute fraction of protein is modified at any given time point, the detection and quantification of PTM by mass spectrometry are significant challenges.

Due to these technological limitations, mass spectrometric PTM analysis requires additional sample preparation steps that enrich the modified peptides. For instance, phosphoproteome analyses rely on the enrichment of phosphorylated peptides using an anti-phosphopeptide antibody, immobilized metal affinity chromatography (IMAC), or titanium-dioxide beads[40,41]. Using an immobilized phosphotyrosine-specific antibody, Glowinski *et al*[42] reported 85 different proteins that exhibited altered phosphorylation upon infection with *Helicobacter pylori* (*H. pylori*), an organism strongly linked with gastric cancer. Holland *et al*[43] also found that the phosphorylation of 20 proteins was differentially regulated by *H. pylori* infection using IMAC enrichment approach.

Additionally, protein glycosylation has been mapped in gastric cancer cells. Asparagine, serine, and threonine residues of proteins are glycosylated in the endoplasmic reticulum and Golgi apparatus. Increased glycosylation has been associated with the proliferation and progression of various cancers, and glycans with specific structures have been associated with tumor malignancy[44-46]. Li *et al*[47] performed quantitative proteomic analysis to identify and quantify eleven cell surface N-glycoproteins with differential expression patterns in multidrug-resistant (MDR) gastric cancer and to define the cell surface glycoproteome that is related to resistance to multiple drugs, including vincristine (also known as leurocristine) or doxorubicin (*e.g.*, Adriamycin), which are used to treat gastric cancer.

Secretome and serum proteome

A systematic assessment of the secretome, *i.e.*, all secretory proteins from a cell, may provide crucial insights into cancer biology, as the composition of proteins that are secreted from cancer tissues differs from the proteins that are secreted from normal tissues[48]. The secretome and serum proteome, *i.e.*, all proteins in the blood serum, are considered a major source of cancer biomarkers, and some important regulatory proteins that are secreted into the serum have been used as tumor biomarkers[49].

In a study seeking to characterize the serum proteome from local and invasive gastric cancer, Abramowicz *et al*[50] analyzed serum samples acquired from patients with locally advanced or metastatic cancers and healthy controls. Several proteins with different abundances were detected in cancer patients, with no evidence of differences between the patients with local and invasive cancers. Loei *et al*[51] have compared the secretomes of AGS and MKN7 cells using iTRAQ labeling and found that 43 protein genes were differentially expressed between the two cell lines. Among these proteins, granulin was confirmed by immunohistochemistry to be frequently found in gastric tumor tissues, but it was not found in the normal gastric epithelia.

Interactome

As tumor has been defined as a disease of pathways[52], interactome analysis may offer some valuable insights into cancer biology by offering information beyond the changes in the abundance of individual proteins[53]. In practice, characterizing alterations in protein-protein interactions in cancer is becoming more relevant, as many studies have reported that patients affected by the same type of cancer display diverse protein expression patterns and activation of oncogenic kinases[54-56]. In these cases, classifications based on protein-protein interaction subnetworks

Table 2 Cell line-based summary of recent proteomic analyses of gastric cancer

Cell line	Ref.	Aim of study	Proteins analyzed	Validation[1]
AGS	Holland *et al*[43], 2011	Phosphoproteome upon *H. pylori* infection	20 altered in abundance by *H. pylori*	No
	Loei *et al*[51], 2011	Secretome of AGS and MKN7	43 differed	IHC/WB
	Guo *et al*[68], 2012	Plasma membrane proteome	1473 identified	IHC
	Hou *et al*[69], 2013	Biomarkers for GC metastasis	19 increased and 34 decreased in metastasis	IHC/WB
	Hu *et al*[72], 2013	Global profile of miR-148a-regulated proteins	55 altered by miR-148a	WB
	Marimuthu *et al*[33], 2013	Secretome	263 increased and 45 decreased in GC	IHC
	Yu *et al*[57], 2013	Interactome of VCP	288 putative partners, including 18 PI3K/Akt proteins	WB
	Glowinski *et al*[42], 2014	Tyrosine signaling upon *H. pylori* transfection	85 altered by *H. pylori*	No
	Goh *et al*[60], 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ⩾ 6 cell lines	WB
	Lin *et al*[61], 2015	Tanshinone IIA regulation	102 altered by tanshinone IIA treatment	WB
BGC-823	Cai *et al*[71], 2013	Effects of curcumin on viability and apoptosis	75 altered by curcumin treatment	No
HGC-27	Goh *et al*[60], 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ⩾ 6 cell lines	WB
	Qiao *et al*[62], 2015	Proteomes of three GC cell lines	9 altered	IHC/WB
	Chen *et al*[77], 2016	Proteome with *FAF1* or *H. pylori*	157 altered by *FAF1*, 500 by *H. pylori* and 246 by both	WB
MGC-803	Qiao *et al*[62], 2015	Proteomes of three GC cell lines	9 altered	IHC/WB
MKN7	Loei *et al*[51], 2011	Secretome of AGS and MKN7	43 differed	IHC/WB
	Guo *et al*[68], 2012	Plasma membrane proteome	1473 identified	IHC
	Yang *et al*[70], 2012	Membrane proteome	175 altered	IHC/WB
MKN45	Hu *et al*[13], 2013	Proteome changes following *CXCR1* knockdown	16 increased and 13 decreased by *CXCR1* knockdown	WB
	Yang *et al*[67], 2014	Proteome changes following *NAIF1* overexpression	5 increased and 3 decreased by *NAIF1* overexpression	WB
	Goh *et al*[60], 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ⩾ 6 cell lines	WB
SGC-7901	Li *et al*[47], 2013	Cell surface glycoproteome of MDR	11 altered in MDR cell lines	WB
	Goh *et al*[60], 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ⩾ 6 cell lines	WB
	Qiao *et al*[62], 2015	Proteomes of three GC cell lines	9 altered	IHC/WB

[1]Validation indicates whether a selected set of altered proteins have been validated using immunohistochemistry (IHC) or Western blot (WB) or have not been (No). The cell line studies are listed in the order of publication year and then in alphabetical order by the first author. CXCR1: C-X-C chemokine receptor type 1; FAF1: Fas-associated factor 1; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; MDR: Multidrug resistance; NAIF1: Nuclear apoptosis-inducing factor 1; VCP: Valosin-containing protein.

offered greater accuracy than classifications based on individual marker genes[55].

For this reason, interactome analysis represents an attractive avenue for understanding gastric cancer biology, although its broad applications remain to be established. The first interactome analysis of gastric cancer was performed with valosin-containing protein (VCP), a protein associated with *H. pylori*-induced gastric cancer[57]. In seeking the interacting partner proteins for VCP, Yu *et al*[57] immunoprecipitated VCP and then performed a quantitative mass spectrometric analysis. The authors identified 288 putative binding partners of VCP in the AGS gastric cancer cell line, providing unexpected new insights into the function of *H. pylori* in gastric cancer.

Targeted proteome

In targeted proteomics, prior knowledge of analytes is necessary, which is an attribute not essential for the aforementioned discovery-based proteomics. In this regard, targeted proteomics is similar to immunoassays, in which antibodies recognize and identify specific proteins. Targeted proteomics is emerging as an alternative approach to discovery proteomics or immunoassays, particularly when pre-defined analytes are present at low levels and no reliable antibodies are available.

Selected reaction monitoring (SRM) is the most common approach used for targeted proteome measurements and requires a triple-quadrupole mass spectrometer, as previously reviewed by Picotti *et al*[58] In SRM, a peptide precursor ion from a specific protein with a particular *m/z* is selected in the first phase of tandem mass spectrometry, and a signature product ion is produced by fragmenting the precursor ion and detected by the second phase of mass spectrometry.

The sensitivity and reproducibility of SRM are greater than conventional discovery-based mass spectrometry because only a set of predefined proteins is programmed to be analyzed by the SRM mass spectrometer. Another advantage of SRM lies in its speed. After SRM assays have been defined, they

are significantly faster than a typical discovery-based mass spectrometry. In addition, the measurements can be multiplexed such that one can measure hundreds or even thousands of peptides in a single mass spectrometric run. The SRM approach has been successfully exploited to test the specificity of afamin, clusterin, haptoglobin, and vitamin D-binding protein as potential serum biomarkers of gastric cancer[59].

Gastric cancer cell lines

Several gastric cancer cell lines have been subjected to recent mass spectrometric analyses as a model mimicking gastric cancer. These studies are summarized in Table 2. In a recent study aimed at defining the proteomes of gastric cancer, Goh *et al*[60] have quantified the membrane proteomes of eleven gastric cancer cell lines, including AGS, HGC-27, MKN45, and SGC-7901 cells. A total of 882 proteins were detected, and 57 proteins were upregulated, with a greater than 1.3-fold change in at least six of the eleven cell lines. Depletion of DLAT, a subunit of the pyruvate dehydrogenase complex that was upregulated, reduced cell proliferation. This study contributed to the recent interest and discussion in cancer energetics and related phenomena, such as the Warburg and reverse Warburg effects.

In another proteomic analysis of AGS cells, the most intensively studied cell line in gastric cancer proteomics, Lin *et al*[61] sought to elucidate the mechanism of tanshinone IIA (TIIA) regulation. TIIA is a plant extract used in traditional Chinese herbal medicine that has been reported to have anti-tumor potential against gastric cancer. The authors reported that the cellular levels of the 102 unique proteins were altered upon TIIA treatment.

Other gastric cancer cell lines have also been adopted for proteomic analysis. Qiao *et al*[62] have quantified the proteomes from SGC-7901, HGC-27, and MGC-803 cells. The authors have found that filamin c and a large actin-cross-linking protein were significantly downregulated, establishing functional roles for these proteins in gastric cancer.

PROTEOMIC BIOMARKERS

Quantitative proteomic analyses can provide information on proteins that are differentially abundant in cancerous tissues. These proteins, if verified, may serve as biomarkers for the diagnosis and prognosis of cancer and could be extremely useful for clinical purposes. A mass spectrometry-based cancer biomarker study typically starts with the aforementioned discovery-based proteomics by assessing the differences in the proteome profiles in small cohorts or model systems.

Once candidate biomarkers are identified, orthogonal methodologies, such as antibody-based assays, are applied for biomarker validation and verification[63]. An increasing number of studies have adopted mass spectrometry to identify gastric cancer biomarkers recently and are reviewed in detail by Tsai *et al*[64], Liu *et al*[65], and Lin *et al*[66] We briefly discuss some cases in which the quantitative proteomic approaches described in this review are applied.

In an effort to identify membrane-originated biomarkers of gastric cancer, Yang *et al*[67] compared the relative abundances of membrane proteins from gastric cancer and control samples using the iTRAQ technique. Upregulation of the plasma membrane protein SLC3A2 in gastric cancer cells was validated by immunoblotting of a panel of thirteen gastric cancer cell lines and immunohistochemistry on tissue microarrays comprising 85 matched pairs of normal and tumor tissues.

Plasma membrane proteomes, including cluster of differentiation proteins and receptor tyrosine kinases, have been the subject of another biomarker search. A proteomic investigation by Guo *et al*[68] showed that four proteins, MET proto-oncogene receptor tyrosine kinase (MET), ephrin type A receptor 2 (EPHA2), fibroblast growth factor receptor 2 (FGFR2), and integrin beta 4 (ITGB4), were upregulated in tumor tissues from 90% gastric cancer patients. Furthermore, three of them, MET, EPHA2, and FGFR2, were upregulated in all intestinal-type gastric cancers from this cohort.

In another attempt to identify potential biomarkers of gastric cancer, Marimuthu *et al*[33] quantified the secretome of gastric cancer by SILAC. The authors were able to identify and validate several gastric cancer biomarkers, including proprotein convertase subtilisin/kexin type 9, lectin mannose binding protein 2, and PDGFA-associated protein 1.

Biomarker candidates for gastric cancer metastasis have also been identified by quantitative proteomics. Hou *et al*[69] compared metastatic and non-metastatic gastric cancer cell lines with iTRAQ methods. The authors discovered that caldesmon was downregulated in metastasis-derived cell lines, which was confirmed by a further analysis of seven gastric cancer cell lines. In this study, knockdown of caldesmon in gastric cancer cells lead to an increase in cell migration and invasion, whereas upregulation of caldesmon resulted in a decrease in the phenotype.

CONCLUSION

Due to the progress made in mass spectrometry and quantitative proteomics over the past decade, it is now possible to probe thousands of proteins in a complex proteome. These technological advances include streamlined sample preparation, novel labeling strategies, and improved instrumentation, all of which contribute to the identification of gastric cancer-specific biomarkers, with increasing sensitivity and accuracy. Armed with these advanced proteomics technologies, research endeavors are seeking precise assessments of protein abundance, PTM, and protein-protein interactions that could help define the molecular

signatures of gastric cancer susceptibility.

One remaining question is how these state-of-art technologies can be used in clinics and can make a bigger impact on the real-world management of gastric cancer. In this regard, one could envision the entrance of targeted proteomics into the realm of personalized diagnostics and medicine. Targeted proteomics can provide sensitivity and reproducibility, the core requirements for the technology to be applied to these new and exciting fields. In this scenario, once the key molecular determinants of gastric cancer are defined with time-consuming, discovery-based proteomics, targeted proteomics approaches, such as SRM, could be utilized for the rapid and reproducible monitoring of these key molecules and their networks for individual diagnostics or analyses of treatment responses.

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MINIREVIEWS

Hepatocellular carcinoma in patients with non-alcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the United States and represents an increasingly important etiology of hepatocellular carcinoma (HCC) with annual cumulative incidence rates ranging from 2% to 12% in cohorts of NAFLD cirrhosis. While the risk of progression of NAFLD to HCC remains higher among patients with fibrosis or cirrhosis, an increasing amount of literature describes NAFLD-HCC as a disease that can occur in the absence of cirrhosis. Efforts to characterize the pathogenesis of NAFLD-HCC have suggested mechanisms that strongly associate with states of hyperinsulinemia and chronic inflammation, cellular mechanisms including adaptive immune responses and hepatic progenitor cell populations, and genetic polymorphisms including mutations of PNPLA3. Current literature describes NAFLD-HCC mostly as a disease of late presentation with lower rates of receipt of curative therapy and worse prognosis. However, a growing body of evidence has reported comparable and potentially more favorable disease-free and overall survival rates among patients with NAFLD-HCC after receipt of curative treatment. This review summarizes current evidence of epidemiology, pathophysiology, disease presentation, demand and receipt of curative therapy, post-treatment outcomes, and overall survival of NAFLD-associated HCC.

Key words: Fatty liver; Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Liver cirrhosis; Liver cancer

Core tip: This review summarizes the epidemiology, pathogenesis, disease presentation, demand and receipt of curative treatment, and post-treatment outcomes of hepatocellular carcinoma (HCC) in nonalcoholic fatty liver disease (NAFLD). The review highlights the developing understanding of NAFLD-HCC pathogenesis, which has broadened to include genetic polymorphisms, adaptive immune responses, and cellular regenerative pathways using hepatic progenitor cell populations. While NAFLD-HCC has been described to have poorer prognosis as compared with other HCC etiologies, this review features summarized evidence that disease-free and survival rates among patients with NAFLD-HCC are comparable and potentially favorable after receipt of curative treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and third most common cause of cancer-related mortality worldwide[1-3]. In the United States, the overall incidence of HCC has increased approximately four-fold from 1.5 cases per 100000 in 1973 to 6.2 cases per 100000 in 2011[3]. Rates of mortality are similar to rates of incidence of HCC in the United States[1,3]. Based on recent analysis using the Surveillance, Epidemiology, and End Results (SEER) 18 database, the estimated annual incidence of HCC is 6.2 cases per 100000 and the incidence-based mortality rate is 5 cases per 100000[3]. A recent United States report revealed that while the overall rate of cancer-related deaths has declined, mortality from liver cancer has increased at the highest rate as compared with all other reported cancers from 2003 to 2012[4]. While most cases of HCC in the United States are attributable to chronic hepatitis C virus (HCV) infection, there has been growing evidence to suggest that nonalcoholic fatty liver disease (NAFLD) is projected to become a leading cause of HCC incidence and mortality[5-7]. A recent study by Younossi *et al*[7] revealed that the annual incidence of NAFLD-HCC based on a SEER- Medicare cohort has grown by 9% per year from 2004 to 2009.

The prevalence of NAFLD-HCC is expected to rise in concurrence with the proportion of NAFLD among adults in the United States population. In the Western world, NAFLD is already the most common chronic liver condition with an estimated prevalence of 30% in the United States, although a previous cohort has reported prevalence as high as 46%[6,8-10].

The prevalence of NAFLD will continue to grow in a setting of increasing rates of obesity, diabetes, dyslipidemia, and other disorders associated with the metabolic syndrome which are closely related to NAFLD[6,8-16].

The risk of progression to cirrhosis among patients with NAFLD is approximately four to 20% depending on the severity of necrosis and fibrosis[6,17]. Although the risk of developing NAFLD-HCC from NAFLD cirrhosis is not clearly delineated, the cumulative incidence of HCC from NAFLD cirrhosis has been reported as 2.4% and 12.8% over a median follow-up of 3.2 to 7.2 years[16,18,19]. A large single-center cohort study reported an annual HCC incidence of 2.6% among patients with NAFLD cirrhosis[18].

NAFLD-HCC without cirrhosis

While previous studies have described fibrosis progression in NAFLD and its risk of cirrhosis and HCC, recent reports increasingly recognize NAFLD-HCC as an entity that can occur in the absence of cirrhosis[6,16,20-27]. In fact, up to 50% of incident NAFLD-HCC may occur without cirrhosis[6,28]. In a recent Veterans Administration (VA) study, 34.6% of patients with NAFLD-HCC did not have diagnostic evidence of cirrhosis[23].

Notably, liver biopsy was performed in 52.4% of the study cohort[23]. While only 8% of patients in this cohort had NAFLD, the study demonstrated that patients with NAFLD had the greatest odds of developing HCC in the absence of cirrhosis in comparison to other etiologies including chronic hepatitis B virus (HBV), HCV, and alcoholic liver disease (ALD) (adjusted odds ratio 3.9 when compared with HCV)[23]. Another large United States study comprised of three tertiary care centers identified 157 patients, who underwent surgical resection for HCC, with histological evidence of non-cirrhotic hepatic steatosis after excluding patients with viral hepatitis, alcohol abuse, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, hemochromatosis, alpha-1-antitrypsin deficiency, and Wilson disease[20]. The study revealed that 80% of cases were associated with stage 0 liver fibrosis and only 15% had steatohepatitis[20].

PATHOPHYSIOLOGY

Although our understanding of the pathogenesis of NAFLD-HCC remains limited, several proposed mechanisms have been described including chronic inflammation in the setting of hyperinsulinemia or metabolic syndrome, cellular mechanisms including hepatic progenitor cells and adaptive immune responses, and genetic polymorphism including variations of patatin-like phospholipase domain-containing protein 3 (PNPLA3) (Figure 1).

Hyperinsulinemia

Carcinogenic features of the metabolic syndrome

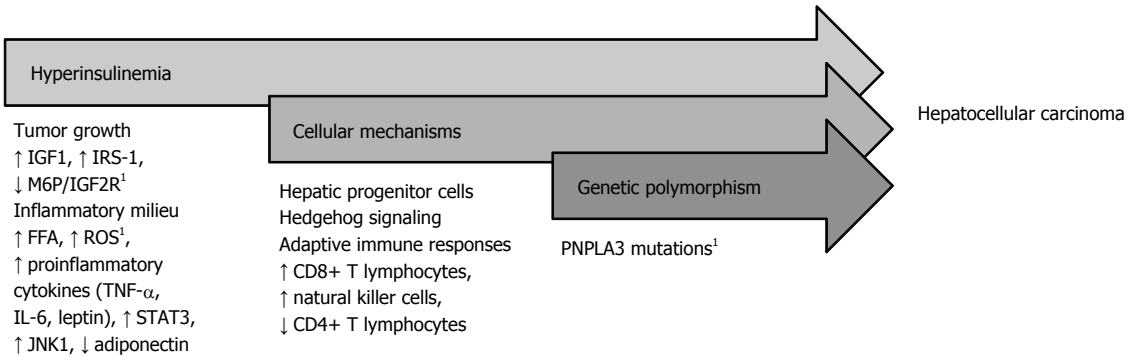

Figure 1 Proposed mechanisms of nonalcoholic fatty liver disease-hepatocellular carcinoma. [1]Mechanism has been identified in nonalcoholic fatty liver disease in the absence of cirrhosis. IGF-1: Insulin-like growth factor-1; IRS-1: Insulin receptor substrate-1; M6P/IGF2R: Mannose 6-phosphate/insulin-like growth factor-2 receptor; FFA: Free fatty acids; ROS: Reactive oxidative species; TNF-α: Tumor necrosis factor-alpha; IL-6: Interleukin-6; STAT3: Signal transducer and activator of transcription; JNK1: c-Jun amino-terminal kinase 1; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

including uninhibited tumor growth, chronic inflammation, increased production of proinflammatory cytokines like c-Jun amino-terminal kinase 1 (JNK1), and reduction of anti-inflammatory proteins like adiponectin are mechanisms seen in NAFLD. NAFLD is a hepatic manifestation of the metabolic syndrome and considered a mechanism through which metabolic syndrome could lead to HCC[29].

Tumor growth: Uninhibited and dysregulated cell growth in the setting of hyperinsulinemia has been described as a function of insulin-like growth factor-1 (IGF-1), mannose 6-phosphate/insulin-like growth factor-2 receptor (M6P/IGF2R), and insulin receptor substrate-1 (IRS-1)[14,30,31]. IGF-1, a peptide hormone upregulated in hyperinsulinemia, facilitates cellular proliferation and inhibits cell death[14]. The M6P/IGF2R, a tumor- suppressor gene, regulates cell growth by inhibiting cell proliferation and promoting apoptosis *via* transforming growth factor-beta and insulin-like growth factor-2, respectively[31]. Interestingly, mutations of M6P/IGF2R are seen in HCC and have been identified even in the absence of viral hepatitis and liver cirrhosis[31]. IRS-1, an intracellular protein that promotes cell growth *via* cytokine signaling, is overexpressed (> 200%) in HCC tumors, which induces a downstream signaling effect that promotes tumor cell growth and enhances tumor progression[30].

Inflammatory cascade: Hyperinsulinemia also triggers an inflammatory milieu involving free fatty acids (FFA), proinflammatory cytokines, reactive oxidative species (ROS), JNK1, and adiponectin[14,32-35]. A high insulin state promotes release of FFA from adipocytes, which promotes steatosis, and in the setting of inflammation fostered by proinflammatory cytokines, can lead to cirrhosis and HCC[14].

Accelerated production of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and leptin, promotes a chronic cycle of hepatocyte injury, apoptosis, and compensatory proliferation in conditions of inflammation and oxidative stress that can lead to mutations, dysplasia, and eventually carcinogenesis[14]. A study by Park *et al*[33] described HCC development in the setting of obesity as a function of enhanced TNF-α and IL-6 expression. In addition to causing hepatic inflammation, both cytokines activate signal transducer and activator of transcription 3 (STAT3), an oncogenic transcription factor, which can enhance proliferation and progression of hepatocytes that can acquire oncogenic mutations that lead to tumor development[33]. Interestingly, leptin has been described in liver carcinogenesis *via* upregulation of telomerase reverse transcriptase leading to immortalization of tumor cells in HCC[34].

ROS are implicated in carcinogenesis through dysregulation of the cell cycle[35]. A mouse model demonstrated that mitochondria in fatty livers have accelerated production of superoxide anions as compared with mitochondria in normal livers[35]. A case report from Japan described a patient without cirrhosis and with NAFLD-associated HCC that had localized markers of oxidative cellular injury (*e.g*., anti-oxidized phosphatidylcholine) in tumorous and non-tumorous steatotic hepatocytes, which suggests that the prevalence and persistence of oxidative injury in the setting of steatosis is related to the development of HCC[36]. Therefore, the presence of increased oxidative species as a suspected function of chronic cell stress is associated with cell injury, apoptosis, and compensatory proliferation, which can enhance mutation formation and cancer cell growth.

JNK1: The combination of proinflammatory cytokines, ROS, and FFA activates JNK1, a protein kinase that has increased activity in the setting of obesity and insulin resistance, and in turn phosphorylates IRS-1[32]. JNK1 has been demonstrated to promote development and proliferation of HCC through epigenetic mechanisms[37]. Chang *et al*[37] described the association of JNK1 activity with alterations in histone H3 methylation in HCC, which leads to sustained

expression of genes that promote cell proliferation and repression of genes responsible for cell differentiation and tumor suppression.

Adiponectin: A decreased amount of adiponectin, an anti-inflammatory polypeptide, in the setting of obesity has also been described in HCC pathogenesis[14,38]. Adiponectin has been identified as a negative regulator of angiogenesis and inhibitor of primary tumor growth *via* decreased neovascularization and enhancement of tumor cell apoptosis[38]. Repression of adiponectin activity in the setting of hyperinsulinemia allows uninhibited tumor cell growth, which can subsequently lead to HCC in NAFLD.

Cellular mechanisms

Hepatic progenitor cell populations: NAFLD-related hepatocyte injury induces Hedgehog signaling, a complex cellular pathway for tissue repair and regeneration. One of the main mechanisms activated through Hedgehog signaling includes mobilization of hepatic progenitor cell populations to replace damaged hepatocytes. While essential for liver repair, aberrations in liver progenitor population activation can lead to impaired repair and dysregulated proliferation of hepatocytes which can potentiate carcinogenesis[39,40].

Current data suggest that greater duration and degree of cell injury as seen in NAFLD lead to overstimulation of Hedgehog signaling, which can result in dysregulated cellular repair and malignant transformation[40,41]. The development of HCC has been described as a function of aberrant Hedgehog hyperactivity as progenitor cells activated through Hedgehog could survive independently from regulation of nuclear localization of nuclear factor-kappa B (NF-κB) and thereby less susceptible to NF-κB-driven apoptosis[42].

Adaptive immune responses: Recent studies have revealed the potential role of the adaptive immune system, specifically the roles of CD8+ T lymphocytes, natural killer cells, and CD4+ T lymphocytes in the development of NAFLD-associated HCC[43,44]. From feeding mouse models with choline-deficient and high-fat diets, Wolf *et al*[44] described the metabolic activation of intrahepatic CD8+ T lymphocytes and natural killer cells, which in turn, synergistically with inflammatory cytokines, cause liver damage and induce carcinogenesis. Ma *et al*[43] demonstrated in mouse models of NAFLD-HCC that selective CD4+ T lymphocyte depletion results from NAFLD and leads to enhanced carcinogenesis. Hepatocytes derived from NAFLD-HCC mice exhibited increased linoleic acid secretion, which promoted selective CD4+ T lymphocyte death through increased mitochondrial-derived ROS generation[43]. While deregulated lipid metabolism and inflammation have been characterized in NAFLD, the role of hepatocyte-derived lipid secretion causing selective CD4+ T lymphocyte loss highlights a novel function of the adaptive immune system in this disease.

Genetic polymorphism

Variation in PNPLA3: Another mechanism that has been associated with the pathogenesis of NAFLD-HCC focuses on genetic risk, specifically variation in PNPLA3. A genome-wide association analysis of 9229 genetic variants of PNPLA3 revealed that homozygous carriers of the I148M variant protein of PNPLA3 had a > 2-fold higher hepatic fat content than noncarriers[45]. Furthermore, the I148M variant protein was more prevalent in the group at highest risk of NAFLD[45]. Similarly, a study by Liu *et al*[46] identified that the PNPLA3 rs738409 C→→G single nucleotide polymorphism, which encodes the I148M variant protein, demonstrated a gene-dosage effect in which an increased number of G alleles present (*i.e.,* homozygous G allele) was associated with an increased incidence of NAFLD-HCC with an odds ratio as high as 12.19 when compared with the general UK population. Interestingly, the PNPLA3 rs738409 C→→G polymorphism was associated with NAFLD-HCC risk independent of the presence of cirrhosis[46].

PRESENTATION AT DIAGNOSIS

Patients with NAFLD-HCC generally have a more advanced presentation of HCC at the time of diagnosis[14,28,47,48]. A VA cohort study by Mittal *et al*[47] revealed that in comparison to HCV-related HCC, fewer cases of NAFLD-HCC were diagnosed in early HCC stages or Barcelona Clinic Liver Cancer (BCLC) stage A (5.8% *vs* 15.7%, $P = 0.04$) and more cases were diagnosed in terminal stages of HCC or BCLC stage D (19.2% *vs* 16.1%, $P = 0.04$). A recent study by Piscaglia *et al*[28] also revealed a significantly greater proportion of patients with NAFLD-HCC who presented in advanced stages as compared with patients with HCV-HCC (BCLC stage C 33.1% *vs* 23.9%, respectively, $P = 0.033$).

Advanced stage at presentation among patients with NAFLD-HCC is likely attributable to delayed diagnosis in the setting of suboptimal HCC surveillance among patients with cirrhosis and no surveillance among patients without cirrhosis or unrecognized cirrhosis[28,47]. In an Italian cohort study, a significantly lower proportion of patients with NAFLD-HCC underwent surveillance as compared with those with HCV-HCC (47.6% *vs* 63.3%, $P = 0.001$)[28]. In the United States, Mittal *et al*[47] reported that fewer patients with NAFLD-HCC had surveillance within three years before their HCC diagnosis as compared with patients with ALD and HCV. Approximately 43% in the NAFLD-HCC group had HCC surveillance within three years of diagnosis as compared with 60% and 87% in the ALD- and HCV-

Table 1 Summary of survival outcomes for presumed nonalcoholic fatty liver disease-hepatocellular carcinoma undergoing curative therapies

First author	Study design	Study period	Study location	Number of NAFLD-HCC cases	Treatment[1]	Survival outcomes	
						Overall survival	Disease-free survival
Reddy	Retrospective cohort	2000-2010	Single-centered (United States)	52	OLT, resection, RFA[2]	1-yr: 90%[3] 3-yr: 72%[3] 5-yr: 65%[3]	1-yr: 84%[3] 3-yr: 70%[3] 5-yr: 60%[3]
Wong	Retrospective cohort	2002-2012	Multi-centered (United States)	NA[4]	OLT	1-yr: 87.5% 3-yr: 79.8% 5-yr: 65.5%	NA
Piscaglia	Prospective cohort	2010-2012	Multi-centered (Italy)	49	OLT, resection, RFA	1-yr: 90%-95%[3] 3-yr: 85%-90%[3]	NA
Malik	Retrospective cohort	NA	Single-centered (United States)	17	OLT	1-yr: 85%-90%[3]	NA
Hernandez-Alejandro	Retrospective cohort	2000-2011	Single-centered (Canada)	17	OLT	NA	1-yr: 95%[3] 3-yr: 95%[3] 5-yr: 85%[3]
Cauchy	Retrospective cohort	2000-2011	Single-centered (France)	62[5]	Resection	1-yr: 83% 3-yr: 75%	1-yr: 83% 3-yr: 70%
Wakai	Retrospective cohort	1990-2007	Single-centered (Japan)	17	Resection	5-yr: 59%	5-yr: 66%
Takuma	Retrospective cohort	1992-2009	Single-centered (Japan)	36[6]	Resection, RFA, PEI, MCT	1-yr: 94% 3-yr: 85% 5-yr: 54%	1-yr: 89% 3-yr: 68% 5-yr: 54%

[1]Total cohort underwent one treatment unless otherwise specified; [2]Four patients had both RFA and resection; [3]Survival rates were not reported in the text; [4]Total number of NAFLD-HCC cases was not reported as analysis of outcomes among HCC cases secondary to NAFLD was done in a subanalysis. Total HCC cases in the cohort was 5326; [5]Metabolic syndrome was used as a surrogate for NAFLD after exclusion of chronic hepatitis B or C infection, excessive alcoholic consumption, alcoholic liver disease, and hemochromatosis; [6]Crytogenic cirrhosis was used as a surrogate for NAFLD after exclusion of chronic hepatitis B or C infection, alcohol consumption, primary biliary cholangitis, autoimmune hepatitis, primary sclerosing cholangitis, alpha 1-antitrypsin deficiency, Wilson's disease, and hemochromatosis. Estimates are determined from the Kaplan Meier figures. NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; OLT: Orthotopic liver transplantation; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; MCT: Microwave coagulation therapy; NA: Data not available.

related HCC groups, respectively[47].

CURATIVE TREATMENTS AND OUTCOMES

Demand and receipt of curative treatment

Recipients of curative treatment, which includes orthotopic liver transplantation (OLT), resection, and radiofrequency ablation (RFA), have favorable survival outcomes[3]. In concurrence with the growing incidence of NAFLD-HCC, studies have reported an increasing demand for curative treatment, especially OLT and resection[49,50]. NAFLD has become the second leading etiology among OLT cases for HCC since 2006[49]. Wong *et al*[49] reported that OLT for NAFLD-HCC has increased four-fold in the United States from 2002 to 2012. Over the past decade, the proportion of resections of HCC in the setting of NAFLD has also increased[50]. A study by Cauchy *et al*[50] demonstrated a continuous increase in the number of patients undergoing resection for HCC from 2.5% in 2000 to more than 15% in 2011.

Interestingly, patients with NAFLD-HCC have been recognized as less likely recipients of curative therapy[7,47]. In a VA cohort, only 10.8% of the NAFLD-HCC group received curative treatment as compared with 21.9% of the HCV-related HCC group[47].

Furthermore, in comparison to the HCV-related group, more patients in the NAFLD- HCC group received no treatment (77.5% *vs* 61.5%)[47]. An Italian study showed that fewer patients with NAFLD-HCC received resection as compared with patients with HCV-HCC (19% *vs* 11%, $P = 0.002$)[28]. A large United States population-based study using SEER data revealed that 5.7% of patients with NAFLD-HCC received OLT as compared with 11.3%, 6.4%, and 6% of patients with HCC secondary to HCV, HBV, and ALD, respectively[7]. Similarly, a European cohort revealed that none of its 45 cases of NAFLD-HCC received OLT as primary therapy[48].

Survival outcomes from curative treatment

Data on outcomes of patients who receive curative therapy for NAFLD-HCC remain scarce as most available studies are limited to small sample sizes, surrogate diagnoses of NAFLD (*i.e.*, metabolic syndrome, cryptogenic cirrhosis), or grouped treatment outcomes (*i.e.*, OLT, resection, RFA combined). Based on the available data, the recurrence and survival rates of NAFLD-HCC appear similar to other etiologies of HCC who undergo treatment with curative intent[28,50-56] (Table 1).

OLT for NAFLD-HCC: A United States cohort study of patients who underwent curative treatment for HCC identified 20 patients who received OLT for NAFLD-HCC and 83 who received OLT for HCC secondary to HCV and/or ALD[52]. No significant difference existed in disease-free survival and overall survival in patients who received OLT for HCC over a median follow-up of 50 mo[52].

In a retrospective cohort using United Network for Organ Sharing data from 2002 to 2012, Wong *et al*[54] reported comparable survival rates at one, three, and five years between those who received OLT for HCC secondary to NAFLD, HCV, ALD, or HCV and ALD (P = 0.99). Survival at one year post-OLT for HCC was 87.5% for nonalcoholic steatohepatitis (NASH) (*vs* 88.5% for HCV, 90.2% for ALD, 88.7% for HCV and ALD)[54]. At three years, survival for NAFLD-HCC was 79.8% as compared with 73.9%, 73.3%, and 75% for HCV, ALD, and HCV and ALD, respectively[54]. Five- year survival data remained similar between all etiologies of HCC in the cohort: 65.5% for NAFLD, 65.7% for HCV, 63.9% for ALD, and 65.7% for HCV and ALD[54]. Rates of graft survival were also not significantly different between all HCC etiologies, and patient survival was similar across groups (84.3%, 77%, and 63.1% at year one, three, and five, respectively for NAFLD-HCC)[54].

Another United States cohort study by Malik *et al*[51] retrospectively reviewed 17 patients with NASH-related cirrhosis who underwent OLT with and without known HCC. When compared with the group of patients with NASH-related cirrhosis without HCC, the group with NAFLD-HCC had similar survival (88%) over a mean follow-up period of 2.5 years[51].

A Canadian retrospective cohort study described disease-free survival in 81 cases of liver transplant recipients, which included 64 with HCV-related HCC and 17 patients with NAFLD-HCC[55]. Over a 10-year follow-up period, disease-free survival in the HCV and NAFLD groups were approximately 65% and 85%, respectively (P = 0.11)[55]. While the NAFLD group had a similar number and cumulative size of tumors as compared with the HCV group, the NAFLD group had a significantly lower proportion of patients with vascular invasion and poorly differentiated HCC[55]. Therefore, NAFLD-HCC can have favorable, and potentially better tumor recurrence rates than their HCV counterparts as demonstrated by their trend towards greater disease-free survival (85% *vs* 65%) in the setting of less aggressive HCC presentation at diagnosis.

OLT for NAFLD-cirrhosis: Literature on post-OLT outcomes among those who undergo transplantation for NAFLD-HCC remains limited. Given the comparable rates of post-OLT survival between those who undergo transplantation for NALFD-related cirrhosis without HCC and those with HCC, inclusion of a few relevant studies that assess post-OLT outcomes in the setting of NASH and not specifically HCC can provide supplementary information[57-59].

A retrospective cohort study at a single large transplant center in the United States compared post-OLT outcomes for NASH cirrhosis and alcoholic cirrhosis[57]. Estimates of patient survival at nine years post-OLT were comparable between those who received transplantation for alcoholic cirrhosis or biopsy- or ultrasound-confirmed NASH (P = 0.30)[57]. Survival at one, three, five, and nine years were 78%, 78%, 78%, and 52%, respectively, in the NASH group *vs* 92%, 86%, 86%, and 76%, respectively, in the alcoholic cirrhosis group. Rates of graft failure were similar between both groups (24% in NASH *vs* 18% in alcoholic cirrhosis group, P value 0.40)[57]. Acute rejection (41% *vs* 23%) and recurrent steatohepatitis (33% *vs* 0%) were significantly more common in the NASH group as compared with the alcohol group; however, neither complication was associated with higher rates of re-transplantation[57].

A United States cohort study by Yalamanchili *et al*[58] retrospectively identified patients who underwent OLT for cryptogenic cirrhosis (n = 239) or NASH-related cirrhosis (n = 18) after biopsy confirmation and exclusion of HCV and alcoholic cirrhosis. In comparison to a miscellaneous group that included other indications for OLT, survival at one, five, 10, and 20 years was similar (85.6% *vs* 86.3%, 71.4% *vs* 69.9%, 56.5% *vs* 52.7%, 12.6% *vs* 20.6%, respectively between the cryptogenic/NASH group *vs* miscellaneous group)[58]. Post-OLT mortality was more likely from cardiovascular disease (21.2% *vs* 14.1% of deaths) and less likely from recurrent liver disease (0.7% *vs* 10.2%) in patients with cryptogenic/NASH cirrhosis as compared with those who underwent transplantation for other indications[58].

An analysis of outcomes for primary OLT from the Scientific Registry of Transplant Recipients database from 2001 to 2009 compared 1959 patients who underwent OLT for NASH with 33822 patients who had OLT for other indications including cryptogenic cirrhosis, HCV, ALD, HBV, primary biliary cholangitis, primary sclerosing cholangitis, and autoimmune hepatitis[59]. One- and three-year survival was not significantly different between post-OLT patients who underwent transplant for NASH (84%, 78%) *vs* cryptogenic cirrhosis (86%, 79%) *vs* others (87%, 78%) (P = 0.67)[59]. Graft survival remained similar and comparable between all groups (76% for NASH at three years)[59].

Liver resection for NAFLD-HCC: In addition to OLT, outcomes of liver resections have been studied for NAFLD-HCC[50,53]. Cauchy *et al*[50] assembled a cohort of 62 patients with HCC in the setting of metabolic syndrome as a surrogate for NAFLD after exclusion of other etiologies of HCC including HCV, HBV, ALD, hemochromatosis, and autoimmune liver disease who underwent surgical resection. Overall

survival and disease-free survival rates were 83% and 83%, respectively, at one year and 75% and 70%, respectively, at three years[50].

In a Japanese cohort study, a group of 17 patients with NAFLD was compared with a group with HCV or HBV who underwent surgical resection for HCC[53]. Overall survival was 59%, 57%, and 63% in the patients with NAFLD-HCC, HCV-related HCC, and HBV-related HCC, respectively, after five years post-resection, and was not significantly different among the three groups[53]. On the contrary, five-year disease- free survival was significantly better in the NAFLD-HCC group as compared with the HCV and HBV groups (66% *vs* 29% *vs* 39%)[53].

Another Japanese study by Takuma *et al*[56] described disease-free and overall survival among HCC cases secondary to cryptogenic cirrhosis as a surrogate for NAFLD *vs* HCV All patients received some form of curative treatment for HCC except for OLT, which included RFA, percutaneous ethanol injection, or microwave coagulation therapy[56]. Over a mean follow-up of 49 mo, those with NAFLD-HCC had significantly lower tumor recurrence rates (39% *vs* 71%, $P < 0.001$) and significantly higher five-year survival rates (80% *vs* 61%, $P = 0.02$) as compared with their HCV counterparts[56]. While the NAFLD-HCC group had a significantly greater proportion of patients with larger tumors (2.0 cm *vs* 2.8 cm) than the HCV-related HCC group, all were within Milan criteria[56]. Therefore, patients with NAFLD-HCC can have improved and better disease-free and overall survival as compared with patients with HCV-associated HCC contingent that they present with early-stage HCC.

Postoperative complications

While long-term disease-free and overall survival among patients with NAFLD-HCC who receive curative therapy is favorable, some studies have reported increased rate of postoperative morbidity and mortality post-hepatectomy in the setting of higher NAFLD activity score (NAS) or cirrhosis[50,53].

In a study by Cauchy *et al*[50], outcome comparisons were made between: (1) patients without severe fibrosis and NAS < 2; and (2) patients with severe fibrosis (stage F3 or F4) or with a NAS $\geqslant$ 2. Aside from having a higher body mass index (BMI) in the latter group (median BMI 31.1 kg/m^2 *vs* 28.4 kg/m^2), no preoperative clinical characteristics or operative characteristics differed between the two groups[50]. The group with severe fibrosis or higher NAS had significantly higher rates of 90-d mortality (18% *vs* 0%), liver-related complications (32% *vs* 4%), and cardiorespiratory complications (37% *vs* 13%)[50]. Interestingly, multivariate analysis showed that severe underlying fibrosis was not a risk factor for major complications; however, a NAS of two or greater was associated with increased major complications[50].

Wakai *et al*[53] reported significantly higher rates of postoperative morbidity and 30-d mortality among patients with NAFLD-HCC as compared with those of other HCC etiologies including HBV or HCV. Postoperative morbidity was seen in 59% of the NAFLD group *vs* 31% and 28% of the HCV and HBV groups, respectively[53]. In the NAFLD group of 10 patients who had postoperative complications, the most common complication was hepatic insufficiency, which was observed in four patients[53].

Mortality at 30 d postoperatively was 12%, 0.7%, and 3.3% in the NAFLD, HCV, and HBV groups, respectively[53]. The two patients who died in the NAFLD group had underlying NAFLD-related cirrhosis[53].

Therefore, postoperative morbidity and mortality may be enhanced in the setting of inflammatory processes as suggested by the association between postoperative complications and higher NAS and cirrhosis.

OVERALL SURVIVAL

Patients with NAFLD-HCC are recognized to have worse prognosis related to advanced stage of HCC at presentation and lower eligibility for curative treatment[7,28]. In a recent SEER cohort study, patients with NAFLD-HCC appeared to have poorer prognosis than patients with viral HCC as demonstrated by a shorter survival time and poorer one year survival rate from time of diagnosis (61% *vs* 50%, $P < 0.0001$)[7].

A large Italian study by Piscaglia *et al*[28] showed that survival was significantly shorter in NAFLD-HCC as compared with HCV-related HCC (25.5 mo *vs* 33.7 mo, $P = 0.017$) which was mainly attributable to later stage of HCC at time of diagnosis.

Interestingly, in the same study, survival difference disappeared after matching NAFLD-HCC and HCV-related HCC by curative treatment (34.2 mo *vs* 40.8 mo, respectively, *P* value 0.073)[28].

A study by Reddy *et al*[52] assessed severity of liver dysfunction and stage of HCC at diagnosis and long-term survival in patients with NAFLD-HCC *vs* HCV- and/or ALD- HCC. At time of HCC diagnosis, patients with NAFLD-HCC had significantly better hepatic function as compared with the HCV- and ALD-related HCC group[52]. There were no significant differences in previous HCC therapy and tumor characteristics at time of diagnosis, including largest tumor size, presence of satellite lesions, stage T3-4 disease (based on American Joint Committee on Cancer staging), and vascular invasion[52]. Subsequently, no differences existed between receipt of curative treatment between the two groups (NAFLD *vs* HCV and/or ALD)[52]. While recurrence-free survival was not significantly different between patients with NAFLD- and HCV/ALD- related-HCC, those with NAFLD-HCC had longer overall survival[52]. While having less liver dysfunction at baseline may have contributed to overall improved

survival in the NAFLD-HCC group, multivariate analysis adjusting for clinical factors and curative treatment continued to demonstrate that patients with NAFLD-HCC had longer overall survival[52]. Therefore, after controlling for disease presentation and receipt of curative therapy, patients with NAFLD-HCC experienced better long-term survival.

A European cohort study showed a non-significant difference of overall survival between patients with NAFLD-HCC *vs* those with non-NAFLD-HCC (median 11.28 mo *vs* 15.5 mo, $P = 0.287$)[48]. In this cohort, there was no significant difference in receipt of curative treatment and no difference of BCLC stage at time of HCC diagnosis[48]. Similarly, a compilation of case reports of NAFLD-HCC of predominantly early-stage HCC (single tumors approximately 3cm in largest size) revealed that all cases that received a form of curative therapy had no tumor recurrence or death over a 5- to 50-mo follow-up period[60]. Therefore, patients with NAFLD-HCC can have favorable survival contingent that they are diagnosed at early stages of HCC and receive curative treatment.

FUTURE DIRECTIONS

Our review highlights recent updates on NAFLD-HCC epidemiology, pathophysiology, disease presentation, demand and receipt of curative treatment, outcomes from curative treatment as compared with other etiologies of HCC, and overall survival. Current literature demonstrate that the incidence of NAFLD-associated HCC is increasing, may often occur in the absence of cirrhosis, and present at a more advanced stage, and thereby patients with NAFLD-HCC are less likely to be candidates of curative treatment modalities relative to other etiologies of HCC. However, OLT for NAFLD-associated HCC has increased four-fold over the past decade, and post-transplant survival appears to be similar to other HCC-based indications for OLT.

Although the literature addressing NAFLD-HCC is growing, there are several areas which represent high priorities for further research. More robust epidemiological studies to identify high-risk groups for NAFLD-HCC incidence and NAFLD-related mortality may help inform future surveillance and treatment strategies. Additional investigation into mechanisms and determinants of HCC development in non-cirrhotic NAFLD *vs* NASH may provide critical insight to support evidence-based guidelines on HCC surveillance. Further studies addressing surgical and transplant outcomes among patients with NAFLD and NASH-associated HCC may also guide clinicians and the transplant community on optimal organ allocation and post- transplant management. Significant opportunity exists to address key deficits in knowledge regarding epidemiology, pathogenesis, surveillance, treatment, and surgical outcomes of NAFLD-associated HCC, which remains a rapidly growing global public health problem.

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MINIREVIEWS

Conversion of laparoscopic colorectal resection for cancer: What is the impact on short-term outcomes and survival?

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Abstract

Laparoscopic resection for colon and rectal cancer is associated with quicker return of bowel function, reduced postoperative morbidity rates and shorter length of hospital stay compared to open surgery, with no differences in long-term survival. Conversion to open surgery is reported in up to 30% of patients enrolled in randomized control trials comparing open and laparoscopic colorectal resection for cancer. In this review, reasons for conversion are anatomical-related factors, disease-related-factors and surgeon-related factors. Body mass index, local tumour extension and co-morbidities are independent predictors of conversion. The current evidence has shown that patients with converted resection for colon cancer have similar outcomes compared to patients undergoing a laparoscopic completed or open resection. The few studies that have assessed the outcomes after conversion of laparoscopic rectal resection reported significantly higher rates of complications and longer length of hospital stay in converted patients compared to laparoscopically treated patients. No definitive conclusions can be drawn when converted and open rectal resections are compared. Early and pre-emptive conversion appears to have more favourable outcomes than reactive conversion; however, further large studies are needed to better define the optimal timing of conversion. With regard to long-term oncologic outcome, overall and disease-free survival in the case of conversion in laparoscopic colorectal cancer surgery seems to be worse than those achieved in patients in whom resection was successfully completed by laparoscopy. Although a worse long-term oncologic outcome has been suggested, it remains difficult to draw a proper conclusion due to the heterogeneity of the long-term outcomes as well as the inclusion of both colon and rectal cancer patients in most of the studies. Therefore, we discuss the currently available evidence of the impact of conversion in laparoscopic resection for colon and rectal cancer on both short-term outcomes and long-term survival.

Key words: Conversion; Laparoscopy; Open surgery;

Colon cancer; Rectal cancer; Morbidity; Mortality; Predictors; Recurrence; Survival

Core tip: Several randomized controlled trials have reported the short-term advantages of laparoscopic resection compared to open resection for both colon and rectal cancer. In addition, there is evidence showing the non-inferiority of the laparoscopic approach in colon and rectal cancer surgery in long-term survival. Conversion to open surgery has been reported in up to 30% of laparoscopic colorectal cancer resections. However, both short and long-term outcomes in these patients are unclear. Therefore, we discuss the currently available evidence of the impact of conversion of laparoscopic resection for colon and rectal cancer on both short-term outcomes and long-term survival.

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INTRODUCTION

Since its introduction in the early nineties[1], laparoscopic resection for colorectal cancer has increasingly gained popularity[2]. Large randomized controlled trials (RCTs) have proved several short-term advantages of this approach, such as less intraoperative blood loss, sooner return to bowel function and shorter hospital stay[3-5] and similar long-term oncologic when compared with open surgery[6-11].

Conversion of laparoscopic colorectal resection to open surgery has been reported in up to 30% of patients enrolled in these RCTs. However, converted patients were mostly analyzed in the laparoscopic group on an "intention-to-treat" basis. The evidence coming from the non-randomized studies that have specifically assessed the impact of conversion on both short-term and long-term outcomes (*i.e.*, local recurrence rate and overall and disease-free survival) is controversial[12-29]. The vast majority of these studies only included a limited number of patients and did not analyze colon and rectal cancer patients separately. As a consequence, the real influence of conversion on both short-term outcomes and long-term survival outcome in colorectal cancer patients is still unclear.

The aim of this review was to summarize all the available literature with regard to the short and long-term outcome in patients who were converted during laparoscopic resection for both colon and rectal cancer and to compare these outcomes with the results in patients in whom resection was successfully completed by laparoscopy.

LITERATURE SEARCH AND STUDY SELECTION

A search strategy of the literature was independently performed by two reviewers (Allaix ME and Furnée EJB) in MEDLINE, using the Pubmed search engine. The following search terms in title and abstract were used as free text words and Medical Subject Headings (MeSH): "colon", "rectum", "colorectal", "rectal", "conversion", "cancer", "laparoscopy" and "laparoscopic". The literature search was performed for all years, up to March 2016. The studies identified by the search strategy were subsequently selected based on title, abstract and full-text by two independent reviewers (Allaix ME and Furnée EJB).

DATA ACQUISITION

Data of the included studies were independently acquired by two reviewers using a standard data extraction form. The study design, number of total, laparoscopic and converted patients, sex ratio, age, body mass index and preoperative (chemo)radiation in rectal cancer patients were extracted from the individual studies. Collected intra-operative data included type of colorectal resection, reason for conversion, amount of blood loss and operative time. With regard to short-term outcome parameters, the number and type of postoperative complications, mortality rate, postoperative transfusion rate, time to return to bowel function, reoperation and readmission rate and hospital stay were collected. Based on the histological assessment of the colorectal specimen, tumor size, number of lymph nodes, presence of a positive resection margin and tumor staging according to TNM classification as well as disease stage were extracted. Regarding long-term oncologic follow-up, the number of patients available for follow-up, time to follow-up, local and distant recurrence rate and overall as well as disease-free survival were collected.

RESULTS

The search strategy in MEDLINE yielded a total of 654 articles eligible for selection. Based on the in- and exclusion criteria, all articles were subsequently selected on title, abstract and full-text. Eventually, 18 studies were selected for inclusion in this review: 12 prospective[12,15-17,19-22,24-26,28], five retrospective cohort studies[13,14,18,23,29] and one prospective case-control study[27].

Most studies included colon as well as rectal cancer patients. Three studies only included colon cancer patients[23,25,29] and five only included rectal cancer patients[14,22,26,20,28]. Overall, a total number of 53329

Table 1 Baseline characteristics of individual studies

Ref.	Study design	Colon and/or rectal	No. patients	Conversion (%)			Men (%)		Age (yr)		BMI (kg/m^2)	
				Overall	Colon resection	Rectal resection	LAP	CONV	LAP	CONV	LAP	CONV
Allaix *et al*[13]	Retrospective	Both	1114	122 (10.9)	77 (10.7)	45 (11.4)	530 (53.4)	69 (56.6)	67.0[1]	68.0	23.0	24.0
Agha *et al*[14]	Retrospective	Rectal	300	26 (8.6)	NA	26 (8.6)	166 (60.5)[1]	21 (80.7)	64.7	64.5	26.2[1]	29.0
Biondi *et al*[12]	Prospective	Both	207	33 (15.9)	14 (42.4)	19 (57.6)	102 (58.6)	23 (69.7)	65.5	66.8	NR	NR
Bouvet *et al*[15]	Prospective	Both	91	38 (41.1)	NR	NR	30 (56,6)	25 (65.8)	65.0	67.0	NR	NR
Chan *et al*[16]	Prospective	Both	470	41 (8.7)	NR (12.3)	NR (7.2)	238 (55.5)[1]	30 (73.2)	69.0	69.1	NR	NR
Franko *et al*[21]	Prospective	Both	174	31 (17.8)	NR	NR	73 (51.0)	21 (51.2)	70.0	69.0	NR	NR
Keller *et al*[22]	Prospective	Rectal	141	25 (17.7)	NA	25 (17.7)	63 (54.3)	17 (68.0)	63.1	63.5	28.7	27.5
Li *et al*[23]	Retrospective	Colon	217	33 (15.2)	33 (15.2)	NA	94 (51.1)	20 (60.7)	62.6	62.9	25.6	24.5
Martínek *et al*[17]	Prospective	Both	243	17 (7.0)	10 (6.3)	7 (8.2)	146 (64.6)	13 (76.5)	64.5	62.8	26.7	28.4
Moloo *et al*[24]	Prospective	Both	359	46 (12.8)	NR	NR	171 (54.6)	25 (54.3)	65.0	65.0	NR	NR
Ptok *et al*[25]	Prospective	Colon	346	56 (16.2)	56 (16.2)	NA	NR	NR	66.5	68.9	NR	NR
Rickert *et al*[26]	Prospective	Rectal	162	38 (23.5)	NA	38 (23.5)	69 (55.7)	27 (71.0)	63.0[1]	69.0	25.1	25.8
Rottoli *et al*[20]	Prospective	Rectal	173	26 (15.0)	NA	26 (15.0)	NR	NR	63.2	64.3	24.9[1]	27.3
Rottoli *et al*[27]	Prospective[2]	Both	93	31 (NA)	NR	NR	37 (59.7)	24 (77.4)	72.0	72.0	26.8[1]	29.6
Scheidbach *et al*[18]	Retrospective	Both	1409	80 (5.7)	41 (8.2)	39 (6.4)	658 (49.5)	46 (57.5)	68.9	69.7	25.2[1]	26.4
White *et al*[19]	Prospective	Both	175	25 (14.3)	NR	NR	70 (46.7)	11 (44.0)	69.7	74.4	27.2	26.9
Yamamoto *et al*[28]	Prospective	Rectal	1073	78 (7.3)	NA	78 (7.3)	625 (62.8)	48 (61.5)	62.9	63.8	22.7[1]	24.6
Yerokun *et al*[29]	Retrospective	Colon	46472	6144 (13.2)	6144 (13.2)	NA	19738 (48.9)[1]	3308 (53.8)	70.0[1]	69.0	NR	NR

[1]*P* value of difference between overall LAP and CONV is < 0.05; [2]Case-control study. BMI: Body mass index; NR: Not reported; NA: Not applicable; LAP: Laparoscopic group; CONV: Converted group.

patients were included in all individual studies. The median conversion rate was 14.3%. The median conversion rate for colon and rectal resections was 12.8% and 10.0%, respectively. Definition of conversion as well as description of surgeon's experience in laparoscopic colorectal surgery was reported in 12 studies (66.7%)[12-17,19-21,23,24,26-28]. The baseline characteristics of the individual studies are reported in Table 1. Three studies reported significantly more male patients in the converted group (CG) compared to the laparoscopic group (LG)[14,16,29]. The body mass index (BMI) was significantly higher in the CG in five studies[14,18,20,27,28]. Neo-adjuvant chemo-radiation in rectal cancer patients was significantly more frequently applied in the CG in the study by Keller *et al*[22] (n = 63, 54.3% *vs* n = 19, 76.0%) and in the LG in the study by Rottoli *et al*[20] (n = 33, 22.4% *vs* n = 1, 3.8%). There were no significant differences between both groups in the other six studies reporting this item[13-16,19,26].

Type of colorectal resection

The type of colorectal resection performed in the individual patients was only reported in eight studies (44.4%). In all patients who underwent a colon resection in the LG, 362 (39.0%) had a right hemicolectomy, 271 (29.2%) a left hemicolectomy and 295 (31.8%) a sigmoid colon resection. The number of different colonic resections in the CG was 60 (42.6%), 40 (28.4%) and 41 (29.1%), respectively. In the rectal resection group, low anterior resection was performed in 1636 patients (82.6%) in the LG and 185 (80.4%) in the CG, abdominoperineal resection in 323 (16.3%) and 39 (17.0%), and Hartmann's procedure in 22 (1.1%) and 6 (2.6%), respectively.

Reasons for conversion and intra-operative data

The reason for conversion was reported in 16 studies (88.9%) (Table 2). In more than half of them, tumor related aspects were the most frequent reason for conversion[12,13,18-20,23,24,25,27].

Intra-operative blood loss was reported in eight studies and ranged from 74 mL to 200 mL in the LG and from 147 mL to 500 mL in the CG. In all eight studies, intra-operative blood loss was significantly less in the LG compared to the CG (Table 2). Duration of surgery was significantly shorter in the LG in 11 of 15 studies reporting the operative time (Table 2).

Short-term postoperative outcomes

Several studies have compared the short-term outcomes of laparoscopic colorectal resections converted to open surgery to those achieved after laparoscopically completed colorectal resection, reporting controversial results. Postoperative complication rate ranged from 6.0% to 36.8% in the LG and from 15.4% to 61.2% in the CG (Table 3). In five studies (21.7%), postoperative complications occurred more frequently in the CG than in the LG[14,16,18,22,28], while 8 studies did not find any significant difference[12,13,15,17,20,25-27]. The wound infection rate was significantly lower in the LG in four out of ten studies reporting this topic[12,14,18,26]. A significant difference in anastomotic leakage rate was found in only one out of 11 studies, in favor of the LG (5.0% *vs* 13.8%)[18]. With regard to prolonged postoperative ileus, there was only one out of six studies reporting a significant difference in favor of the LG (1.1% *vs* 9.1%)[12]. Urologic and cardiopulmonary complications were reported in six studies (33.3%) and no significant differences were found between both groups[13,14,16,18,22,26,28]. Return to

Table 2 Reason for conversion, intra-operative blood loss and operative time

Ref.	Tumor related (%)			Anatomic related (%)	Intra-operative complication (%)	Obesity (%)	Adhesions (%)	Other reason (%)	Intra-operative blood loss (mL)		Duration of operation (min)	
	Overall	Colon	Rectal						LAP	CONV	LAP	CONV
Allaix *et al*[13]	59 (48.4)	44 (57.1)	15 (33.3)	6 (4.9)	5 (4.1)	23 (18.8)	18 (14.8)	11 (9.0)	100[1]	150	140[1]	180
Agha *et al*[14]	0 (0)	0 (0)	0 (0)	0 (0)	5 (19.2)	10 (38.5)	4 (15.4)	7 (26.9)	NR	NR	215[1]	258
Biondi *et al*[12]	17 (51.5)	NR	NR	3 (9.1)	6 (18.2)	0 (0)	6 (18.2)	1 (3.0)	96.4[1]	184	162.0[1]	187.9
Bouvet *et al*[15]	6 (15.8)	NR	NR	10 (26.3)	2 (5.3)	0 (0)	12 (31.6)	8 (21.1)	NR	NR	240	270
Chan *et al*[16]	11 (26.9)	NR	NR	4 (9.8)	6 (14.7)	0 (0)	14 (34.1)	6 (14.7)	191.2[1]	461.9	179.4	187.2
Franko *et al*[21]	4 (12.9)	NR	NR	18 (58.1)	5 (16.1)	0 (0)	NR	4 (12.9)	NR	NR	160[1]	182
Keller *et al*[22]	NR	NR	NR	NR	NR	NR	NR	NR	74[1]	253	242.6[1]	260
Li *et al*[23]	15 (45.5)	15 (45.5)	NA	4 (12.1)	4 (12.1)	0 (0)	10 (30.3)	0 (0)	90[1]	147	165[1]	188
Martínek *et al*[17]	3 (17.6)	NR	NR	6 (35.3)	5 (29.4)	NR	NR	3 (17.6)	177[1]	415	152[1]	224
Moloo *et al*[24]	13 (28.3)	NR	NR	12 (26.1)	4 (8.7)	0 (0)	5 (10.9)	11 (23.9)	NR	NR	NR	NR
Ptok *et al*[25]	15 (26.8)	15 (26.8)	NA	8 (14.3)	7 (12.5)	0 (0)	9 (16.1)	17 (30.4)	NR	NR	178.9	186.7
Rickert *et al*[26]	7 (18.4)	NA	7 (18.4)	11 (28.9)	4 (10.5)	6 (15.8)	6 (15.8)	4 (10.5)	NR	NR	345	363
Rottoli *et al*[20]	7 (26.9)	NA	7 (26.9)	10 (23.5)	6 (23.1)	0 (0)	2 (7.7)	1 (3.8)	NR	NR	285[1]	342
Rottoli *et al*[27]	12 (38.7)	NR	NR	6 (19.3)	2 (6.5)	0 (0)	11 (35.5)	0 (0)	NR	NR	NR	NR
Scheidbach *et al*[18]	24 (30.0)	NR	NR	8 (10.0)	14 (17.5)	0 (0)	15 (18.8)	19 (23.7)	200[1]	500	180[1]	232
White *et al*[19]	18 (72.0)	NR	NR	0 (0)	4 (12.0)	0 (0)	3 (12.0)	0 (0)	NR	NR	145.6[1]	172
Yamamoto *et al*[28]	13 (16.7)	NA	13 (16.7)	26 (33.3)	7 (9.0)	12 (15.4)	10 (12.8)	10 (12.8)	80[1]	265	270[1]	295
Yerokun *et al*[29]	NR	NR	NA	NR	NR	NR	NR	NR	NR	NR	NR	NR

[1]*P* value of difference between LAP and CONV is < 0.05. NR: Not reported; NA: Not applicable; LAP: Laparoscopic group; CONV: Converted group.

Table 3 Postoperative data

Ref.	Postoperative complications (%)		Wound infection (%)		Anastomotic leakage (%)		Mortality (30-d) (%)		Hospital stay (d)	
	LAP	CONV	LAP	CONV	LAP	CONV	LAP	CONV	LAP	CONV
Allaix *et al*[13]	156 (15.7)	20 (16.4)	9 (0.9)	3 (2.5)	49 (4.9)	4 (3.3)	3 (0.3)	1 (0.8)	7	9
Agha *et al*[14]	101 (36.8)[1]	16 (61.2)	33 (12.0)[1]	6 (23.0)	23 (8.3)	3 (11.5)	0 (0)	0 (0)	10	10.5
Biondi *et al*[12]	10 (6.0)	11 (33.3)	1 (0.6)[1]	2 (6.1)	3 (1.7)	1 (3.0)	NR	NR	8.4[1]	10.6
Bouvet *et al*[15]	13 (24.5)	10 (26.3	NR	NR	NR	NR	1 (1.9)	0 (0)	6[1]	8
Chan *et al*[16]	72 (16.7)[1]	23 (56.1)	8 (1.9)	6 (2.4)	10 (2.3)	1 (2.4)	1 (0.2)	1 (2.4)	6[1]	10
Franko *et al*[21]	NR	NR	NR	NR	NR	NR	1 (0.7)	2 (6.5)	4.0[1]	6.8
Keller *et al*[22]	25 (21.5)[1]	8 (32.0)	2 (1.7)	4 (20.0)	3 (2.6)	0 (0)	1 (0.9)	0 (0)	4.4[1]	6.8
Li *et al*[23]	NR	NR	4 (2.2)	3 (9.1)	14 (7.6)	3 (9.1)	NR	NR	4[1]	8
Martínek *et al*[17]	65 (28.8)	6 (35.3)	NR	NR	NR	NR	7 (3.1)	0 (0)	11.3	12.5
Moloo *et al*[24]	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Ptok *et al*[25]	75 (25.9)	22 (39.3)	NR	NR	NR	NR	1 (0.3)	1 (1.8)	NR	NR
Rickert *et al*[26]	42 (33.9)	19 (50.0)	6 (4.8)[1]	7 (18.4)	18 (16.4)	7 (22.6)	0 (0)	1 (2.6)	12[1]	15
Rottoli *et al*[20]	34 (23.1)	4 (15.4)	NR	NR	17 (11.6)	4 (15.4)	0 (0)	0 (0)	8	9
Rottoli *et al*[27]	13 (21.0)	7 (22.6)	NR	NR	1 (1.6)	1 (3.2)	1 (1.6)	0 (0)	NR	NR
Scheidbach *et al*[18]	389 (28.5)[1]	41 (51.3)	138 (10.4)[1]	16 (20.0)	67 (5.0)[1]	11 (13.8)	20 (1.5)[1]	4 (5.0)	NR	NR
White *et al*[19]	NR	NR	14 (9.3)	5 (20.0)	NR	NR	0 (0)	1 (4.0)	8.3[1]	14.4
Yamamoto *et al*[28]	210 (21.1)[1]	34 (43.6)	56 (5.6)	14 (17.9)	72 (7.2)	14 (17.9)	0 (0)	0 (0)	14[1]	20
Yerokun *et al*[29]	NR	NR	NR	NR	NR	NR	419 (1.0)[1]	115 (1.9)	5[1]	6

[1]*P* value of difference between LAP and CONV is < 0.05. NR: Not reported; LAP: Laparoscopic group; CONV: Converted group.

bowel function was only reported in four studies[12,13,18,23], and in two of them[18,23] bowel function returned significantly sooner in the LG (2.3 d *vs* 3.4 d and 3 d *vs* 4 d, respectively).

In four out of six studies, postoperative transfusion was necessary in significantly more patients in the CG[13,14,18,21], whereas no significant difference between both groups was found in the other two studies[20,27]. A previous pooled analysis of these data showed that converted patients were more likely to require postoperative blood transfusion than patients undergoing completed laparoscopic resection[30]. The 30-d mortality rate was not significantly different between both groups in most studies, although a significant difference in favor of the LG was found in three studies[18,21,29]. A previous meta-analysis of seven studies including 1655 patients showed that converted patients had a higher risk of 30-d mortality than patients with laparoscopically completed colorectal resection[30].

There was a range in reoperation rate in the LG from 4.9% to 17.7% and from 0% to 15.0% in the CG. Only one study reported a significant difference between both groups, in favor of LG (4.9% *vs* 15.0%)[18]. In 10 out of 14 studies, the hospital stay

was significantly shorter in the LG[12,15,16,19,21-23,26,28,29], whilst no significant difference was present in the remaining studies[13,14,17,20]. The readmission rate was only reported in three studies[21,22,29]. A significant difference was found in one, in favor of the LG (5.0% *vs* 7.5%)[29]. The interpretation of these data is made difficult by several factors, including heterogeneous definitions of conversion, size and nature of the studies that in most cases did not separately analyze colon and rectal cancer patients.

Only a few studies assessed factors that might affect morbidity after conversion of laparoscopic colorectal resections. Aytac *et al*[31] performed a retrospective review of 2483 patients undergoing laparoscopic colorectal resection. A total of 270 were converted to open surgery. A high ASA score, previous abdominal surgery but not conversion were independently associated with postoperative complications. Among patients who required conversion to open surgery, smoking, cardiac co-morbidities, hypertension, previous abdominal surgery and intra-operative adhesions were factors significantly associated with increased postoperative complications. Patients who suffered postoperative complications had a significantly shorter time to conversion. However, it is worth to note that patients undergoing conversion within 50 min from the beginning of the operation were older and were more likely to have co-morbidities.

Conversion in colon cancer: When considering colon cancer patients, three large studies did not report significant differences in short-term outcomes[23,25,29]. Guillou *et al*[5] analyzed the short-term results of the Medical Research Council (MRC) CLASICC trial reporting similar rates of postoperative morbidity among 61 converted patients and 185 who had a completed laparoscopic colon resection (38% *vs* 34%). Our group[13] recently compared the early outcomes in 641 patients who had a completed laparoscopic colon resection and in 77 converted patients. No significant differences were observed in complication rate: 12.9% *vs* 14.5%, respectively (P = 0.864). Median length of hospital stay was 7 d *vs* 8 d, respectively (P = 0.303). Similar results were reported by Ptok *et al*[25] in a prospective study comparing 290 patients with completed laparoscopic colon resection and 56 converted patients: morbidity rate was 26% *vs* 39% and mortality rate was 1.8% *vs* 0.3%, respectively. Conversely, a retrospective review of the United States National Cancer Data Base including 40328 patients treated by completed laparoscopic colon resection and 6144 converted patients, found a slightly longer hospital stay (median 4 d *vs* 3 d) and higher rates of both readmission within 30 d (7.5% *vs* 5%) and mortality at 30 d (1.9% *vs* 1%) in the group of converted patients[29].

Conversion in rectal cancer: Only one RCT[5] and a few studies[14,22,28] focused on patients undergoing laparoscopic resection for rectal cancer, reporting worse short term outcomes in case of conversion to open surgery. The short-term results of the MRC CLASICC trial showed a higher 30-d postoperative complication rate in 82 converted patients compared to 160 patients who had a laparoscopic completed rectal resection: transfusion requirement, wound infections, pulmonary infection and anastomotic leakage rate were increased in converted patients[5]. Hospital stay was longer in these patients. Agha *et al*[14] analyzed rectal cancer patients undergoing elective laparoscopic rectal surgery. The overall complication rate was higher in the group of converted patients. Perioperative blood transfusions were needed in 11.5% of converted patients and in 1.9% of patients undergoing a completed laparoscopic rectal resection (P = 0.001). Wound infections occurred more frequently in converted patients (23% *vs* 12%, P = 0.01). Yamamoto *et al*[28] retrospectively reviewed 1073 patients undergoing laparoscopic surgery for rectal cancer. The postoperative morbidity rate was significantly higher after conversion: 43.6% *vs* 21.1%. The most common complications in converted patients were wound infection (17.9%), anastomotic leakage (17.9%) and small bowel obstruction (10.3%). The rate of these complications in the group of patients who had a completed laparoscopic rectal resection was 7.2% for anastomotic leakage, 5.6% for wound infection and 3.0% for small bowel obstruction. Resumption of gastrointestinal functions was significantly prolonged in the case of conversion and median length of hospital stay was significantly longer (20 d *vs* 14 d, P = 0.010). Similar results were observed by others[22].

Reactive *vs* pre-emptive conversion: The outcomes after a reactive or a pre-emptive conversion are poorly studied. Yang *et al*[32] matched 30 patients who had undergone a reactive conversion for several reasons including bleeding, bowel injury, ureter damage or splenic injury with 60 patients who had pre-emptive conversion and 60 patients who had a laparoscopically completed colorectal resection. After a reactive conversion, patients more frequently had a postoperative complication (50% *vs* 26.7%, P = 0.028), later resumption of a regular diet (6 d *vs* 5 d, P = 0.033) and a trend towards a longer hospital stay (8.1 d *vs* 7.1 d, P = 0.08) than patients who had a pre-emptive conversion. Aytac *et al*[31] found that reactive conversion was associated with a higher risk of postoperative pneumonia, bleeding and need for reoperation compared to patients requiring pre-emptive conversion to open surgery. Overall morbidity, length of hospital stay and readmission rates were slightly worse in patients with reactive conversion, even though the differences did not reach statistical significance.

The increased rate of postoperative morbidity after reactive conversion might be related to the sequelae of the intraoperative complication that leads to a

reactive conversion, suggesting that a low threshold for conversion is advisable in challenging cases, thus avoiding a dangerous and lengthy dissection. However, the best timing of conversion is unclear. Belizon *et al*[33] found that the postoperative morbidity rate significantly decreased if the decision to convert was taken within or after the first 30 min of the procedure (40% *vs* 86.9%, $P < 0.05$). Conversely, Aytac *et al*[31] reported that timing of conversion did not adversely affect the postoperative outcomes: 72 patients converted within the first 30 min had similar overall morbidity, reoperation and readmission rates as 198 patients who had late conversion. However, the interpretation of these findings is biased by the fact that several patients in both groups underwent reactive conversion. Further studies are needed to better clarify the real impact of conversion timing on the postoperative course.

Converted *vs* open colorectal resection: While several studies have compared the short-term outcomes after conversion in laparoscopic colorectal resection to those observed after laparoscopically completed colorectal resection, there are limited data on the effects of conversion compared to planned open surgery. The evidence is controversial with some studies showing a worse postoperative course in converted patients and others reporting no significant differences[34]. In particular, among 4 studies that included only rectal cancer patients, one found better results in converted patients[35], one reported better outcomes after planned open rectal resection[36] and two found no differences[26,37]. Recently, Giglio *et al*[34] conducted a systematic review and meta-analysis of short term outcomes after laparoscopic converted or open colorectal resection. The aim was to determine if conversion is a drawback or a complication of laparoscopic colorectal resection burdened by additional postoperative morbidity. The authors identified 20 studies including a total of 30656 patients undergoing open surgery and 11085 having laparoscopic colorectal resection. A total of 1935 patients (17%) in the laparoscopic group were converted to open surgery. Colorectal cancer was the indication for surgery in 13 of the studies included in this meta-analysis, while both benign and malignant diseases were included in seven. A total of five studies only included rectal cancer patients, while other five only analyzed colon resections. The risk of bias was moderate to high in 11 studies. No differences in 30-d mortality and 30-d morbidity rates were found. While a higher rate of wound infection was observed among converted patients, no significant differences were observed between both groups in length of hospital stay, anastomotic leakage, postoperative bleeding, sepsis, cardiac complications, deep venous thrombosis and reoperation rates. Subgroup analyses on mortality, overall morbidity and length of hospital stay according to the site of disease (colon *vs* rectum), and indication for surgery (cancer *vs* benign disease) showed that the short-term outcomes in converted patients were comparable to those observed in patients treated by open surgery. The learning curve of the surgeon and the reason for conversion did not show any adverse impact on the postoperative course of converted patients.

Very recently, Masoomi *et al*[38] published the results of a retrospective analysis of 646414 patients from the United States National Inpatient Sample undergoing laparoscopic (27.7%) or open colorectal resection for both benign and malignant diseases. The conversion rate of laparoscopic to open surgery was 16.6%. They found that the group of converted patients had significantly better outcomes compared to the open group, with a lower in-hospital mortality rate. Even though the length of hospital stay was similar between the two groups, the median total hospital charge was $2800 higher in the converted group compared to the open group. Similar results were reported by Yerokun *et al*[29] who used the United States National Cancer Data Base to identify patients undergoing elective colon resection for stage I-Ⅲ colon cancer between 2010 and 2012. Of 104400 patients, 40328 (38.6%) underwent laparoscopic colectomy, 57928 (55.5%) open colectomy and 6144 (5.9%) converted colectomy. After adjustment for patient, clinical and treatment characteristics, conversion to open surgery was associated with a significantly reduced 30-d mortality (1.9% *vs* 2.8%, $P < 0.001$), a shorter hospital stay and a reduced 30-d readmission rate (5.9% *vs* 7.5%, $P < 0.001$) when compared to open surgery.

Since converted colon resection is still associated with better outcomes than planned open colon resection, the authors concluded that the laparoscopic approach in patients with colon cancer should be attempted in all cases with no contraindications to laparoscopy. Even though there is increasing evidence showing that conversion of laparoscopic colon resection does not impair short-term outcomes, the data are not definitive and robust data regarding conversion of laparoscopic rectal resection are missing. While waiting further studies to confirm these results and to shed more light on the impact of conversion of laparoscopic rectal resections, several studies have been conducted aiming to identify risk factors for conversion. Algorithms to predict conversion from laparoscopic colorectal resection to open surgery have been proposed; however, most of them are not specific for colorectal cancer[39-41]. To date, only a few studies have been focused on predictors of conversion in patients with colon or rectal cancer. Thorpe *et al*[42] analyzed the data from the MRC Conventional *vs* Laparoscopic-Assisted Surgery in Colorectal Cancer (CLASICC). They found that locally advanced tumor, BMI, and ASA score greater than 3 were independent risk factors for conversion in colon cancer patients. Similarly, BMI and male sex were independently

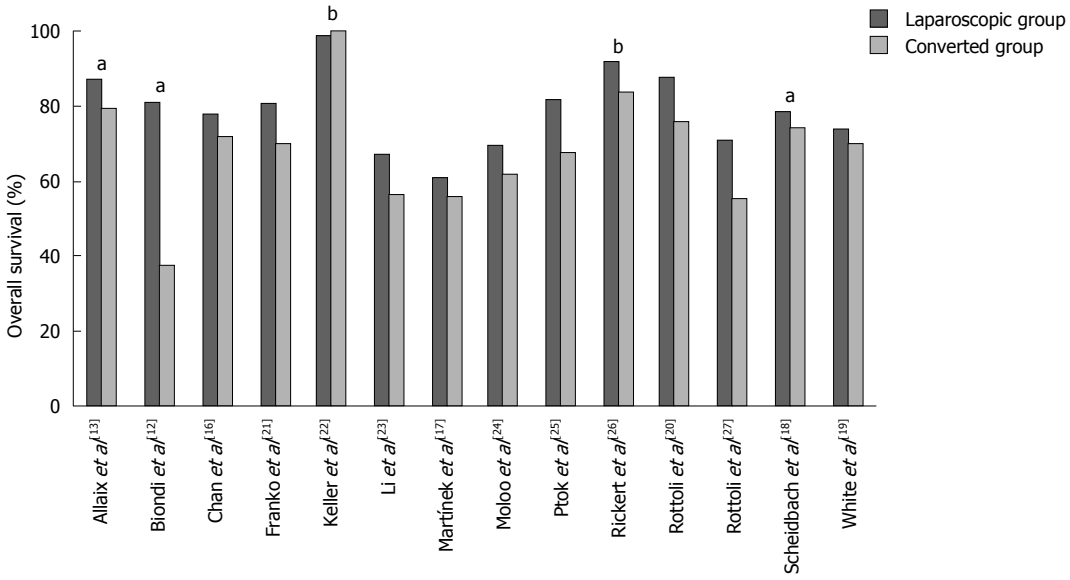

Figure 1 Overall survival rates reported in the individual studies. [a]*P* value of difference between the laparoscopic and converted group is < 0.05; [b]3-yr overall survival rates, the other studies reported 5-yr overall survival rates.

associated with conversion in rectal cancer patients. BMI was also identified as risk factor for conversion in rectal cancer patients by Yamamoto *et al*[28] in a retrospective analysis of 1073 rectal cancer patients.

A model to predict conversion to open surgery during laparoscopic rectal resection for cancer was proposed by Zhang *et al*[43]. Six possible risk factors for conversion were extracted from a review of the literature and a series of 602 laparoscopic rectal resections, including male sex, surgical experience (at least 25 previous laparoscopic rectal resections), previous open abdominal surgery, BMI ⩾ 28, tumor diameter ⩾ 6 cm, and tumor invasion, for which a score of relevance was assigned. Further studies however are needed to confirm this model.

Histological outcome of the colorectal specimen

The tumor (T) stage was reported in 10 studies (55.6%)[12-17,19,23,25,27]. In most of them (*n* = 7), no significant differences in T-stage were found between both groups. In the study by Allaix *et al*[13], T1 was significantly more frequent in the LG (*n* = 345, 34.8% *vs n* = 9, 7.4%), whilst T3 and T4 were significantly more frequent in the CG (*n* = 446, 45.0% *vs n* = 85, 69.7% for T3, and *n* = 37, 3.7% *vs n* = 15, 12.3% for T4, respectively). Agha *et al*[14] found significantly more T2-tumors in the LG (*n* = 87, 31.7% *vs n* = 5, 19.2%) and significantly more T4-tumors in the CG (*n* = 10, 3.6% *vs n* = 6, 23.0%). In the study by Bouvet *et al*[15], T4-tumors were significantly more frequent in the CG (*n* = 1, 1.9% *vs n* = 5, 13.2%), whilst the other T-stages were comparable between both groups in this study.

The number of lymph nodes harvested was similar between both groups in all 13 studies reporting this item[12,13,15-17,20-23,26-29]. The number of harvested lymph nodes ranged from 8 to 20.2 in the LG and from 9 to 22.4 in the CG. The N-stage was reported in five studies (27.8%)[12,13,20,25,26]; in two of these studies[12,13], the N0-stage was significantly more frequent in the LG (*n* = 679, 68.4% *vs n* = 67, 54.9% and *n* = 92, 52.9% *vs n* = 8, 24.2%, respectively).

The tumor size ranged from 3.5 to 5.1 cm in the LG and from 3.5 to 5.6 cm in the CG. In three out of seven studies (42.9%), the tumor size was significantly larger in the CG (5.3 cm *vs* 3.9 cm, 5.0 cm *vs* 4.0 cm and 4.0 cm *vs* 3.7 cm, respectively)[12,16,29]. Tumor margin status was also reported in seven studies[13,14,17,20,26,27,29] and in one of these, tumor margin positivity was significantly more frequent in the CG (*n* = 319, 5.2% *vs n* = 1075, 2.7%)[29].

Disease stage was reported in all studies, although incomplete in two[20,18]. In seven studies, stage Ⅰ-Ⅲ patients were included[12,13,19,21,23,25,29], in three stage Ⅰ-Ⅳ[14,17,24], in four studies stage 0-Ⅳ[15,16,22,28] and in two studies stage 0-Ⅲ[26,27]. In three studies[12,13,29], disease stage Ⅰ was significantly more frequent in the LG, whilst stage Ⅲ was more frequent in the CG. In the studies by Biondi *et al*[12] and Yerokun *et al*[29], stage Ⅱ was also significantly more frequent in the LG. In the studies by Agha *et al*[14] and Rottoli *et al*[20], stage Ⅳ disease was significantly more frequent in the CG as well.

Long-term oncologic outcome

Survival: Most of the studies reported one or more long-term oncologic outcome measures. Overall survival (OS) was reported in fourteen studies[12,13,16-27] (Figure 1). The median 5-year OS was 79.7% (61.0%-99.1%) in the LG and 70.2% (38.0%-100%) in the CG. OS was in favor of the patients in the LG in all studies, except in the study by Keller *et al*[22],

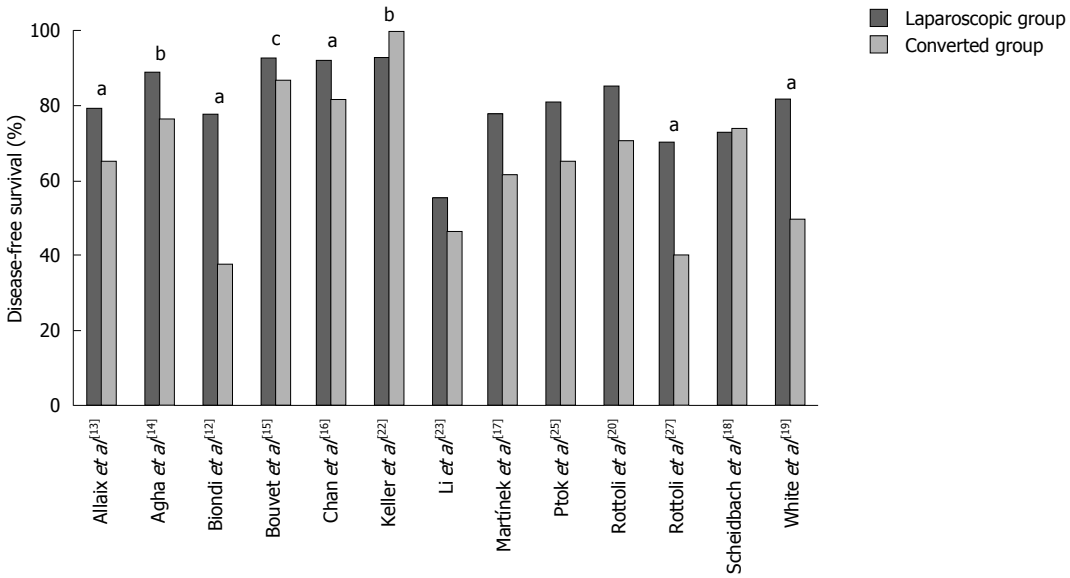

Figure 2 Disease-free survival rates reported in the individual studies. [a]*P* value of difference between the laparoscopic and converted group is < 0.05; [b]3-yr and [c]2-yr disease-free survival rates, the other studies reported 5-yr disease-free survival rates.

who reported an extremely high OS in both groups. However, there was a statistically significant difference in OS in only three studies in favor of the LG[12,13,18]. The lack of significance in OS between LG and CG in the other series might be caused by the small number of patients included, as two of the studies reporting a significant difference in OS were very large studies including more than one thousand patients[13,18]. Additionally, the interpretation of the outcome with regard to OS is also complicated by the fact that in most studies colon as well as rectal cancer patients were included. OS was reported in three of the five studies only including rectal cancer patients[20,22,26] and in two of the three studies in which only colon cancer patients were included[23,25]. In none of these studies, there was a significant difference in OS found between the laparoscopic and converted group.

The 5-year disease-free survival (DFS) was reported in ten studies[12,13,16-20,23,25,27], whilst 3 and 2-year DFS was reported in two[14,22] and one study[15], respectively (Figure 2). The median DFS was 81.3% (55.5%-93.1%) in the LG and 65.6% (38.0%-100%) in the CG. DFS was in favor of the LG in all, except two studies[18,22]. A statistical significant difference was found in five of the studies reporting a favorable outcome in the LG (Figure 2).

The significant worse OS and DFS in the CG might be related to several factors other than conversion itself, including a locally advanced tumor[30]. We performed a multivariate analysis of predictors of survival in our study and found pT4 tumor stage and a positive lymph node ratio $\geqslant$ 0.25, but not conversion itself as independent predictors of poor OS and DFS[13]. In addition, an adverse survival in converted patients might be explained by a more extensive inflammatory response due to more tissue damage as well as a higher postoperative complication rate compared to the laparoscopic group of patients[30].

Recurrence: The local and distant recurrence rate was also reported in some studies. Median duration of follow-up in the studies reporting these recurrence rates was 35 (22.5-120) mo in the LG and 34.1 (23.6-120) mo in the CG. At follow-up, local recurrence rate ranged from 2.6% to 15.8% in the LG and from 0% to 26.3% in the CG. A statistically significant difference in local recurrence rate between both groups was only found in one of the studies: Chan *et al*[16] reported a significant difference in local recurrence rate of 2.8% in the LG and 9.8% in the CG after 31 mo of follow-up ($P < 0.001$). Four studies reported the local recurrence rate in a study population of only rectal cancer patients. In three of these studies, the local recurrence rate was comparable: 3% in both groups after 34 months of follow-up in the study by Rickert *et al*[26], 4.8% in the LG and 3.8% in the CG ($P = 0.875$) after almost two years of follow-up in the study by Agha *et al*[14]; in the study by Keller *et al*[22] local recurrence was only present in the LG, in 2.6% of patients after 38.2 mo of follow-up ($P = 0.07$). Rottoli *et al*[20] reported a large difference in local recurrence rate between both groups; 11.4% in the LG and 26.3% in the CG, although this did not reach statistical significance ($P = 0.07$). However, this large difference in recurrence rate between both groups might be explained by the difference in duration of follow-up of 10 mo between both groups in this study; 36 months in the LG and 46 months in the CG.

The rate of distant metastases in the LG en CG was reported in seven studies[13,14,17,22,23,26,27], ranging from 4.3% to 17.3% in the LG and from 0% to 22.6% in the CG. In three of these seven studies[13,14,27],

the distant metastasis rate was noteworthy higher in the CG, even though the difference did not reach the statistical significance. In two of these studies both colon and rectal cancer patients were included: Rottoli *et al*[27] reported a distant metastasis rate of 9.9% in the LG and 21.8% in the CG after a follow-up of approximately four years (P = 0.79), and in the study by our group[13] the distant metastasis rate was 16.1% in the LG and 22.6% in the CG after ten years of follow-up (P = 0.244). Agha *et al*[14] only included rectal cancer patients and reported a distant recurrence rate of 13.1% in the LG and 19.2% in the CG (P = 0.390). The distant metastasis rate was comparable between both groups in the other four studies.

CONCLUSION

This review of the literature has demonstrated that conversion of laparoscopic colon resection does not seem to significantly increase the postoperative morbidity, while the results after converted laparoscopic resection for rectal cancer are less favorable than those achieved by patients who had a completely laparoscopic surgery. With regard to long-term oncologic outcome, OS and DFS in the case of conversion in laparoscopic colorectal cancer surgery seems to be worse compared to the group of patients in whom resection was successfully completed by laparoscopy. However, it remains difficult to draw a proper conclusion due to the heterogeneity of the long-term oncologic outcomes as well as the inclusion of both colon and rectal cancer patients in most of the studies. Poorer survival in converted patients seems to be multifactorial, including tumor stage as well as inflammatory response secondary to more tissue damage and sequelae of postoperative complications.

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MINIREVIEWS

Prediction models of hepatocellular carcinoma development in chronic hepatitis B patients

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Abstract

Chronic hepatitis B virus (HBV) infection is a major cause of cirrhosis and hepatocellular carcinoma (HCC). Applying the same strategies for antiviral therapy and HCC surveillance to all chronic hepatitis B (CHB) patients would be a burden worldwide. To properly manage CHB patients, it is necessary to identify and classify the risk for HCC development in such patients. Several HCC risk scores based on risk factors such as cirrhosis, age, male gender, and high viral load have been used, and have negative predictive values of $\geqslant$ 95%. Most of these have been derived from, and internally validated in, treatment-naïve Asian CHB patients. Herein, we summarized various HCC prediction models, including IPM (Individual Prediction Model), CU-HCC (Chinese University-HCC), GAG-HCC (Guide with Age, Gender, HBV DNA, Core Promoter Mutations and Cirrhosis-HCC), NGM-HCC (Nomogram-HCC), REACH-B (Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B), and Page-B score. To develop a noninvasive test of liver fibrosis, we also introduced a new scoring system that uses liver stiffness values from transient elastography, including an LSM (Liver Stiffness Measurement)-based model, LSM-HCC, and mREACH-B (modified REACH-B).

Key words: Chronic hepatitis B; Hepatocellular carcinoma; Development; Prediction models

Core tip: This is the summary about prediction models of hepatocellular carcinoma development in chronic hepatitis B patients.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is the main cause of cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) globally[1]. Of chronic HBV carriers, approximately 15%-40% develop chronic hepatitis B (CHB)[2]. Around 90% of CHB patients undergo seroconversion of HBeAg to anti-HBe and become inactive carriers. However, approximately 10% of CHB patients have chronic active hepatitis and develop liver cirrhosis at a rate of 2% per year. Because progression of liver disease in CHB patients is closely associated with active viral replication, a high level of HBV DNA has been known as an independent risk factor for disease progression. Therefore, suppression of HBV with antiviral therapy could reduce the risk of developing cirrhosis and HCC.

The development of potent antiviral drugs has an important role in the management of patients with CHB. The natural course of the disease could be modified by HBV therapy and risk for HCC could be reduced[3-5]. Antiviral therapy reduces, but does not completely eliminate risk for HCC[6,7]. The annual incidence of HCC range from 0.01% to 5.4% in CHB patients treated with entecavir or tenofovir[8]. Therefore, applying a standardized policy for antiviral therapy and HCC surveillance to all CHB patients may not be cost-effective[9]. Thus, stratification of the risk for HCC development is important for the management of CHB patients.

This review summarizes the prediction models of HCC development in CHB patients.

RISK FACTORS FOR HBV-RELATED HCC

Risk factors for disease progression in CHB can be classified into three categories: host factors, viral factors and liver factors[4,10,11]. Host factors include older age, male gender, family history of HCC, obesity, genetic susceptibility such as single-nucleotide polymorphisms, cirrhosis, smoking, alcohol, diabetes mellitus and immune status[11-16]. Viral factors include a high level of HBV DNA, positive hepatitis B virus e antigen (HBeAg), HBV genotype, HBV mutants, and a high serum level of hepatitis B surface antigen (HBsAg)[16-22]. Particularly, an increasing viral load is a strong predictor of the risk for HCC independent of HBeAg, aminotransferase, and cirrhosis[12,13,18,23]. Liver factors consist of advance fibrosis and cirrhosis, poor liver function, active hepatitis, and other concomitant liver diseases such as co-infection with hepatitis C virus or, alcoholic and nonalcoholic fatty liver diseases[11-13,24-26].

The progression of liver disease in chronic HBV infection is mediated by active virus replication. The annual incidence of cirrhosis in the overall population with CHB is 2%-7%, depending on viral replication status[27]. In particular, disease progression is markedly accelerated in patients with active viral replication by up to 15%-20%. Currently, a complete virological response (CVR) can be achieved even in CHB patients using potent antiviral therapy. Thus, the prognostic value of the baseline level of HBV DNA, which was suggested by large-scale studies to be a robust prognostic indicator of the "natural" course of chronic HBV infection before the era of antiviral treatment, is limited[28]. In the era of antiviral therapy, the prognostic significance of serum levels of HBV DNA has substantially diminished, because most treated patients achieve a virological response[28]. More importantly, the risk for developing liver-related events cannot be completely eliminated even in those who achieve a complete virological response; thus, caution is required in so-called "high-risk" patients who may experience disease progression.

APPROACHES TO DEVELOPING RISK SCORES

Factors independently associated with HCC are first identified in a training or derivation cohort[4,16]. Second, scores are assigned to different parameters in the equation to generate the final score. This score is validated in a validation cohort to demonstrate its applicability and reproducibility. If no independent cohort is available, external validation can be applied to assess the performance of the score in new data. This validation involves using a single observation from the original sample as the validation data, and the remaining observations as the training data. This is repeated such that each observation is used as training and validation data.

Validation of the score usually includes discrimination and calibration. Discrimination can be assessed using a receiver operating characteristic (ROC) curve, sensitivity, and specificity[29,30]. Calibration is evaluated by estimating the observed HCC risk using the Kaplan-Meier method with the same cumulative risk scores. A combination of neighboring groups of cumulative risk scores will be performed if the observed HCC risk in a group with the same cumulative risk score is low[4,13].

HCC RISK SCORES

Until now, several HCC risk prediction scoring systems have been derived to estimate the risk for HCC development in CHB from baseline parameters[11-13,31]. Almost all the scores were derived from and internally validated in treatment-naïve Asian CHB patients[23]. Besides, external validation has been limited to Asian CHB patients or those undergoing treatment with entecavir. Studies including European Caucasian and American patients have shown the models to be somewhat less predictive; however, rates of HCC were very low, significantly limiting the conclusions[6,32].

Individual prediction model

Based on the risk factors of 4339 Korean patients, the individual prediction model (IPM) was developed by calculating the relative weights of risk factors, and a screening program for HCC was established[33]. Old age, male gender, initial serum AFP level, platelet count, serum albumin, severe liver parenchymal echogenic pattern in ultrasonography and heavy alcohol consumption were significant risk factors for HCC. Based on these risk factors, the IPM was calculated using the following formula: risk index (RI) for HCC = e^A, A = -6.2543 + (1.7219 × liver cirrhosis) + (1.3145 × old age over 40 years) + (1.2631 × chronic HCV infection) + (0.8257 × AFP > 20 ng/mL) + (0.7754 × chronic HBV infection) + (0.7339 × chronic hepatitis) + (0.5840 × heavy alcoholics) + (0.3 × man) + (0.2830 × ALT > 40 IU/L) + (0.221 × unknown alcohol history). Probability for HCC = RI/(1 + RI). The authors prospectively applied the screening program to 833 patients with chronic liver disease stratified into three groups [a low-risk group (< 5% probability), an intermediate group (5%-15% probability), and high-risk group (> 15% probability)] by IPM. The patients were followed, at intervals that varied according to the risk index. According to IPM, 2 of 324 patients in the low-risk group (0.62%), 20 of 413 patients in the intermediate-risk group (4.8%), and 22 of 96 patients in the high-risk group (22.9%) were diagnosed with HCC. Thus, the screening program based on IPM enabled cost-effective prediction of the risk of developing of HCC by focusing on the high-risk group.

CU-HCC score

The Chinese University (CU)-HCC score was first derived using a cohort of 1005 Chinese CHB patients that had undergone HCC surveillance at the Chinese University of Hong Kong[4,12,25]. It was validated in an independent cohort of 424 Chinese CHB patients[34]. All patients were treatment-naïve at baseline. Among the patients in the training and validation cohort, 15.1% and 25.0%, respectively, received antiviral therapy during the long-term follow-up. The CU-HCC score is composed of five factors: age, albumin, bilirubin, HBV DNA, and cirrhosis; it ranges from 0 to 44.5. Two cutoff values (5 and 20) discriminated HCC risk into three categories. In all, 105 (10.4%) patients in the training cohort and 45 (10.6%) patients in the validation cohort developed HCC during a median of 10 years of follow-up. The 5-year HCC-free survival rates were 98.3%, 90.5%, and 78.9% in the low-, medium-, and high-risk groups, respectively. Using the lower cutoff of 5 points, this score has a high negative predictive value (97.8%) for excluding future HCC development.

GAG-HCC score

The Guide with Age, Gender, HBV DNA, Core Promoter Mutations and Cirrhosis (GAG-HCC) score was developed from a cohort of 820 Chinese CHB patients from tertiary referral clinics[4,13]. All patients were treatment-naïve at baseline and followed-up for a median of 77 mo. There are two versions of the score. The original version is composed of five parameters: gender, age, core promoter mutations, levels of HBV DNA, and cirrhosis. Because the test for core promoter mutations may not be available in some centers, the score was simplified to omit such mutations. The score can be above 100, as age is one of the components. A cutoff value of 100 had a sensitivity and specificity of 84.1% and 76.2% for 5-year prediction, and 88.0% and 78.7% for 10-year prediction, respectively. The negative predictive values for excluding future HCC development were 98.3%-100%.

NGM1-HCC and NGM2-HCC

The risk evaluation of viral load elevation and associated liver disease (REVEAL)-HBV investigators first suggested easy-to-use nomograms based on noninvasive clinical characteristics using data from 3653 patients[31]. Previously confirmed independent risk predictors were sex, age, family history of HCC, alcohol consumption habit, ALT level, HBeAg serostatus, levels of HBV DNA, and HBV genotype. Regression coefficients were rounded to integer risk scores, and the predicted risk over 5- and 10-year periods for each risk score was calculated and depicted as nomograms. Nomogram 1 and Nomogram 2 hepatocellular carcinoma (NGM1-HCC and NGM2-HCC) were used to calculate individual baseline risk scores for each patient[31]. The patients were categorized into low-, medium- and high-risk groups to facilitate comparison of the risk scores using the different prediction models, and to simplify their use in the clinical setting. The correlation coefficients between observed HCC risk and the nomogram-predicted risk were greater than 0.90.

REACH-B score

The risk estimation for hepatocellular carcinoma in chronic hepatitis B (REACH-B) score was derived using 3584 Chinese CHB patients from the Taiwanese REVEAL cohort, and validated in a cohort of 1505 patients from

three tertiary referral clinics in Hong Kong and South Korea[4,11]. The patients in the training cohort did not have cirrhosis at the time of recruitment, and remained treatment-naïve throughout the 12-year follow-up period. Variables included in the risk score were sex, age, serum levels of alanine aminotransferase, HBeAg status, and levels of HBV DNA level. In all, 131 (3.7%) patients in the training cohort and 111 (7.4%) patients in the validation cohort were developed HCC. A 17-point risk score was developed and HCC risk ranged from 0% to 23.6% at 3 years, 0% to 47.4% at 5 years, and 0% to 81.6% at 10 years for patients with the lowest and highest HCC risks, respectively. The score accurately estimated the risk for developing HCC at 3, 5, and 10 years in patients with CHB. A revised version of REACH-B that includes serum levels of qHBsAg is also available[35].

PAGE-B score

Previous risk scores have been developed mainly in Asians. Therefore, these scores may not be suitable for Caucasian patients with CHB. A new score named PAGE-B has recently been developed for Caucasian CHB patients[36]. A nine-center cohort study was performed in Caucasian CHB patients treated with oral antivirals[36]. They included 1815 adult CHB patients without baseline HCC who received entecavir or tenofovir for more than 1 year. The PAGE-B score was developed based on age, gender, and platelets. During a median of 50 months of follow-up, 51 (3.8%) patients in the derivation group and 34 (6.9%) patients in the validation group developed HCC. Patients with PAGE-B scores of $\leqslant$ 9, 10-17, and $\geqslant$ 18 had 5-year cumulative HCC incidences of 0%, 3% and 17%, respectively. In the validation cohort, the negative predictive value to exclude HCC using at a cut-off of 10 points approached 100%. This was the first study to develop an HCC risk score for Caucasian CHB patients and was the first score for patients treated with current first-line antiviral therapies.

LIVER STIFFNESS MEASUREMENT-BASED MODELS

The degree of liver fibrosis is significantly related to risk for HCC development[37,38]. To date, the gold standard for evaluating the degree of fibrosis is liver biopsy. However, liver biopsy cannot be performed in all CHB patients in a clinical setting due to its invasiveness and complications[39]. Transient elastography (TE, FibroScan®, Echosens, Paris, France) has been validated as a noninvasive method for assessing fibrosis in chronic liver disease[40]. The advantage of TE include its noninvasiveness, highly reproducibility, and accuracy. TE is used as a reliable surrogate for liver biopsy to detect early cirrhosis in patients with CHB[41].

For patients with ascites or high BMI, the use of XL probe could be helpful to check liver stiffness[42,43]. Especially, the presence of nonhepatic ascites does not affect underlying liver stiffness by TE[42]. A liver stiffness value > 12 kPa or 13 kPa by TE can be used to detect histologic cirrhosis in patients with CHB[44,45]. Furthermore, recent studies have reported TE can predict the development of portal hypertension-related complications and HCC[46-48].

Liver stiffness measurement-based Model

Stratified baseline liver stiffness values in patients with CHB are independent predictors of HCC development[38]. The 3-year cumulative incidence of HCC is significantly higher in patients with a higher liver stiffness value[38]. Kim *et al*[49] prospectively analyzed 1110 patients with CHB who received a transient elastography and were available for inclusion criteria from May 2005 to December 2007.

A previous multivariate analysis showed that age, male gender, and liver stiffness values independent predictors of HCC (all $P < 0.05$). In addition, HBV DNA levels $\geqslant$ 20000 IU/L showed borderline significance. Using these four variables, a predictive model was developed (AUROC 0.806, 95%CI: 0.738-0.874). The formula for a 3-year probability of HCC occurrence is as follows: Probability = 1 - P^A [A = exp (0.05306 × age + 1.106 × male gender + 0.04858 × liver stiffness values + 0.50969 × HBV DNA $\geqslant$ 20000 IU/L)]. In bootstrap analyses, the AUROC remained largely unchanged between iterations, with an average value of 0.802 (95%CI: 0.791-0.812). The predicted risk for HCC development calibrated well with the observed risk, with a correlation coefficient of 0.905 ($P < 0.001$).

LSM-HCC score

Wong *et al*[50] developed a new liver stiffness measurement (LSM)-HCC score composed of LSM, age, serum albumin, and levels of HBV DNA. Because diagnosis of cirrhosis based on ultrasonography may be incorrect, cirrhosis as a factor of CU-HCC score was substituted by LSM. Among 1555 CHB patients, 1035 and 520 were assigned to the training and validation cohort, respectively. During a mean of 69 months of follow-up, 38 (3.7%) patients in the training cohort and 17 (3.4%) patients in the validation cohort developed HCC. The LSM-HCC score ranged from 0 to 30. Using 11 as the cutoff value, 706 (68.2%) and 329 (31.8%) patients were in the low- and high-risk categories; 4 (0.6%), and 29 (8.8%) patients developed HCC over 5 years. The AUROCs of the LSM-HCC score were higher than those of the CU-HCC score (0.83-0.89 *vs* 0.75-0.81). The sensitivity for identifying HCC was 87.9% and the NPV was 99.4% at 5 years.

Modified REACH-B score

The REACH-B scoring system, which was developed and validated as a simple HCC prediction model prior to the era of antiviral therapy, showed suboptimal

Table 1 Summary of hepatocellular carcinoma prediction models

	IPM	CU-HCC	GAG-HCC	REACH-B	LSM-HCC	mREACH-B	PAGE-B
Full name	Individual Prediction Model	Chinese University-HCC	Guide with Age, Gender, HBV DNA, Core Promoter Mutations and Cirrhosis-HCC	Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B	Liver Stiffness Measurement-HCC	Modified Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B	
Calculation	Risk Index (RI) for HCC = e^A, A = -6.2543 + (1.7219 × liver cirrhosis) + (1.3145 × old age over 40 yr) + (1.2631 × chronic HCV infection) + (0.8257 × AFP > 20 ng/mL) + (0.7754 × chronic HBV infection) + (0.7339 × chronic hepatitis) + (0.5840 × heavy alcoholics) + (0.3 × man) + (0.2830 × ALT > 40 IU/L) + (0.221 × unknown alcohol history)	Age (> 50 yr = 3; ≤ 50 = 0) + albumin (≤ 35 g/L = 20; > 35 = 0) + bilirubin (> 18 μmol/L = 1.5; ≤ 18 = 0) + HBV DNA (< 4 log copies/mL = 0; 4-6 = 1; > 6 = 4) + cirrhosis (yes = 15; no = 0)	14 × sex (male = 1; female = 0) + age (in years) + 3 × HBV DNA (log copies/mL) + 33 × cirrhosis presence = 1; absence = 0)	Male sex: 2 points Age: 1 point for every 5 yr from 35 to 65 yr of age (0-6 points) ALT (IU/L): 15-<45 (1 point), ≥ 45 (2 points) Positive HBeAg: 2 points HBV DNA (log copies/mL): 4-< 5 (3 points), 5-< 6 (5 points), ≥ 6 (4 points)	Age (> 50 yr = 10; ≤ 50 = 0) + albumin (≤ 35 g/L = 1; > 35 = 0) + HBV DNA (> 200000 IU/mL = 5; ≤ 200000 = 0) + liver stiffness (≤ 8.0 kPa = 0; < 8.0-12.0 = 8; > 12.0 = 14)	Male sex: 2 points Age: 1 point for every 5 yr from 35 to 65 yr of age (0-6 points) ALT (IU/L): 15-< 45 (1 point), ≥ 45 (2 points) Positive HBeAg: 2 points Liver stiffness values: < 8.0 kPa (0 point), 8.0-13.0 (2 points), > 13.0 kPa (4 points)	Age; < 30 (-4 points), 30-39 (-2 points), 40-49 (0 point), 50-59 (2 points), 60-69 (4 points), ≥ 70 (6 points) Male sex: 5 points Platelets (mm^3): ≥ 200 × 10^3 (0 point), 100× 10^3-< 200 × 10^3 (6 points), < 100 × 10^3 (11 points)

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

predictive performance. Therefore, an alternative predictor of long-term prognosis is required particularly in CHB patients who had achieved CVR from antiviral treatment, because levels of HBV DNA are no longer useful at the time of CVR.

In the modified REACH-B model (mREACH-B model), the serum levels of HBV DNA were substituted for the LS value, and had better predictive performance among patients who were at CVR following entecavir therapy[28]. The authors reassessed the scores at CVR, using LS values instead of suppressed HBV DNA. The AUROC value for risk at the 3-year follow-up was 0.805, compared to 0.629 using the original REACH-B scoring system, when 0, 1, and 2 points were assigned to LS values of < 8.0, 8.0-13.0, and > 13.0 kPa, respectively (referred to as the modified REACH-B I), and 0.814 (95%CI: 0.709-0.912) when 0, 2, and 4 points were assigned to LS values of < 8.0, 8.0-13.0, and > 13.0 kPa, respectively (referred to as the modified REACH-B II).

The performance of conventional HCC prediction models (CU-HCC, GAG-HCC, REACH-B, and LSM-HCC scores) and the mREACH-B score has been assessed[51]. During the follow-up (median, 75.3 mo), HCC developed in 125 (9.6%) of 1308 subjects. The mREACH-B score had a significantly higher AUROC for prediction of HCC development at 3/5 years (0.828/0.806), compared to the LSM-HCC (0.777/0.759), GAG-HCC (0.751/0.757), REACH-B (0.717/0.699), and CU-HCC (0.698/0.700) scores (all *P* values < 0.05 *vs* mREACH-B). Thus, the prognostic performance of the mREACH-B score was superior to that of the conventional models.

OTHER HCC RISK MODELS

Existing prediction models were mostly developed in Asia. There were limited data about HCC risk models for people at high risk in the United States or European countries. France group suggested *PNPLA3* rs738409 (GG) genotype had an effect on the occurrence of HCC[52]. They created the following model: age × 0.05085 - 1.88790 × female gender + BMI × 0.09712 + rs738409 (GG) × 0.78377.

When applied to 250 patients with alcoholic cirrhosis, scores ranged from 2.20-9.25. The cut-off values for calculated score were below 5, between 5 and 7, and above 7, respectively. 6-year incidence of HCC increased according to stratification of three risk groups.

There was another risk prediction model suggested from United States[53]. By Cox proportional hazards regression model, clinical and demographic data (including age, sex, smoking status, alkaline phosphatase level, and platelet count) and Epidermal Growth Factor Gene genotype (GG) was used to predict HCC risk. The cohort was stratified into three groups depending on the risk of HCC development.

CONCLUSION

This review summarizes prediction models of HCC development in CHB patients (Tables 1 and 2). HCC

Table 2 Comparisons of published hepatocellular carcinoma prediction models

	IPM	CU-HCC	GAG-HCC	REACH-B	LSM-HCC	mREACH-B	PAGE-B
Number of patients	994	1005	820	3584	1035	1308	1325
Place of development	South Korea	Hong Kong	Hong Kong	Taiwan	Hong Kong	South Korea	Europe
Race	Asian	Asian	Asian	Asian	Asian	Asian	Caucasian
Age (yr)		48	40.6	45.7	46	50	52
HBeAg-negative (%)			56.6	84.8	75	60.3	84
Cirrhosis (%)		38.1	15.1	0	32	17.8	20
Follow-up (yr)	2.7	9.94	5.62	12	5.8	6.3	3.6
Antiviral therapy (%)		15.1	0	0	38	64.8	100
HCC (%)	90 (0.1)	105 (10.4)	40 (4.9)	131 (3.7)	38 (3.7)	125 (9.6)	51 (3.8)
Components of the risk scores	Age Male Platelet Cirrhosis Albumin AFP Heavy alcoholics	Age Albumin Bilirubin Cirrhosis HBV DNA	Age Male BCP mutation Cirrhosis HBV DNA	Age Male ALT HBeAg-positive HBV DNA	Age Albumin HBV DNA LS value	Age Male ALT HBeAg-positive LS value	Age Male Platelet
Risk scores	Low (< 5) Intermediate (5-15) High (> 15)	Low (< 5) Intermediate (5-19) High (> 19)	Low (< 100) High (≥ 100)	Low (0-5) Intermediate (6-11) High (12-18)	Low (< 11) High (≥ 11)	Low (< 10) High (≥ 10)	Low (≤ 9) Intermediate (10-17) High (≥ 18)
NPV (%)		97% at 10 yr	99% at 10 yr	98% at 10 yr	99.4% at 5 yr	96.8% at 5 yr	100% 5 yr

HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; LS: Liver stiffness; AFP: α-fetoprotein; NPV: Negative predictive value.

risk scores can accurately predict subsequent HCC development in CHB patients. Different levels of care and different intensities of HCC surveillance should be offered according to the patient's risk profile. Patients in the high-risk category should be offered antiviral therapy, as well as appropriate HCC surveillance. Effective suppression of HBV replication by antiviral therapy can reduce risk for HCC development. However, antiviral therapy does not eliminate the HCC risk completely, because of the presence of virus integrated into the host genome. The HCC risk is higher in cirrhotic than non-cirrhotic patients. Antiviral therapy with no risk of resistance such as entecavir or tenofovir should be initiated before cirrhosis occurs.

HCC prediction models can help optimize antiviral therapy based on the level of HCC risk. It should be adjusted for patients who are already on treatment. Decisions regarding who needs treatment and regular surveillance should be individualized using HCC risk prediction models.

FUTURE PERSPECTIVES

In the future, a more accurate risk model that incorporates newly identified risk factors and somatic and inherited biomarkers (*e.g.*, single-nucleotide polymorphisms, proteomics) is required for more accurate estimation of risk. Various plasma proteins have been proposed as new biomarkers of genetic background to predict development of HCC. These biomarkers are expected to guide individual surveillance or treatment for CHB patients. However, further functional studies are needed to validate these biomarkers. In addition, simple, user-friendly models for primary care providers would facilitate referral of high-risk patients.

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ORIGINAL ARTICLE

Basic Study

Rhubarb extract partially improves mucosal integrity in chemotherapy-induced intestinal mucositis

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Abstract

AIM

To investigate the effects of orally gavaged aqueous rhubarb extract (RE) on 5-fluorouracil (5-FU)-induced intestinal mucositis in rats.

METHODS

Female Dark Agouti rats (n = 8/group) were gavaged daily (1 mL) with water, high-dose RE (HDR; 200 mg/kg) or low-dose RE (LDR; 20mg/kg) for eight days. Intestinal mucositis was induced (day 5) with 5-FU (150 mg/kg) *via* intraperitoneal injection. Intestinal tissue samples were collected for myeloperoxidase (MPO) activity and histological examination. Xenopus oocytes expressing aquaporin 4 water channels were prepared to examine the effect of aqueous RE on cell volume, indicating a potential mechanism responsible for modulating net fluid absorption and secretion in the gastrointestinal tract. Statistical significance was assumed at $P < 0.05$ by one-way ANOVA.

RESULTS

Bodyweight was significantly reduced in rats administered 5-FU compared to healthy controls ($P < 0.01$). Rats administered 5-FU significantly increased intestinal MPO levels ($\geqslant$ 307%; $P < 0.001$), compared to healthy controls. However, LDR attenuated this effect in 5-FU treated rats, significantly decreasing ileal MPO activity (by 45%; $P < 0.05$), as compared to 5-FU controls. 5-FU significantly reduced intestinal mucosal thickness (by $\geqslant$ 29% $P < 0.001$) as compared to healthy controls. LDR significantly increased ileal mucosal thickness in 5-FU treated rats (19%; $P < 0.05$) relative to 5-FU controls. In xenopus oocytes expressing AQP4 water channels, RE selectively blocked water influx into the cell, induced by a decrease in external osmotic pressure. As water efflux was unaltered by the presence of extracellular RE, the directional flow of water across the epithelial barrier, in the presence of extracellular RE, indicated that RE may alleviate water loss across the epithelial barrier and promote intestinal health in chemotherapy-induced intestinal mucositis.

CONCLUSION

In summary, low dose RE improves selected parameters of mucosal integrity and reduces ileal inflammation, manifesting from 5-FU-induced intestinal mucositis.

Key words: Fluorouracil; Inflammation; Mucositis; Rats; Rheum

Core tip: Aqueous rhubarb extract partially improved selected parameters of 5-fluorouracil (5-FU)-induced intestinal mucositis in rats. Exposure to 5-FU decreased bodyweight, yet high-dose rhubarb extract (RE) and low-dose RE (LDR) showed no changes. Myeloperoxidase activity was significantly decreased in rats treated with LDR and 5-FU when compared to the intestinal mucositis control group. Ileal mucosal thickness was significantly improved (19%) in animals with intestinal mucositis and treated with LDR. In xenopus oocytes expressing AQP4 water channels, RE blocked swelling induced by a decrease in external osmotic pressure which indicated that water influx across the epithelial barrier was selectively blocked by RE.

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INTRODUCTION

Traditional herbal medicines have been used for centuries in the maintenance and improvement of health or the treatment of illnesses. Globally, ancient herbal remedies have been created based on theories, beliefs and experiences representing various cultures at different times throughout history[1]. Consequently, traditional herbal medicines are being investigated increasingly for their potential to treat and reduce the symptoms of a wide variety of diseases and disorders, specifically cancer and its treatment-related side-effects. Many cancer patients seek alternative medicines that will complement their standard-care treatments with the hope that they will improve symptoms associated with either the cancer or their anti-cancer treatments[2].

Cancer is a life-threatening illness affecting millions of individuals world-wide. In westernized countries approximately 50% of the population will develop cancer before the age of 85[3]. Chemotherapy forms one of the most common strategies for cancer treatment. Cytotoxic chemotherapy drugs, such as 5-fluorouracil (5-FU), act by inhibiting DNA synthesis of not only malignant cells, but also rapidly dividing cells lining the intestinal mucosa[4]. An increase in cell apoptosis stimulates the production of reactive oxygen species (ROS) and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-4 resulting in further tissue and blood vessel damage[5,6]. This cascade of events results in a range of debilitating clinical side-effects, from nausea and vomiting to inflammation and ulceration of the gastrointestinal tract; and sepsis may occur if untreated[7,8]. These painful and life-threatening side-effects collectively form a disorder known as intestinal mucositis which affects approximately 60% of patients undergoing chemotherapy[9]. Current therapies for intestinal mucositis seek to reduce the severity of symptoms rather than acting as a curative

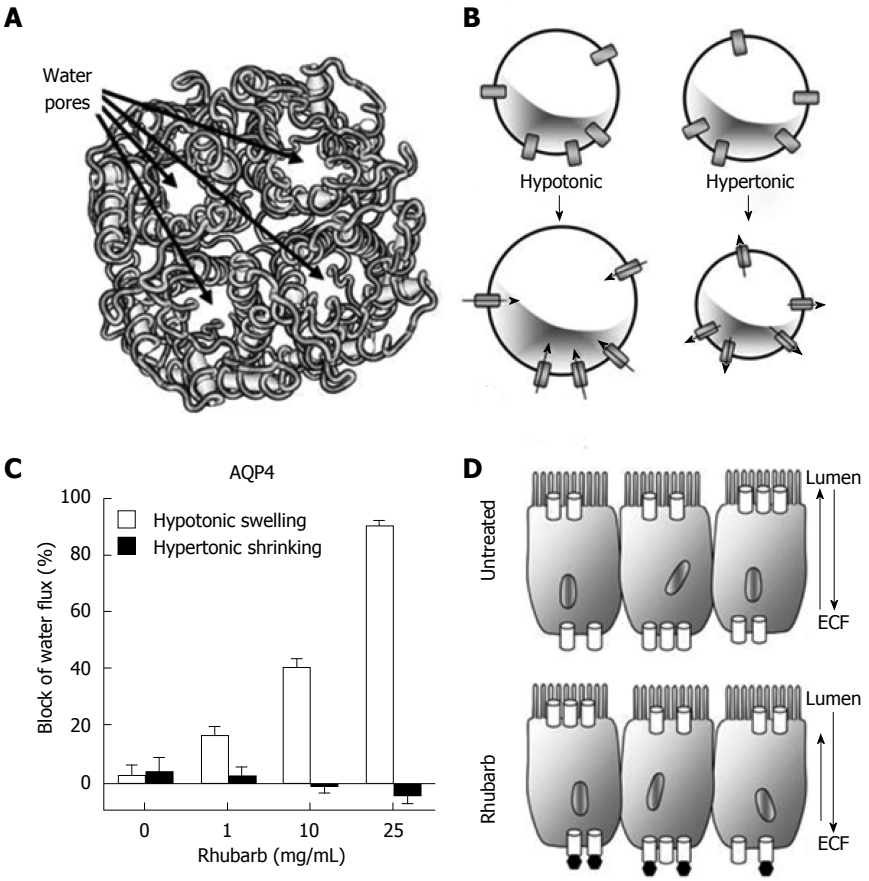

Figure 1 Directional blockade of water flux through an aquaporin-4 channel by reconstituted aqueous rhubarb extract. A: Diagram of a water channel illustrating the intra-subunit water pores in each subunit of the tetramer; B: Illustration of the volume changes induced by osmotic gradients in mammalian AQP4-expressing Xenopus oocytes; C: Dose-dependent blockade of swelling but not shrinking responses by rhubarb extract (RE) in AQP4-expressing oocytes; D: Diagram of the hypothesized effect of blockade by extracellular RE at AQP4 channels present in the basolateral side of intestinal barrier epithelial cells, predicted to result in enhanced net fluid absorption.

or preventative measure[10,11]. Thus, treatments are required with the potential to eliminate or reduce the adverse side-effects of cancer chemotherapy.

Recently, in experimental systems, plant extracts such as grape seed extract (GSE) and Iberogast® have been investigated as potential treatments for intestinal mucositis on the basis of their anti-inflammatory and antioxidant constituents[12-14]. Indeed, plant-sourced molecules and compounds are commonly perceived to be safer therapeutics compared to synthetic compounds[15]. There are limited studies on the pharmacology of herbal medicines, yet such extracts may offer protection against intestinal mucositis in an experimental setting. The scientific study of further plant-based extracts is therefore warranted.

Rhubarb, *Rheum spp.,* is a herbaceous perennial plant with a long, fleshy stalk, commonly used for cooking and medicine. Dried rhubarb rhizomes were traditionally used in Chinese medicine as a natural remedy for gastrointestinal complications including diarrhoea, constipation and inflammation[16]. The pharmacological effects have been attributed to the stalk of the plant[17,18]. Two main active constituents (ethanol-soluble and water soluble) have been classified in rhubarb stalks. Anthraquinones form the main ethanol-soluble active constituent of rhubarb stalks[14]. These constituents have exhibited a diarrhoeal effect in mice providing a possible purgative mechanism of action[18]. In contrast, the aqueous extract of rhubarb has recently demonstrated anti-diarrhoeal properties, believed to be mediated by tannins through regulation of intestinal water secretion and absorption[18]. Importantly, chemotherapy recipients experiencing intestinal mucositis have altered membrane integrity and impaired water absorption and secretion[7,19].

Aquaporins (AQPs) are integral membrane proteins responsible for the regulation of water transport across a membrane *via* an osmotic gradient[20,21]. Aquaporin channels are tetramers with a water pore located in each subunit of the channel (Figure 1A). Water molecules move in single file through aquaporin pores, down osmotic and hydrostatic gradients. As one molecule enters *via* the extracellular region of the channel, another molecule is displaced into the cytoplasm and vice versa[22]. Currently, 13 mammalian AQPs have been identified (AQP 0-12). AQPs are abundant in tissues reliant on high water permeability

to maintain correct function[21,23] and are involved in metabolic processes such as kidney, lung, brain and gastrointestinal function[24-26]. In the human gastrointestinal tract, AQPs 3, 7 and 8 are expressed throughout the mucosal epithelia, and AQP1 is present in endothelial cells of the vasculature. In early stage inflammatory bowel disease, tight junctions and transport systems are impaired, leading to a leaky epithelium. Clinical human biopsies showed that levels of expression of AQPs1 and 3 are reduced in Crohn's Disease and AQPs 7 and 8 are decreased in ulcerative colitis, based on quantitative PCR and immunolabelling assays[27]. As well, the typical apical localisation of AQP8 in bowel was lost, and the appearance of a faint basolateral signal suggested intestinal epithelial cell polarity was disrupted.

Aquaporin-4 (AQP 4) is believed to provide the principal mechanism for bidirectional water transport across the basolateral membrane of small intestinal enterocytes[28]. These water channels ensure that efficient water absorption and secretion is maintained, thus allowing for adequate hydration and optimal stool consistency[29]. Liu *et al*[17] demonstrated that the anti-diarrhoeal effect of rhubarb tannins extract occurred *via* the inhibition of AQP 2 and 3 expression *in vitro* and in a mouse model of magnesium sulphate-induced diarrhoea. In addition, the water-soluble polysaccharides of rhubarb have protected the gastrointestinal tract against inflammation resulting from 2,4,6-trinitrobenzene sulfonic acid-induced colitis[17]. The anti-inflammatory mechanism of action underlying rhubarb extract (RE) remains unclear; however, it is thought that tannins may reduce the production of pro-inflammatory cytokines such as IL-4 and IFN-γ[17]. Consequently, RE was explored for its anti-inflammatory potential in intestinal mucositis and its potential to influence water transport across the intestinal mucosa[17,18].

In the current study, an aqueous fraction of rhubarb was investigated for its potential to reduce intestinal damage induced by the antimetabolite chemotherapy drug, 5-FU in rats. It was hypothesised that RE would decrease the severity of intestinal mucositis by improving histopathological parameters and potentially regulate faecal output *via* water secretion into the intestinal lumen.

MATERIALS AND METHODS

RE preparation

Rhubarb stems (2.5 kg) were sectioned (1 cm) and boiled with absolute ethanol to remove alcohol-soluble components. Once cooled, the liquid was discarded and the residues were further boiled with water. The aqueous rhubarb components were retained for dehydration to obtain a concentrated powder[17]. Dehydration was conducted by freeze-drying at the South Australian Research and Development Institute, West Beach, South Australia. Four grams of powder were obtained for every 500 g of fresh rhubarb. Based on fractionation of the extract, the active agent appears to be a water-soluble ethanol-insoluble glycopeptide. Lectin array profiling has indicated that mannose and N-acetylglucosamine are predominant components of the carbohydrate structure. The precise chemical structure and possible presence of more than one isoform with biological activity remains to be determined.

Animal trial, metabolism data and disease Activity index

Six week old female Dark Agouti rats (n = 32; 110-150 g) were sourced from the Animal Resources Centre (Western Australia) and Laboratory Animal Services (The University of Adelaide, South Australia). All animal experimentation was approved by the Animal Ethics Committee of the University of Adelaide (S-2010-111). The animal protocol described in this study was designed to minimise pain or discomfort to the animals and complied with the National Health and Medical Research Council Code of Practice for Animal Care in Research and Teaching. Prior to the experimentation period, rats were individually housed in Tecniplast™ (PA, United States) metabolism cages for 48 hours to acclimatise. Rats received *ad libitum* water and 18% Casein diet[30] and were exposed to a 12 h light-dark cycle in a temperature controlled room (22 ℃). After the acclimatisation phase, rats were randomly allocated to four treatment groups (n= 8/group): Water + Saline, Water + 5-FU, Low-Dose Rhubarb (LDR; 20 mg/kg BW) + 5-FU and High-Dose Rhubarb (HDR; 200 mg/kg BW) + 5-FU. Water, HDR and LDR (1 mL) were administered daily *via* orogastric gavage on days 0 to 7. LDR dose for gavage was based on the estimated dose required to block aquaporin water channel activity in the oocyte expression system, and the dose HDR was selected as a 10 fold higher concentration for comparison.

Daily recordings of body weight, feed and water intake and faecal and urine output were conducted. Faecal pellets were collected daily, weighed and placed in a drying oven at 70 ℃ for 72 h. The percentage weight loss was used as an indication of moisture content in the faecal samples. On day 5, rats were injected with 5-FU (150 mg/kg BW; Hospira Australia Pty Ltd, Melbourne, Victoria) to induce intestinal mucositis. The single high dose of 5-FU used in the current study was determined from previous studies in our laboratory[31]. Following 5-FU administration, daily disease activity index (DAI) scoring was performed by a blinded researcher based on overall condition, weight loss and stool consistency. Each parameter was scored based on a scale of 0 (normal) to 3 (maximal severity) giving a maximum daily total of 9 for severely affected rats[32,33].

Blood, organ and tissue collection

Rats were humanely euthanized on day 8 *via* carbon dioxide asphyxiation. Day 8 of the experimental period

represented 3 d post 5-FU exposure and due to the acute nature of 5-FU-induced intestinal mucositis, this was determined to be the optimal day when histological damage in the intestine was most evident. The gastrointestinal tract was removed and emptied, then the lengths of each section [duodenum, jejunum, jejuno-ileum junction (JI), ileum and colon] were recorded and weighed.

Segments (2 cm and 4 cm) of the small intestine tract were collected at approximately 10% (jejunum) and 90% (ileum) of the total small intestine length for histological and biochemical analysis, respectively. Samples for histological analysis were fixed in 10% buffered formalin for 24 h and transferred to 70% ethanol for preservation. Segments for biochemical analysis were weighed and snap-frozen in liquid nitrogen prior to storage at -80 ℃. The remaining thoracic and abdominal organs (thymus, lungs, heart, spleen, kidneys, liver, stomach and caecum) were weighed and discarded.

Biochemical analysis

Myeloperoxidase (MPO) is an enzyme present in the intracellular granules of neutrophils and provides a quantitative analysis of acute inflammation. The assay was performed with slight modification from Beyer *et al*[34]. Segments of the small intestinal tract (jejunum, JI and ileum; 4 cm) were thawed and prepared for MPO assay *via* homogenization in 10 mmol/L phosphate buffer (pH 6.1). Homogenised samples were centrifuged at 13000 rpm for 12 min and the supernatant was discarded. The remaining pellet was resuspended with 0.5% hexadecyltrimethyl ammonium bromide buffer and vortexed prior to a final centrifuge (13000 rpm for 2 min). Supernatant from each sample (50 μL aliquot) was dispensed into a 96-well plate and the MPO reaction was initiated with an *O*-dianisidine dihydrochloride solution (200 μL/well; 4.2 mg *O*-dianisidine dihydrochloride, 12.5 μL hydrogen peroxide (30%) in 2.5 mL potassium phosphate buffer (50 mmol/L, pH 6.1) and 22.5 mL distilled H_2O). A spectrometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore) measured absorbance (450 nm) at one minute intervals over a 15 min period. The change in absorbance was used to calculate MPO activity within a tissue sample (MPO units/g of intestinal tissue).

Histological analysis

Intestinal samples stored in 70% ethanol were embedded with paraffin wax and cross-sectioned at 4 μm. Histological slides were stained with haematoxylin and eosin for qualitative and quantitative analysis. Qualitative measurements of 40 villus and crypts per intestinal section (jejunum, JI and ileum) were performed blinded using Image ProPlus software for Windows (version 5.1.1; Media Cybernetics, Silver Spring MD, United States) connected to a Nikon Eclipse 50i light microscope (Nikon Cooperation, Japan) and a ProGres C5 digital camera (Jenoptik, Germany). Intestinal sections were also analysed quantitatively using disease severity scores based on 11 criteria described by Howarth *et al*[32]. Each criterion was scored on a scale of 0 (normal) to 3 (severely damaged) for five cross-sections of each intestinal region. The median score for each criterion was calculated and the scores of all criteria were summed to give an overall disease severity score; with a score of 33 indicating maximal tissue damage[32,33].

Xenopus oocyte preparation

Unfertilized oocytes from *Xenopus laevis* were prepared as described previously[35,36] and maintained in ND96 saline (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, and 5 mmol/L HEPES, pH 7.55) supplemented with 100 μg/mL penicillin,100 U/mL streptomycin, and 10% horse serum. Oocytes were injected with 50 nL of water containing 1 ng of rat AQP4 wild-type cRNA and were incubated for 2 or more days at 16-18 ℃ prior to osmotic swelling and shrinking assays in saline without antibiotics or serum. Hypotonic saline (50%) was prepared by diluting isotonic saline with an equal volume of water, whilst 200% hypertonic saline was prepared by doubling the NaCl concentration of the saline. Volume change rates were measured by videomicroscopy at 0.5 frames/s over 30 s using NIH ImageJ software (http://rsbweb.nih.gov/ij/), as described previously[35,36].

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 19 for Windows (SPSS Inc., Chicago, IL, United States) and GraphPad Prism 6.02 for Windows (GraphPad Software Inc., San Diego, CA, United States). Normality tests were performed on all data sets to determine parametric and non-parametric data. All parametric data (metabolic data, MPO activity and villus height/crypt depth measurements) was analysed using one-way ANOVA with Tukey *post hoc* test. Non-parametric data (DSS and DAI) was analysed using Kruskal-Wallis with Mann Whitney *U post hoc* test. All data were expressed as mean ± SEM with the exception of disease severity scores which were expressed as medians and range. Values of $P < 0.05$ were considered significant.

RESULTS

Dose-dependent blockade of AQP4 water channel activity by extracellular aqueous RE

Cloned rat AQP 4 water channels expressed in Xenopus oocytes were analysed quantitatively for osmotically-driven changes in cell volume in the presence and absence of dried reconstituted aqueous RE. Decreased external osmotic pressure (50% hypotonic saline) induced a volume increase (swelling) that was blocked

Table 1 Total daily food (g) and water (mL) intake, and faecal (g) and urine (mL) output for the trial period prior to the administration of 5-FU (days 1 to 5)

	Water	LDR	HDR
Food intake (g)	51.0 ± 0.7	52.3 ± 2.0	53.8 ± 1.0
Water Intake (mL)	122.5 ± 7.3	129.4 ± 12.0	115.0 ± 7.1
Wet faecal output (g)	6.2 ± 0.3	6.8 ± 0.3	6.6 ± 0.4
Urine output (mL)	79.3 ± 5.6	79.8 ± 6.1	85.8 ± 5.0

Rats were gavaged daily with water, LDR or HDR (1 mL); data expressed as mean (g or mL) ± SEM. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

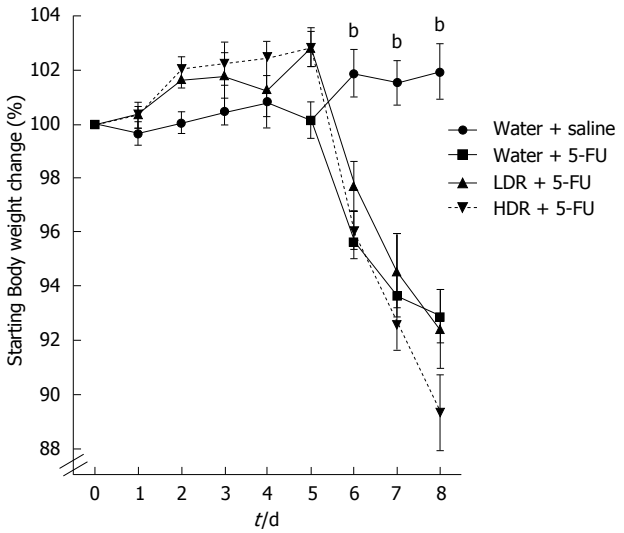

Figure 2 Daily change in starting bodyweight (%) from days 0 to 8 in rats gavaged with water, LDR or HDR and intraperitoneally injected with saline or 5-FU on Day 5. Data are expressed as mean ± SEM. Mean values of 5-FU controls and 5-FU + LDR and 5-FU + HDR were significantly different when vs water + saline controls; $^{b}P < 0.01$. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

by RE (Figure 1B and C). In contrast, the volume decrease (shrinking) induced by 200% hypertonic saline was not significantly altered by RE (Figure 1B and C), indicating that the blocking effect of RE was directional. In the presence of extracellular RE, water influx into the cell mediated by AQP4 was selectively blocked, whereas water efflux was not altered, providing a potentially useful tool for differentially modulating net fluid absorption and secretion in the gastrointestinal tract. The current *ex vivo* study predicted that RE would act on basolateral AQP 4 channels and alleviate water loss across the barrier epithelium (Figure 1D), thereby promoting intestinal health in the experimental setting of chemotherapy-induced intestinal mucositis.

Metabolic data and faecal moisture content

Low dose rhubarb (LDR) and high dose rhubarb (HDR) had no significant effect on metabolic parameters (bodyweight, feed and water intake and faecal and urine output) when compared to controls

Table 2 Total daily food (g) and water (mL) intake, and faecal (g) and urine (mL) output for the trial period after the administration of 5-FU (days 6 to 8)

	Water + saline	Water + 5-FU	LDR + 5-FU	HDR + 5-FU
Food intake (g)	29.1 ± 0.6	11.5 ± 1.6^{e}	7.8 ± 0.6	5.2 ± 1.4^{d}
Water intake (mL)	75.0 ± 4.3	94.4 ± 7.7	107.2 ± 5.1	90.6 ± 12.9
Wet faecal output (g)	3.3 ± 0.2	2.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.3^{c}
Urine output (mL)	47.5 ± 4.7	64.5 ± 6.7	71.0 ± 2.1	70.6 ± 10.4

Rats were gavaged daily with water, LDR or HDR (1 mL) and received an intraperitoneal injection of either saline or 5-FU on day 5. $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* water + 5-FU; $^{e}P < 0.001$ *vs* water + saline. All values are expressed as mean [% relative to bodyweight ($\times 10^{-2}$)] ± SEM. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

prior to administration of 5-FU (Table 1). After 5-FU administration, feed intake was significantly decreased (by 60%; $P < 0.001$) in comparison to healthy controls (Table 2). Furthermore, in 5-FU treated rats administered HDR, feed intake was further reduced by 55% when compared to 5-FU controls ($P < 0.01$). However, normal feed intake was maintained in 5-FU treated rats administered LDR. Although feed intake was significantly reduced in 5-FU controls, there was no reduction in wet faecal output compared to healthy controls. However, in 5-FU treated rats administered HDR, faecal output was reduced by 41% in comparison to 5-FU controls. There were no significant effects on water intake and urine output between control and RE treatment groups (Table 2). Similarly, no significant effects on faecal moisture content were evident among all treatment groups, before or after 5-FU administration (data not shown).

Bodyweight change

A reduction in feed intake was consistent with decreased bodyweight after 5-FU administration (Figure 2). Prior to inducing intestinal mucositis with 5-FU, RE had no significant effect on bodyweight. Treatment with 5-FU resulted in a significant reduction in bodyweight compared to normal controls ($P < 0.01$). However, compared to 5-FU controls, HDR and LDR had no effect on mean bodyweight following 5-FU administration.

DAI score

Administration of 5-FU significantly increased DAI scores in comparison to healthy controls ($P < 0.01$; Figure 3). Days 6 and 8 produced significantly greater DAI scores in 5-FU treated rats administered HDR and LDR, respectively, compared to 5-FU controls; otherwise, RE treatments had no significant effect on symptomatic disease activity.

Visceral gastrointestinal organ weights and lengths

Visceral and gastrointestinal organ weights were expressed as a proportion of bodyweight (Tables 3 and 4). Reductions in relative thymus (by ⩾ 35%; $P < 0.001$) and relative spleen weight (by ⩾ 23%; P

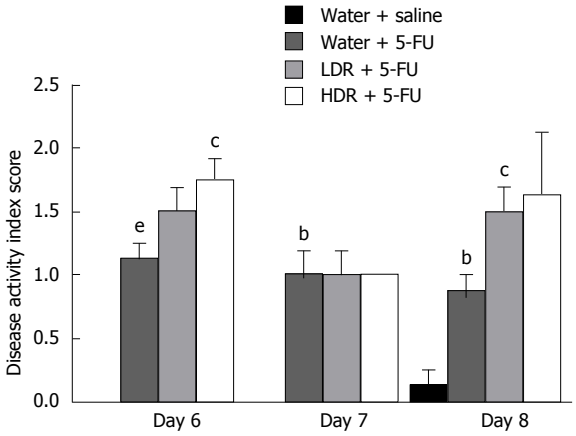

Figure 3 Effects of rhubarb extract and 5-fluorouracil on disease activity scores on days 6 to 8 of the experimental period. Rats received a daily water, HDR or LDR gavage for an 8-d trial period and an intraperitoneal injection of 5-FU or saline on day 5. Disease activity scores were assigned on Days 6 to 8 based on overall condition, weight loss, stool consistency and rectal bleeding. $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* water + saline; $^{c}P < 0.05$ *vs* water + 5-FU. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

Table 3 Visceral organ weights of rats gavaged daily with water, low-dose or high-dose rhubarb extract (1 mL) during an 8-d trial period and administered with an intraperitoneal injection of saline or 5-fluorouracil on day 5

	Water + saline	Water + 5-FU	LDR + 5-FU	HDR + 5-FU
Thymus	14.6 ± 1.3	6.6 ± 0.5[e]	9.4 ± 0.6	9.5 ± 0.9
Heart	37.5 ± 0.8	39.0 ± 1.0	39.4 ± 0.8	39.1 ± 0.7
Lung	60.0 ± 2.2	63.0 ± 2.5	67.3 ± 4.7	71.9 ± 3.0
Liver	362.9 ± 6.5	362.7 ± 11.0	358.8 ± 7.2	339.7 ± 7.2
Spleen	20.3 ± 0.5	15.6 ± 0.3[e]	15.2 ± 0.4	14.6 ± 0.5
Kidneys	75.6 ± 5.3	86.5 ± 1.7	88.7 ± 1.1	89.4 ± 2.7
Caecum	39.7 ± 1.1	43.7 ± 2.4	49.2 ± 2.5	47.0 ± 2.1
Stomach	57.3 ± 2.6	55.3 ± 1.1	58.8 ± 0.9	61.9 ± 1.2

$^{e}P < 0.001$ *vs* water + saline. All values are expressed as mean [% relative to bodyweight ($\times 10^{-2}$)] ± SEM. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

< 0.001) were apparent in all rats treated with 5-FU when compared to healthy controls (Table 3). In 5-FU treated rats, HDR and LDR had no significant effect on visceral organ weights compared to 5-FU controls.

A significant decrease in the combined jejunum and ileum relative weight (by ⩾ 10%; $P < 0.01$) was evident in all 5-FU treated rats (Table 4). However, this effect was not present in the duodenum. There was also no effect of HDR or LDR on relative duodenum weight and the combined relative weights of jejunum and ileum in 5-FU treated rats, compared to 5-FU controls. Administration of 5-FU had no effect on relative colon weight in comparison to healthy controls. However, when compared to 5-FU controls, administration of LDR to 5-FU treated rats significantly increased colon weight (29%; $P < 0.01$). Additionally, 5-FU significantly reduced the combined jejunum and ileum length in comparison to healthy controls (Table 5). However,

Table 4 Gastrointestinal organ weights of rats gavaged daily with water, low-dose and high-dose rhubarb extract (1 mL) during an 8-d rial period and administered an intraperitoneal injection of saline or 5-fluorouracil on day 5

	Water + saline	Water + 5-FU	LDR + 5-FU	HDR+ 5-FU
Duodenum	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Jejunum and ileum	2.1 ± 0.1	1.9 ± 0.0[b]	1.9 ± 0.2	1.9 ± 0.1
Colon	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.0[d]	0.6 ± 0.0

$^{b}P < 0.01$ *vs* water + saline; $^{d}P < 0.01$ *vs* water + 5-FU. All values are expressed as mean (% relative to bodyweight) ± SEM. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

Table 5 Gastrointestinal organ lengths of rats gavaged daily with water, low-dose and high-dose rhubarb extract (1 mL) during an 8-d trial period and administered an intraperitoneal injection of saline or 5-fluorouracil on day 5

	Water + saline	Water + 5-FU	LDR + 5-FU	HDR+ 5-FU
Duodenum	5.5 ± 0.2	4.8 ± 0.1	5.1 ± 0.2	4.8 ± 0.2
Jejunum and ileum	71.6 ± 2.3	64.8 ± 0.9[a]	62.9 ± 1.8	63.5 ± 1.7
Colon	11.1 ± 0.3	10.6 ± 0.4	11.2 ± 0.2	10.8 ± 0.4

$^{a}P < 0.05$ *vs* water + saline. All values expressed as mean (cm) ± SEM. LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

this effect was not evident in the duodenum and colon. The administration of HDR and LDR to 5-FU treated rats had no effect on gastrointestinal organ lengths in comparison to 5-FU controls.

Disease severity score

Healthy small intestinal sections achieved median disease severity scores of ⩽ 2. Administration of 5-FU caused significant damage to intestinal structure in the jejunum, JI and ileum; achieving median (range) scores of 21 (18-30), 21 (14-27) and 22 (17-25), respectively, when assessed by semi-quantitative histological scores based on 11 parameters (Figure 4). However, RE had no significant effect on intestinal structure, relative to 5-FU controls.

MPO activity

Increased intestinal MPO activity is a common feature of chemotherapy-induced intestinal mucositis[31]. When compared to healthy controls, 5-FU resulted in increased MPO activity by 780% in the jejunum and 310% in the JI and ileum (Figure 5). RE had no significant effect on MPO activity within the jejunum and the JI in 5-FU treated rats. However, administration of LDR to 5-FU treated rats resulted in reduced MPO activity by 45% ($P < 0.05$) in the ileum, compared to 5-FU controls.

Villus height, crypt depth and mucosal thickness

The combined measurements of villus height and crypt depth provided an overall indication of mucosal

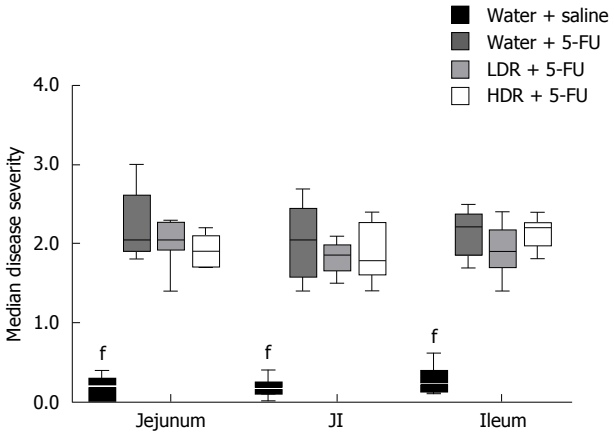

Figure 4 Histological damage assessed by semi-quantitative disease severity score of the jejunum, jejuno-ileum and ileum of rats. Data are expressed as median score (range). Mean values were significantly different *vs* water + 5-FU ($^{f}P < 0.001$). JI: Jejuno-ileum; LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

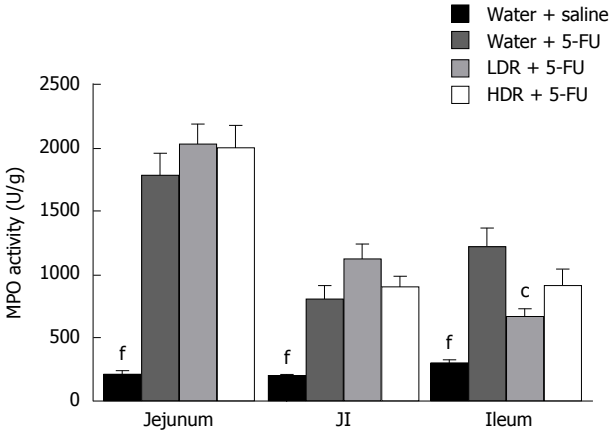

Figure 5 Myeloperoxidase activity present in the jejunum, jejuno-ileum and ileum of rats gavaged with water, low-dose or high-dose rhubarb extract (1 mL) for an 8-d trial period. Rats received an intraperitoneal injection of saline or 5-FU on day 5. Data were expressed as mean [MPO Units (U)/g] ± SEM. Mean values were significantly different ($^{f}P < 0.001$) *vs* water + 5-FU. $^{c}P < 0.05$ *vs* water + 5-FU. JI: Jejuno-ileum; LDR: Low-dose RE; HDR: high-dose RE; RE: Rhubarb extract.

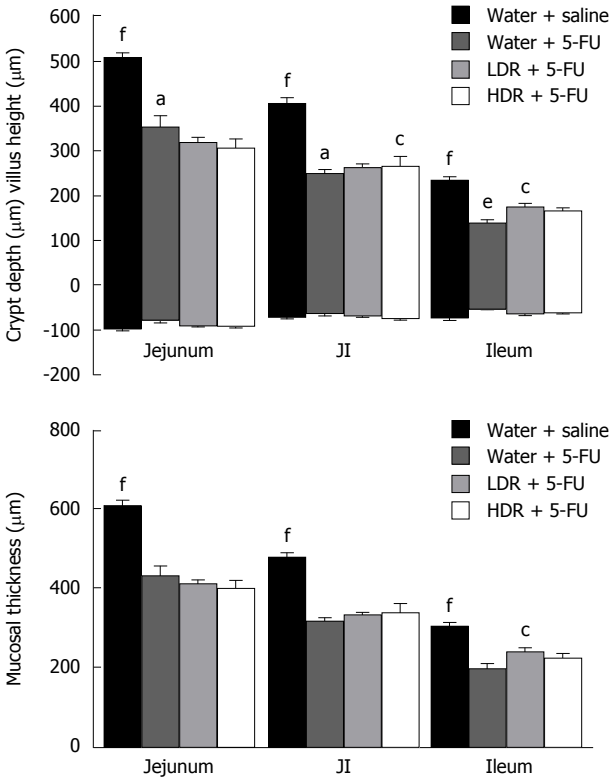

Figure 6 Combination of villus height and crypt depth as a representation of overall mucosal thickness in female Dark Agouti rats. Effects of RE and 5-FU on villus height and crypt depth in female Dark Agouti rats. Rats received a daily water, HDR or LDR gavage for an 8-d trial period and an intraperitoneal injection of 5-FU or saline on Day 5. Mean values were significantly different *vs* water + 5-FU ($^{c}P < 0.05$, $^{f}P < 0.001$). $^{a}P < 0.05$, $^{e}P < 0.001$ *vs* water + saline. JI: Jejuno-ileum; LDR: Low-dose RE; HDR: High-dose RE; RE: Rhubarb extract.

thickness and thus, damage (Figure 6). Administration of 5-FU significantly decreased mucosal thickness by 29% in the jejunum, and 34% in both the JI and ileum when compared to healthy controls. RE had no significant effect on villus height and crypt depth in the jejunum, compared to 5-FU controls. This effect was mirrored in the JI, with the exception of crypt depth which was significantly greater ($P < 0.05$) in 5-FU treated rats receiving HDR. More importantly, administration of LDR to 5-FU treated rats resulted in significantly greater ileal villus heights and crypt depths relative to 5-FU controls; significantly increasing overall ileal mucosal thickness by 19% (Figure 7).

DISCUSSION

Intestinal mucositis remains a debilitating side-effect of chemotherapy treatment. The current study utilised a rat model of intestinal mucositis to investigate the potential for aqueous RE to protect against damage to the intestinal mucosa and regulate water transport in the intestine. The water-soluble components of rhubarb appeared to target more distal regions of the alimentary tract, partially improving selected parameters of the ileum, such as mucosal thickness and MPO activity associated with the clinical manifestations of 5-FU-induced intestinal mucositis.

Administration of 5-FU significantly decreased feed intake and bodyweight as previously described[12,31,37]. A reduction in feed intake and bodyweight is observed in cancer patients due to nausea and pain associated with chemotherapy treatment[38,39]. Interestingly, in the current study, daily administration of HDR to 5-FU treated rats further reduced appetite but maintained bodyweight. It is therefore plausible that the caloric index of HDR may have been contributing to the reduced appetite, yet maintenance of bodyweight in the rats receiving high dose RE.

In the current study, intraperitoneal administration of 5-FU caused significant damage to small intestinal structure, further impacting on intestinal weight and length. Previous studies of experimental intestinal mucositis have noted a correlation between small

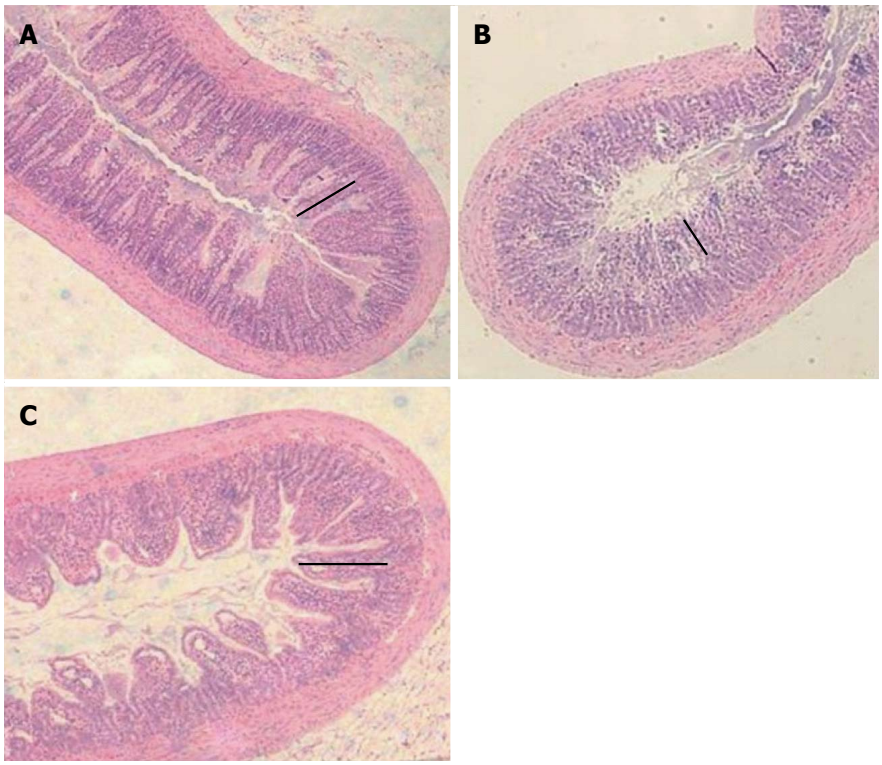

Figure 7 A comparison of the histological structure of ileal sections in a healthy rat (A), after administration of 5-FU (B) and rats treated with LDR + 5-FU (C). Ileum sections of rats from the LDR + 5-FU treatment group (C) exhibited improved mucosal integrity as demonstrated by more defined villi and crypts in comparison to water + 5-FU controls (B). The black line on each diagram represents villus height in each section which was significantly shorter in 5-FU controls. Sections were stained with haematoxylin and eosin and mucosal thickness was analysed by quantitative measurements of villus height and crypt depth. Original photographs were captured at 4 × magnification. LDR: Low-dose rhubarb extract.

intestinal weight and mucosal integrity which was also demonstrated in the current study[13,31]. Jejunum and ileum weights were significantly decreased in 5-FU treated rats, accompanied by increased villus and crypt damage when compared to healthy controls. Enterocyte apoptosis in 5-FU treated rats was likely responsible for the reduced small intestinal weight. However, RE administered to 5-FU treated rats had no significant effect on intestinal weight, compared to 5-FU controls, which suggested that RE did not enhance cell regeneration after 5-FU toxicity.

Administration of 5-FU may result in exposure of the submucosa to harsh luminal conditions[6]. As a compensatory mechanism, the muscularis externa contracts to reduce submucosal contact with the luminal environment in an attempt to prevent bacterial translocation. In the current study, the length of the total jejunum and ileum was reduced by 5-FU treatment, as described previously by Mashtoub *et al*[31]. However, consistent with previous studies, this effect was not present in the duodenum and colon as 5-FU damage was less severe in these regions of the intestine[12,13,31].

In the current study, LDR treatment resulted in a significant increase in ileal villus height and crypt depth; possibly representing LDR promoted crypt cell regeneration and hence, increased migration of rejuvenated cells to the villus. Alternatively, LDR may have exerted an anti-oxidative effect, mediated by the water soluble polysaccharides of rhubarb which may have protected the intestinal mucosa against cell apoptosis; maintaining villus and crypt structure. A reduction in ileal MPO activity by LDR in 5-FU treated rats indicated a decrease in neutrophil activity which further supports the anti-oxidative and anti-inflammatory properties of RE. These results are consistent with previous studies which have exploited plant polysaccharides for their anti-inflammatory and antioxidant properties[12,40,41]. Cheah *et al*[12,14] examined grape seed extract (GSE), a tannin rich by-product of the wine and grape juice industries, in the setting of chemotherapy-induced intestinal mucositis. It was discovered that GSE could partially ameliorate small intestinal inflammation and mucosal damage caused by 5-FU cytotoxicity. Tannins, an active constituent of GSE and possibly RE, possess the ability to prevent the overproduction of ROS or decrease the production of pro-inflammatory cytokines such as IL-4 and IFN-γ[12,17]. Further investigations are therefore required to understand the protective and anti-inflammatory mechanism of action of RE in improving acute intestinal inflammation and damage to the mucosa.

A significant improvement in ileal mucosal integrity and inflammation was observed in 5-FU rats treated with LDR, but not HDR. Limited RE studies have been

conducted, therefore the low and high dose range of 20 mg/kg and 200 mg/kg were selected in the current study to determine the effects of RE across a broad dose range. The efficacy of RE in the current study may therefore have been dose-dependent. Prior to this study, the effects of RE on 5-FU-induced mucosal damage and inflammation were unknown and accordingly, the RE optimal concentration remains undefined. The present study suggested that the effectiveness of RE at varying concentrations may follow a normally distributed relationship. Potentially, at high concentrations ($\geqslant$ 200 mg/kg BW), no significant effects may have been observed due to steric involution of bioactive binding sites. Further studies are therefore required to determine the optimal concentration to attain maximal mucosal protection.

Chemotherapy recipients experiencing intestinal mucositis have altered membrane integrity and impaired water absorption and secretion[7]. Any molecule of a similar size or shape possesses the capability to attach to the pore vestibule and block the transport of water through AQP channels. Pharmacological blockers of aquaporin fluid fluxes are thought to occlude the pore vestibule and impede the bi-directional transport of water through the channel[42-44]. In the current study, RE present in the circulatory system may have targeted AQP 4 channels within enterocytes, resulting in a unidirectional blockade, and thereby decreased water secretion into the lumen of the small intestine. This hypothesised theory is further explained in Figure 1D. Wang *et al*[29] determined that AQP 4 knockout mice had significantly higher stool moisture content in comparison to wild-type ($P < 0.05$). This suggested that stool consistency was dependent on the functionality of AQP 4 channels. This study also established that AQP 4 channels are scarce within the large intestine. Furthermore, within the large intestine, AQP 4 channels are only present on the initial section of the proximal colon[29]. Therefore, it is probable that fluid absorption and secretion across AQP 4 channels in the small intestine may have been partly responsible for the moisture content of the faeces in the current study. Further *in vivo* studies should identify the expression levels of AQP 4 and other aquaporins to determine morphological and potential functional changes after 5-FU exposure. Qin *et al*[18] demonstrated that aqueous RE improved stool consistency in mice with castor oil and magnesium sulphate-induced diarrhoea. Furthermore, aqueous RE caused constipation when administered to normal mice suggesting that RE may have been acting on AQP 4 channels to alter water absorption in the intestine. Consequently, further studies are required to determine the moisture content of caecal fluid to confirm or refute the hypothesis that RE affects stool consistency. This would allow for comparison of water absorption and secretion in the small intestine, independent of the colon. A reduction in caecal moisture content would suggest that RE was preventing fluid secretion across small intestinal AQP 4 channels.

In summary, the present study demonstrated that the ancient herbal remedy RE in its aqueous form, at relatively low dose, offers partial protection to the distal intestinal mucosa against tissue damage and inflammation associated with 5-FU-induced intestinal mucositis. Further studies are warranted to identify the anti-inflammatory and antioxidant properties of RE *via* examination of inflammatory cytokines in blood and tissue. This provides preliminary information regarding the potential use of RE as an adjunct to chemotherapy to improve particular histological manifestations of intestinal mucositis. Moreover, the reduced ileal inflammation and improved mucosal thickness suggests further therapeutic potential for other gastrointestinal inflammatory disorders that ultimately affect the more distal regions of the alimentary tract. However, the potential drug-drug interactions of RE and chemotherapy drugs, such as 5-FU should be thoroughly investigated as recent studies have highlighted concern over such interactions[45]. Future research should also focus on analysing moisture content of caecal fluid to determine whether RE acts as a unidirectional blocker of AQP 4 channels in the small intestine. Finally, further investigation into the active constituents of RE would be beneficial to improve our understanding of its potential utility in bowel disease and its associated mechanism of action.

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COMMENTS

Background

The need to discover effective treatment approaches for chemotherapy-induced intestinal mucositis is growing as cancer incidence continues to increase and thus, the incidence of treatment-related side-effects increases. Traditional medicines are continually being examined for their therapeutic potential in cancer and chemotherapy settings. Accordingly, the aqueous extract of rhubarb (*Rheum Spp.*) was investigated for its potential to improve intestinal integrity and acute inflammation in experimentally-induced intestinal mucositis in rats.

Research frontiers

To our knowledge, this is the first study of its kind to identify the therapeutic effect of aqueous rhubarb extract (RE) in experimentally-induced intestinal mucositis.

Innovations and breakthroughs

This is the first study examining the potential for aqueous RE to improve intestinal integrity and acute inflammation in a rat model of 5-FU-induced intestinal mucositis.

Applications

The promising findings presented in the current study indicate that a low dose of aqueous RE improves selected parameters of 5-fluorouracil (5-FU)-induced

intestinal mucositis. Future studies should determine the active factor of the compound so that it can be extracted and further examined for clinical efficacy.

Terminology

5-FU is a widely utilised chemotherapy drug used to treat a range of cancer types from colon to breast cancer. It may be used independently however, is most commonly used in combination with other chemotherapy drugs, such as Methotrexate. RE was obtained from the stalks of the traditional herbal medicine Rheum spp. The low dose of RE (LDR) was based on the estimated dose required to block aquaporin water channel activity in the oocyte expression system, and the high dose (HDR) was selected as a 10 fold higher concentration for comparison. Aquaporins (AQPs) are integral membrane proteins responsible for the regulation of water transport across a membrane *via* an osmotic gradient. Currently, 13 mammalian AQPs have been identified (AQP 0-12). AQPs are abundant in tissues reliant on high water permeability to maintain correct function and are involved in metabolic processes such as kidney, lung, brain and gastrointestinal function.

Peer-review

This manuscript is well written. The scientific hypothesis and the appropriate tests are well explained and conducted. Results are fairly discussed, notably the question of the need for further experiments investigating an optimal dose.

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ORIGINAL ARTICLE

Basic Study

Characterisation of colonic dysplasia-like epithelial atypia in murine colitis

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Abstract

AIM

To determine if exacerbation of pre-existing chronic colitis in Winnie (*Muc2* mutant) mice induces colonic dysplasia.

METHODS

Winnie mice and C57BL6 as a genotype control, were administered 1% w/v dextran sulphate sodium (DSS) orally, followed by drinking water alone in week-long cycles for a total of three cycles. After the third cycle, mice were killed and colonic tissue collected for histological and immunohistochemical evaluation. Inflammation and severity of dysplasia in the colonic mucosa were assessed in H&E sections of the colon. Epithelial cell proliferation was assessed using Ki67 and aberrant β-catenin signalling assessed with enzyme-based immunohistochemistry. Extracted RNA from colonic segments was used for the analysis of gene expression using real-time quantitative PCR. Finally, the distribution of Cxcl5 was visualised using immunohistochemistry.

RESULTS

Compared to controls, Winnie mice exposed to three cycles of DSS displayed inflammation mostly confined to the distal-mid colon with extensive mucosal hyperplasia and regenerative atypia resembling epithelial dysplasia. Dysplasia-like changes were observed in 100% of Winnie mice exposed to DSS, with 55% of these animals displaying changes similar to high-grade dysplasia, whereas high-grade changes were absent in wild-type mice. Occasional penetration of the muscularis mucosae by atypical crypts was observed in 27% of Winnie mice after DSS. Atypical crypts however displayed no evidence of oncogenic nuclear β-catenin accumulation, regardless of histological severity. Expression of *Cav1*, *Trp53* was differentially regulated in the distal colon of Winnie relative to wild-type mice. Expression of *Myc* and *Ccl5* was increased by DSS treatment in Winnie only. Furthermore, increased *Ccl5* expression correlated with increased complexity in abnormal crypts. While no overall difference in *Cxcl5* mucosal expression was observed between treatment groups, epithelial Cxcl5 protein appeared to be diminished in the atypical epithelium.

CONCLUSION

Alterations to the expression of *Cav1*, *Ccl5*, *Myc* and *Trp53* in the chronically inflamed Winnie colon may influence the transition to dysplasia.

Key words: Mice; Mucin-2; Colon; Colitis; Dysplasia; Dextran sulphate sodium

Core tip: Patients with ulcerative colitis (UC) are at increased risk of developing colonic cancer. Understanding progression to early dysplastic change in the UC-associated inflammation in the colon required a suitable animal model. Winnie mice develop a UC-like chronic colitis and endoplasmic reticulum stress due to a *Muc2* mutation encoding a misfolded mucin-2. We hypothesised that exacerbation of pre-existing chronic inflammation using colitogenic dextran sulphate sodium in a model of spontaneous colitis would induce colorectal tumourigenesis. This study demonstrated that exacerbation of colitis resulted in epithelial hyperplasia in the distal colon and crypt abnormalities resembling dysplasia. Altered expression of genes known to modify tumour growth, specifically *Cav1*, *Ccl5*, *Myc* and *Trp53*, in *Muc2* mutants may predispose to early neoplastic change in the inflamed colon.

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INTRODUCTION

Ulcerative colitis (UC) is a life-long immune disorder that presents as a chronic remitting and relapsing inflammation of the colon, which is also associated with an increased risk of colorectal cancer. Furthermore, the likelihood of developing cancer in the chronically inflamed colon is increased by both the duration and histologic severity of the inflammation[1,2]. Although the association between chronic colitis and colorectal cancer is well-known, the underlying mechanisms have yet to be defined, and due to the heterogeneous nature of the disease, a range of animal models are required to replicate the various aspects of its pathogenesis.

The origins of the chronic inflammation associated with UC may lie in an intrinsic defect in the secreted mucus barrier that lines the colonic surface epithelium. Disruption to the organisation of the colonic mucus, allows the penetration of bacteria into the normally uncolonised inner mucus layer adjacent to the surface epithelium, and possibly deeper into the crypts themselves[3]. Similar permeability defects are observed in patients with active UC, where bacterial-sized material penetrates the normally impenetrable inner mucus layer, a defect which, in a small subset of patients, may remain unimproved during remission[3].

The organised, meshwork-like structure of the secreted colonic mucus in both mice and humans is predominantly formed by the gel-forming mucin-2 (MUC2 and Muc2 in humans and mice respectively). Homozygous *Muc2* gene deletion abolishes Muc2 synthesis and the formation of a sterile inner colonic mucus layer, making Muc2-deficient animals susceptible to colitis caused by colonisation of the colonic mucosa by both pathogenic and normally

commensal bacterial species[4,5]. Homozygous *Muc2* deletion abrogates the formation of a protective colonic mucus initiating a chronic inflammation of the colonic mucosa similar to that observed in UC[6]. While Muc2-deficient mice have demonstrated the importance of Muc2-based mucus in preventing colitis, homozygous *Muc2* deletion is unlikely to reflect the nature of mucus abnormalities observed in UC. The colonic mucus in patients with UC while reduced in thickness, and though it remains penetrable by the luminal microbiota during active disease, the mucus layer is not totally abolished[3,7]. In this regard, the *Muc2* mutation of Winnie may better represent the situation in the inflamed colon of patients with UC. In Winnie mice, missense mutation of an N-terminal domain in *Muc2*, results in abnormal oligomerization of Muc2 and a diminished colonic mucus secretion[8]. Aberrant Muc2 in Winnie also undergoes misfolding and accumulates within the goblet cell, triggering endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR). Activation of the UPR and markers of ER-stress in the colonic epithelium has also been observed in patients with both active and inactive UC[8-10]. The combination of a defect in mucus secretion and concomitant ER-stress makes the Winnie mouse an interesting experimental model of the pathogenesis of UC. Unlike Muc2-deficient mice however, no incident of colonic neoplasia has yet been reported in Winnie mice.

While itself not a tumour suppressor gene, the protective function provided by the gel-forming mucin Muc2 is also necessary to prevent tumourigenesis in the large bowel. Homozygous Muc2 deletion in mice (C57BL6/J × 129SvOla background) has been associated an increased incidence of tumours in the small intestine, colon and rectum over a twelve-month span[11]. Distinct from the Muc2-deficient genotype described previously, the Winnie strain of mice also develop a spontaneous colitis due to Muc2 mutation.

We hypothesised that the defective mucus layer and resulting chronic colitis in Winnie would increase the incidence of colonic neoplasia. However, given the mild inflammation observed in the $Muc2^{-/-}$ and the low incidence of colonic tumours at twelve months, we intended to aggravate the existing inflammation in Winnie to accelerate the process of tumourigenesis. To accomplish this, we employed dextran sulphate sodium (DSS), widely used to induce colonic inflammation in normal (wild-type) and susceptible mouse strains. Administered in a cyclic pattern, DSS-induced inflammation can be used to promote neoplasia in mice already possessing initiating mutations in tumour suppressor genes such as those encoding APC/β-catenin, or p53[12-14] and alone may be sufficient to increase the incidence of colonic dysplasia and carcinoma in certain mouse strains[15,16]. Here we describe a dysplasia-like pathology of the distal colon in Winnie mice after three cycles of DSS administration.

MATERIALS AND METHODS

Ethics statement

All animal procedures were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes of the National Health and Medical Research Council. The study was approved by the Animal Ethics Committee of the University of Tasmania (protocol #13329).

Animals

Eleven to twelve week-old Winnie mice (homozygous Muc2 mutant; C57BL6/J background) and age-matched C57BL/6J (Muc2 wild-type) mice of both sexes were used in this study. Animals were housed within individually ventilated cages containing a corncob bedding (Andersons, Maumee, OH, United States), in a room with a temperature maintained at 21 ℃, with a 12 h photoperiod. Mice were allowed access to radiation-sterilised rodent feed (Barastoc Rat and Mouse, Ridley AgProducts, Australia) and autoclaved tap water *ad libitum*.

Exacerbation of colitis with dextran sulphate sodium

Colitis was exacerbated though the oral administration of a 40000-50000 Da DSS (USB, Affymetrix Inc., Cleveland, OH, United States) through the drinking water. Stock solutions of DSS were prepared by dissolving DSS in sterile tap water and delivered in autoclaved water bottles at a concentration of 1% DSS (w/v). Pairs of Winnie and C57BL6J littermates were randomly allocated to one of two groups. A group of 12 Winnie and 6 C57BL6/J mice received 1% DSS dissolved in drinking water, whereas a control group of 6 Winnie and 4 C57BL6/J animals, was administered sterile drinking water only as a vehicle control. Each cycle of the experimental DSS regimen consisted of the administration of DSS for seven days before substitution of 1% DSS for water for an additional seven days. Mice in the experimental group received DSS in a total of three cycles over 42 d, with a single Winnie mouse euthanased prior to the conclusion of the DSS regimen. Mouse body weight and disease symptoms (*e.g.,* diarrhoea, rectal bleeding) were monitored daily during the experiment. During the course of the experiment one mouse displayed excessive weight loss and morbidity following the first DSS cycle and required euthanasia. At the day of experimental termination (day 42), all animals were killed *via* CO_2 asphyxiation before the abdomen was dissected and the colon removed.

Histopathological evaluation of colitis

The length of the colon from ileocaecal junction to the rectum was recorded. The colon was subsequently opened along its longitudinal axis and the luminal contents were removed prior to weighing the organ. The colon was bisected longitudinally and one half was prepared using the Swiss roll technique described

by[15], whereas the remaining colonic tissue was dissected and snap-frozen for molecular analyses. Swiss rolls 24 h fixation in 10% (v/v) neutral-buffered formalin. Swiss rolls were subsequently transferred to 70% ethanol prior to progressive dehydration, clearing and infiltration with HistoPrep paraffin wax (Fisher Scientific, Philadelphia, PA, United States). Swiss rolls were then embedded in wax and 5 μm sections cut at least three levels 50 μm apart using a rotary microtome. Sections were stained with haematoxylin and eosin Y (H&E; HD Scientific, Sydney, Australia). Slides stained with H&E were evaluated for inflammatory features and neoplasia. Histological inflammation was graded in a blinded fashion by SRD based on previously used criteria[16]. Briefly, crypt architectural distortion was graded 0-5, frequency of crypt abscesses graded 0-3, crypt hyperplasia graded 0-4, extent of mucosal damage graded 0-4, goblet cell depletion graded 0-4, extent of inflammatory infiltration graded 0-4 and frequency of lamina propria neutrophils graded 0-3. The inflammation score for each individual region (distal, middle and proximal colon) was derived from the sum of the score for each of the aforementioned criteria. Dysplastic change and submucosal invasion were graded as no change, low-grade dysplasia, high-grade dysplasia and invasive carcinoma. Crypts involved in glandular profunda were classified as non-dysplastic lesions. Assessment of dysplasia was performed independently by two pathologists (RF and TB) blinded to experimental groupings.

Immunohistochemistry

Slides were dewaxed and exposed to heat-induced epitope retrieval (4 min at 121 ℃) in a 10 mmol/L sodium citrate buffer, pH 6.0 in a decloaking chamber (Biocare Medical, Concord, CA, United States). Slides were cooled to room temperature and washed in 0.1 mol/L Tris-buffered saline (TBS) for 2 min per wash. Endogenous peroxidase activity was blocked by incubating slides in 3% H_2O_2 in methanol for 20 min, followed by 3 × 2 min washes (twice with dH_2O, followed by one wash with TBS). Background sniper (Biocare Medical) was applied to the slides for 20 min and washed off with 3 × 2 min washes with TBS. Slides were incubated with either anti-human β-catenin (clone E247; Abcam, Cambridge, United Kingdom), at a 1:500 dilution, rabbit anti-human Ki67 (clone SP6; Abcam) or rabbit anti-mouse Cxcl5 (Bioss Inc., Woburn, MA, United States) at a dilution of 1:100 was incubated with the slides for 1 h. Excess primary antibody was removed with 3 × 2 min washes with TBS prior to application of HRP-conjugated anti-rabbit secondary antibody (Biocare Medical) for 30 min. Slides were thoroughly rinsed with TBS for 3 × 2 min washes before the addition of a diaminobenzidine (DAB) chromogen solution (Biocare Medical) for 4 min. Tissue was subsequently counterstained with haematoxylin, dehydrated and mounted with DPX mountant (Sigma-Aldrich, Sydney, Australia). Slides were examined through an Olympus IX71 microscope (Olympus Australia, Melbourne, Australia) and images captured using the attached DP21 microscope camera (Olympus).

RNA extraction and RT-qPCR

Colonic tissue was homogenised using rotor-stator generator probes (Omni Scientific) and RNA extracted using the RNeasy Mini spin column kit (Qiagen, Melbourne, Australia) according to the manufacturer's instructions. To minimise genomic DNA contamination DNAse I (Qiagen) digestion was performed during the RNA extraction. Integrity and concentration of extracted RNA was assessed using the Experion Eukaryotic Total RNA electrophoretic system (Bio-Rad Laboratories). Samples with an RNA integrity number (RIN) > 7 were deemed suitable for RT-qPCR. Complementary DNA (cDNA) was synthesised from RNA samples using the iScript Reverse Transcription enzyme and reagents (Bio-Rad) using reaction conditions suggested by the manufacturer. Two-hundred nanograms of cDNA from each sample was added to a PCR reaction including TaqMan Fast Master Mix (Applied Biosystems, Foster City, CA, United States) and a single gene-specific TaqMan probe/primer set. Thermal cycling was performed using a StepOnePlus RT-qPCR instrument (Applied Biosystems). Primer sets used are specified in (Supplementary Table 1). Gene expression was quantified using the comparative ($\Delta\Delta C_T$) method where the threshold cycle (C_T) for each gene was normalised to reference gene *Gapdh*[17]. Relative gene expression in the DSS-treated animals was presented as $2^{-\Delta CT}$.

Statistical analysis

Change in body weight over time was compared using repeated-measures analysis of variance (ANOVA). Incidences of graded histological lesions were compared between treatment groups stratified within genotype using the Mantel-Haenszel χ^2 statistic. Comparisons between means of each unique combination of genotype and treatment were performed using a two-way ANOVA model. Differences in histological scores between anatomical regions were tested post-ANOVA using Tukey's multiple pairwise comparisons test. Non-parametric Spearman's rank correlation was used to test for monotonic relationships between relative transcript abundance and dysplasia scores (non-dysplastic = 1, low-grade = 2, high-grade = 3, dysplasia with submucosal component = 4). In all cases, a *P* value less than 0.05 were deemed to be statistically significant.

RESULTS

Clinical observations

Throughout the experiment, mice were monitored

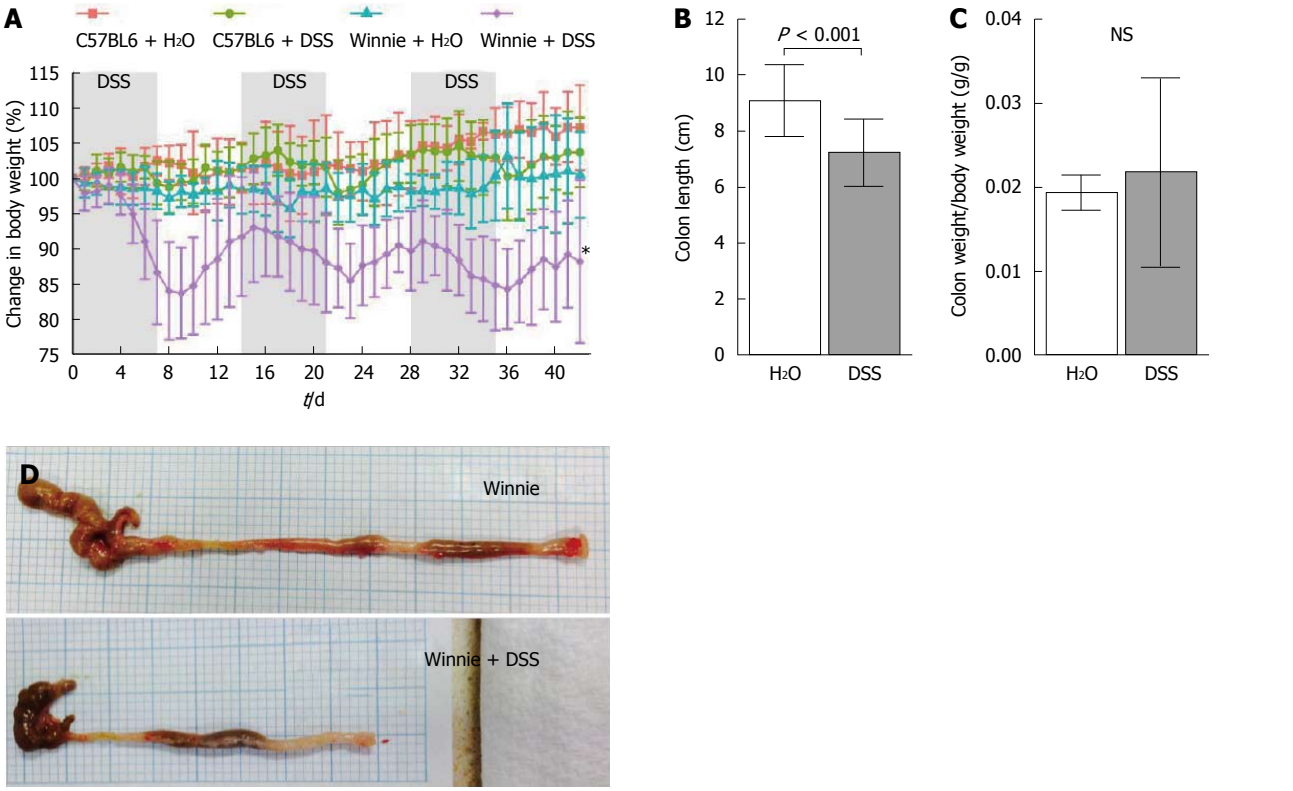

Figure 1 Clinical features of dextran sulphate sodium-induced colitis in Winnie. A: Daily change in body weight of wild-type; B: Winnie mice during the three cycle experimental dextran sulphate sodium (DSS) regimen. Body weight was recorded each day and recorded as percentage change from the starting body weight prior to commencement of the experiment (day 0). Each point represents the mean percentage change in body weight relative to the initial body weight (n = 4). Error bars depict standard deviation (SD) from the mean. Asterisk signifies difference (P < 0.05) between control Winnie mice and Winnie mice exposed to DSS; C: Mean colon length at termination. Error bars depict SD from the mean; D: Gross appearance of the Winnie colon at termination. Representative images of Winnie colon after receiving three cycles of water only (top) and the colon of Winnie receiving three cycles of DSS (bottom).

for the clinical symptoms of colitis. Prior to DSS administration individual Winnie mice of twelve weeks of age, displayed a mild, episodic, non-watery diarrhoea often accompanied by slight loss of body weight, which was usually resolved in 24 h. Clinical symptoms of colitis in eighteen week-old Winnie mice receiving water only did not differ discernibly from those exhibited at twelve weeks of age. Body weight in the control group, on average, remained stable, throughout the experiment (Figure 1A). Compared to the wild-type C57BL6/J, Winnie mice were more sensitive to the administration of DSS in the drinking water. DSS at 1% w/v for 7 d was sufficient to induce a bloody, watery diarrhoea accompanied by considerable weight loss (Figure 1B), which continued 1-2 d after cessation of DSS administration. Colon length in Winnie mice exposed to the DSS regimen was decreased by an average of 24% compared to Winnie mice receiving drinking water alone (Figure 1C). In contrast, colonic tissue from Winnie mice administered DSS was, even when standardised to body weight was no heavier than Winnies receiving only drinking water (Figure 1D). Upon gross post-mortem examination at experimental termination on day 42, the colons of Winnie mice administered DSS remained noticeably inflamed, with an apparent whitening and thickening of the colonic wall, and enlargement of mesenteric lymph nodes compared to control Winnie mice (Figure 1E).

Histopathology

Histological examination of the colon was undertaken to characterise the pathology induced by DSS in the Winnie colon. Initially the inflammation and mucosal damage induced by DSS in the Winnie colon was assessed. Cyclic DSS exacerbated the severity of inflammation in both middle and distal segments of Winnie mice relative to untreated Winnie mice, while DSS had only a minor influence on inflammation in the proximal colon (Figure 2). Compared to the wild-type C57BL6/J (Figure 3A) and wild-type exposed to three DSS cycles (Figure 3B), diffuse leukocytic infiltration into the mucosa, and to a lesser extent the submucosa, was common in the distal and mid-colon of Winnie mice (Figure 3C). In these mice, crypt architecture was occasionally irregular and frequently elongated, and infrequent crypt abscesses were observed. At the time of termination (day 42), severe damage to the distal and mid-colon of Winnie mice exposed to DSS remained evident (Figure 3D). DSS administration in Winnie resulted in an increased influx of leukocytes into the mucosa and submucosa. Large mucosal aggregates of mononuclear leukocytes were frequent, and often extended into the expanded submucosal compartment. Glandular profunda was

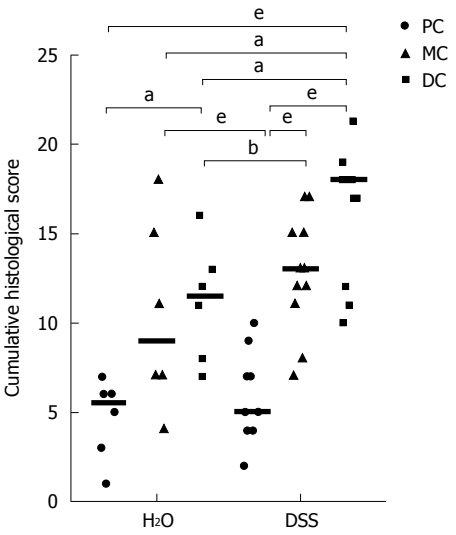

Figure 2 Three cycles of dextran sulphate sodium exacerbated Winnie colitis. Comparison of summed inflammation scores between control Winnie mice and Winnie mice receiving dextran sulphate sodium (DSS). PC, proximal colon, MC, middle colon, DC, distal colon. Bars represent medians of each group, individual animals represented as single points. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$.

occasionally observed in the distal colon involving the larger lymphoid aggregates, which expanded beyond the muscularis mucosae. Areas of superficial mucosal erosion and crypt loss were observed, frequently with foci of crypt fission adjacent the erosion (Figure 3D). Generally, the mucosa of the Winnie distal colon was extensively thickened and contained hyperplastic crypts following repeated DSS exposure. In addition to the typical inflammatory effects observed in Winnie, we assessed the colon for early neoplastic changes. Crypt hyperplasia within the distal and mid-colon of Winnie mice exposed to DSS was often accompanied by foci of abnormal crypt architecture resembling dysplasia. Forty-five percent of Winnie mice displayed crypt abnormalities consistent with a low-grade dysplasia after administration of DSS (Table 1), whereas aberrant crypts were only observed in one untreated Winnie mouse. Low-grade lesions displayed marked architectural distortion, with subtle cytological features such as crowding of epithelial nuclei and increased ratio of nucleus-to-cytoplasm (Figure 3D). Low-grade lesions were absent from all wild-type animals except for one of six C57BL6/J mice exposed to DSS (Table 1). High-grade lesions were observed in the middle and distal colon of 55% of Winnie mice exposed to DSS but were absent from the colon of untreated Winnie and all wild-type mice (Table 1). High-grade crypt lesions displayed the severe architectural distortions such as cribriform (Figure 4A and B) or back-to-back (Figure 4C and D) glandular arrangements associated with colonic dysplasia. In 27% of Winnie mice exposed to DSS, crypt epithelium could be observed within the submucosa underlying apparently dysplastic lesions separated by an intact muscularis mucosae (Table 1). Submucosal glands were lined

Table 1 Incidence and severity of dysplasia-like atypia

Group	Non-dysplastic	Low	High	Submucosal
C57BL/6J	4/4 (100%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
C57BL/6J + DSS	5/6 (83%)	1/6 (17%)	0/6 (0%)	0/6 (0%)
Winnie	5/6 (83%)	1/6 (17%)	0/6 (0%)	1/6 (17%)
Winnie + DSS	0/11 (0%)	5/11 (45%)	6/11 (55%)	3/11 (27%)

Incidence of graded histological lesions in the distal colon of wild-type C57BL/6 and Winnie mice administered three cycles of DSS. Crypt lesions were graded as either non-dysplastic, low-grade or high-grade dysplasia and dysplasia with submucosal extension. The maximum grade observed was recorded for each animal. The incidence of dysplasia-like lesions in Winnie mice was increased by administration of DSS (Mantel-Haenszel $\chi^2 = 6.667$, $P < 0.01$).

with columnar or flattened cuboidal enterocytes but displayed minimal nuclear atypia (Figure 4A-D). Serial sections demonstrated continuity between abnormal mucosal glands and those in the submucosa. No obvious stromal reaction was associated with the submucosal glands. Notably, submucosal glands were usually observed in close proximity to the submucosal vasculature and mirrored the laterally spreading growth pattern seen in the mucosa.

Goblet cell hyperplasia was rare, occurring in a single animal in the Winnie group exposed to DSS (Figure 4C and D). Within a hyperplastic mucosal lesion with apparently high-grade architectural aberrations and mucus-retention cysts, hyperplastic goblet cells were observed in numerous crypts of lengths > 1 mm in length (Figure 4C).

Epithelial cell proliferation in the Winnie colon

Since colonic dysplasia may be associated with abnormal proliferation of the crypt epithelium we assessed the localisation of the Ki67 marker of proliferating cells. Normal Ki67-labelling in C57BL6/J mice extended uniformly for approximately a third of the crypt's total length (Figure 5A). Three cycles of 1% DSS in C57BL6 mice produced a slight expansion of the proliferative zone in the distal colon (Figure 5B). In contrast, the proliferative zone of the colonic crypt was expanded to approximately half to two-thirds of the total crypt length in Winnie mice (Figure 5C). Chronic exacerbation of colitis with DSS extended the Ki67-reactive proliferative zone toward the surface epithelium further than that typically seen in Winnie. Dysplasia-like lesions in Winnie displayed diffuse Ki67 labelling throughout the crypt length (Figure 5D). Notably, the base of some mucosal crypts contained a diffuse pattern of proliferative cells, a feature common to glands penetrating into the submucosa.

Beta-catenin localisation in the Winnie colon

To assess whether oncogenic perturbations in epithelial Wnt/β-catenin signalling were present in the chronically inflamed mucosa, we analysed the intracellular distribution of β-catenin immunohistochemically.

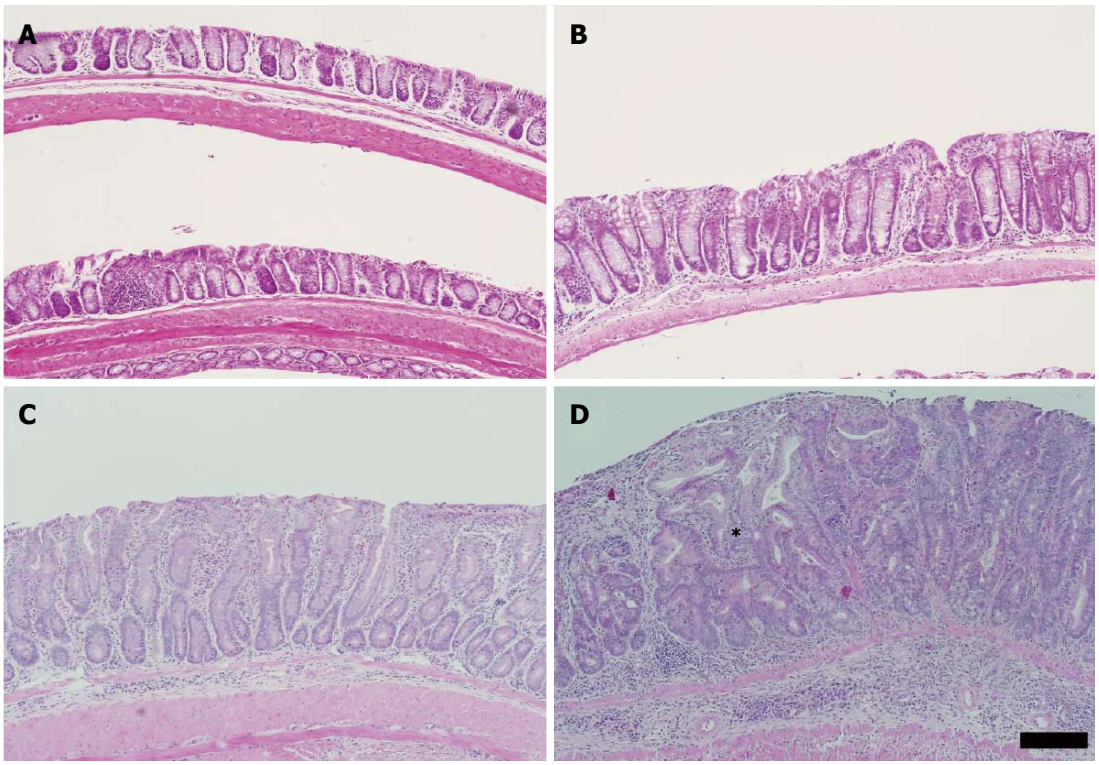

Figure 3 Distal colonic mucosal alterations induced by three cycles of dextran sulphate sodium. A: Representative image of the distal colon obtained from one of four untreated C57BL/6 mouse; B: C57BL/6 distal colon exposed to dextran sulphate sodium (DSS). Image representative of six eighteen week-old C57BL/6 mice exposed to three cycles of 1% DSS; C: Distal colon of Winnie mouse without exposure to DSS. Crypts are hyperplastic and the mucosa displays a marked leukocytic infiltration. Image representative of six untreated Winnie mice examined; D: Distal colon of Winnie mouse displaying colonic hyperplasia and mild focal dysplasia following three cycles of 1% DSS. Mucosa displays features of an active chronic inflammation, with prominent submucosal leukocytic infiltrate. Extensive crypt hyperplasia is visible with a focus of atypical glandular architecture (asterisk). Note the loss of surface epithelium. Numerous mitotic figures are evident. Image representative of the hyperplasia and dysplastic foci total of eleven Winnie mice exposed to three cycles of 1% DSS. All sections stained with HE, scale bar is equivalent to 100 μm.

Localisation of β-catenin in the colonic mucosa of Winnie mice was restricted to the cell membrane of epithelial cells, and was visible to a lesser extent in the cytoplasm of epithelial cells (Figure 6A). Distribution of β-catenin was also predominantly membranous in Winnie mice following chronic exacerbation of colitis with DSS (Figure 6B). No nuclear translocation of β-catenin was evident in any of the dysplasia-like lesions in Winnie mice after DSS administration.

Gene expression analysis

To investigate potential mechanisms resulting in an abnormal epithelial regenerative or dysplastic response, we quantified transcription of a panel of genes associated with carcinogenesis. Included in our analysis were genes likely to accumulate in the chronically inflamed mucosa with known pro-tumourigenic effects such as cyclooxygenase-2 (*Ptgs2)* and interleukin-6 (*Il6*), and the chemokines *Ccl5*, *Cxcl5* and *Cxcl12*[18-20]. Other genes implicated in both inflammation and carcinogenesis included *Spp1* and *Vim*[21,22]. Central regulators of cellular proliferation and survival (*Cdkn2a, Myc* and *Trp53)*, cellular adhesion (*Cav1*), metabolism (*Pparg)*, cell surface glycoproteins (*Muc1* and *Prom1*), all of which have been implicated in cancers were also analysed[23-27]. Relative abundance of transcript for each gene varied considerably within individual mice with DSS-induced colitis, particularly in Winnie mice (Figure 7). Of the genes analysed, those encoding caveolin-1 (*Cav1*), C-C-motif-containing chemokine ligand 5 (*Ccl5*), myelocytomatosis oncogene (*Myc*) and the murine transformation-related protein p53 (*Trp53*), displayed significant alterations to expression in DSS-treated animals. Transcription of *Cav1* was altered by DSS in a manner that was dependent on the mouse strain. While *Cav1* expression in wild-type C57BL6 mice was increased by cyclic administration of 1% DSS, no increase in *Cav1* expression was observed between control Winnie mice and Winnie mice exposed to DSS. While *Ccl5* was unaltered between untreated Winnie and wild-type animals, DSS treatment increased *Ccl5* expression in Winnie relative to C57BL6 exposed to DSS. Expression of Myc in the distal colon was increased only in wild-type mice after the repeated administration of DSS whereas the same treatment had no observed effect on *Myc* expression in Winnie. Expression of *Trp53* was higher in Winnie mice than in the wild-type, but the DSS regimen did not produce a detectable effect on *Trp53* transcription in either wild-type or Winnie. The existence of a potential relationship between the relative abundance of the measured mRNA transcript in the distal colon of pooled Winnie and

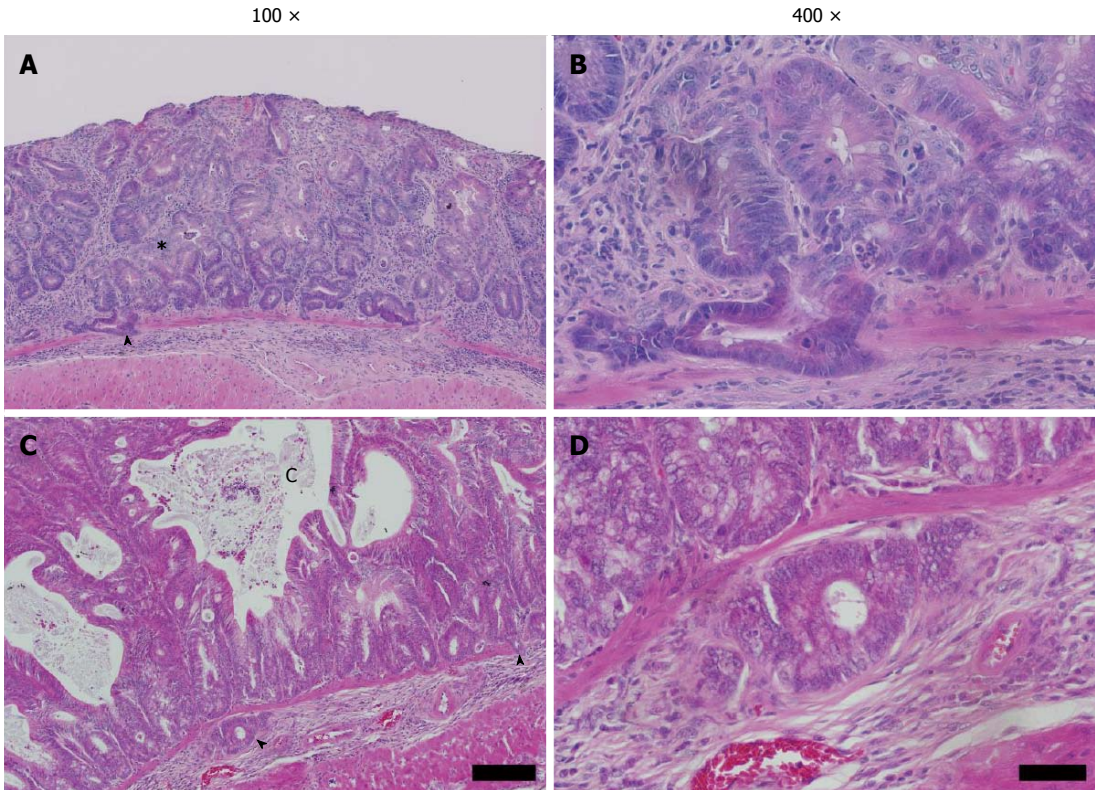

Figure 4 High-grade dysplasia and submucosal penetration in Winnie. A: Winnie distal colonic mucosa after DSS exposure. Distorted and atypical hyperplastic glands (asterisk), with focal infiltration into underlying submucosa through an otherwise intact muscularis mucosae (arrowhead); B: Higher magnification of A. Atypical mucosal and submucosal glands display relatively bland cytology; nuclear polarity mostly intact, though nucleus: cytoplasm ratio is increased. Mitotic figures are common beyond the crypt base. Region of crypt epithelium displaying loss of nuclear polarity (arrowheads). Note the stroma surrounding submucosal glands; C: Regenerative distal colonic mucosa featuring abnormal hyperplastic crypts. Multiple large mucus-containing cysts have formed, apparently formed by confluent dilated crypts lined with hyperplastic goblet cells. Crypts displayed back-to-back arrangement associated with high-grade dysplasia; D: Higher magnification of E. Focal penetration of the submucosa by atypical mucosal crypt. Multifocal penetration of the muscularis mucosae by the overlying abnormal crypts (arrowheads). Stained with HE, scale bar represents 100 μm in A and C, 20 μm in B and D.

Table 2 Spearman's correlation between mRNA transcript abundance and dysplasia severity

Gene	Restimate	*P* value
Cav1	-0.19	0.34
Ccl5	0.46	0.017[a]
Cdkn2a	0.11	0.61
Cxcl5	0.29	0.15[a]
Cxcl12	0.022	0.92
Il6	0.27	0.20
Muc1	-0.13	0.52
Myc	-0.26	0.20
Pparg	0.18	0.39
Prom1	0.18	0.39
Ptgs2	0.18	0.42
Spp1	0.40	0.049
Trp53	0.15	0.47
Vim	-0.14	0.51

Relationship between relative mRNA abundance (ΔΔCT) and severity of dysplasia in the distal colon was tested using Spearman's rank correlation. Estimate of Spearman's co-efficient of correlation (ρ) and corresponding *P* value for each gene are given, [a]$P < 0.05$.

C57BL6/J mice and the grade of dysplasia was tested using Spearman's rank correlation (Table 2). Monotonic increases in the gene expression of *Ccl5* (ρ = 0.46) and *Spp1* (ρ = 0.40) were weakly correlated with increasing severity of dysplasia. All other genes tested displayed no discernible statistical association between gene expression and histological severity.

Cxcl5 localisation in the Winnie colon

Given the large variance in increased abundance of Cxcl5 mRNA in the distal colon lysates, we sought to investigate the source and distribution of the Cxcl5 protein within colonic tissue. Immunolabelling of Cxcl5 protein revealed an altered pattern of expression subsequent to induction of colitis (Figure 8). Wild-type mice displayed a relatively low level of Cxcl5 in the colon, with cytoplasmic staining visible in colonic epithelial cells only. Cxcl5 production also appeared to be increased in the epithelial cells approaching the crypt apex when compared to epithelial cells located near the crypt base. Administration of 1% (w/v) DSS solution to C57BL6/J mice resulted in a slight increase in Cxcl5-specific staining in the crypt epithelium. Similarly, colitis induced by the Muc2 mutation in Winnie mice was associated with an increase in Cxcl5 protein levels within the intestinal epithelium. Additional insult of 1% DSS in Winnie mice produced

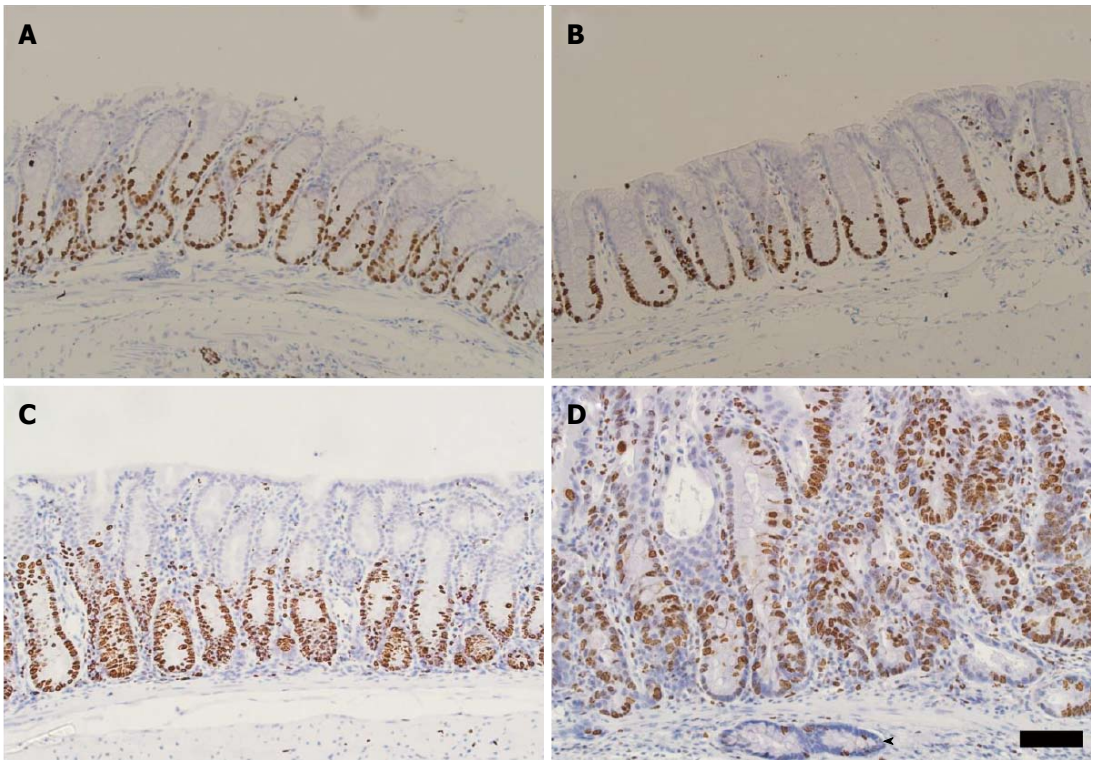

Figure 5 Immunohistochemical detection of Ki67 in Winnie distal colonic mucosa. A: Immunostaining of Ki67 in the distal colon of untreated C57BL6 mice. Image representative of Ki67 localisation in the distal colon of the four C57BL6 mice examined; B: Distal colonic Ki67 localisation representative of six C57BL6 mice exposed to three cycles of 1% dextran sulphate sodium (DSS); C: Distal colon of Winnie mouse without exposure to three cycles of DSS. Ki67-labelling in the epithelium is visible apically approximately half the crypt length; D: Ki67 immunolabelling of the Winnie distal colon exposed to three cycles of DSS. Crypt base proliferative zone extends approximately two-thirds of the crypt length. Submucosal gland (arrowhead) displays few positive nuclei. Scale bar represents a distance of 50 μm.

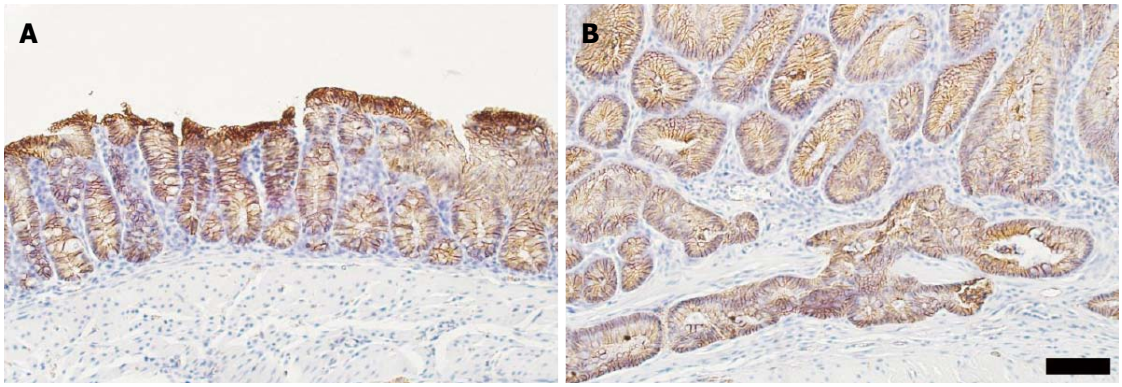

Figure 6 Localisation of β-catenin in Winnie distal colonic mucosa. A: Representative image of Winnie distal colon of the six animals without DSS exposure immunostained for β-catenin. B-catenin in the colonic epithelium is primarily associated with the epithelial cell membrane; B: Immunostained dysplastic distal colonic mucosa from a Winnie mouse exposed to three cycles of DSS (representative of *n* = 11). B-catenin localised to the cell membrane of epithelial cells without any nuclear accumulation. Scale bar is equivalent to 100 μm.

still higher levels of Cxcl5 in the intestinal epithelium. Notably the production of Cxcl5 was also increased in mucosal and submucosal leukocytes.

DISCUSSION

The pathology of UC is associated with a depletion of the protective colonic mucus layer, suggesting a reduction in mucus secretion from colonic goblet cells[7]. Experimental evidence suggests however, that a partial reduction in mucin secretion may be insufficient to permit the development of colitis[28]. Disruption of the normal colonic mucus structure through Muc2 ablation or altered post-translational modification, permeabilises the normally impenetrable inner mucus layer to bacteria[3]. A similar permeabilisation of the colonic mucus was observed in the inflamed colonic mucosa of UC patients, suggesting that Muc2 mutation may

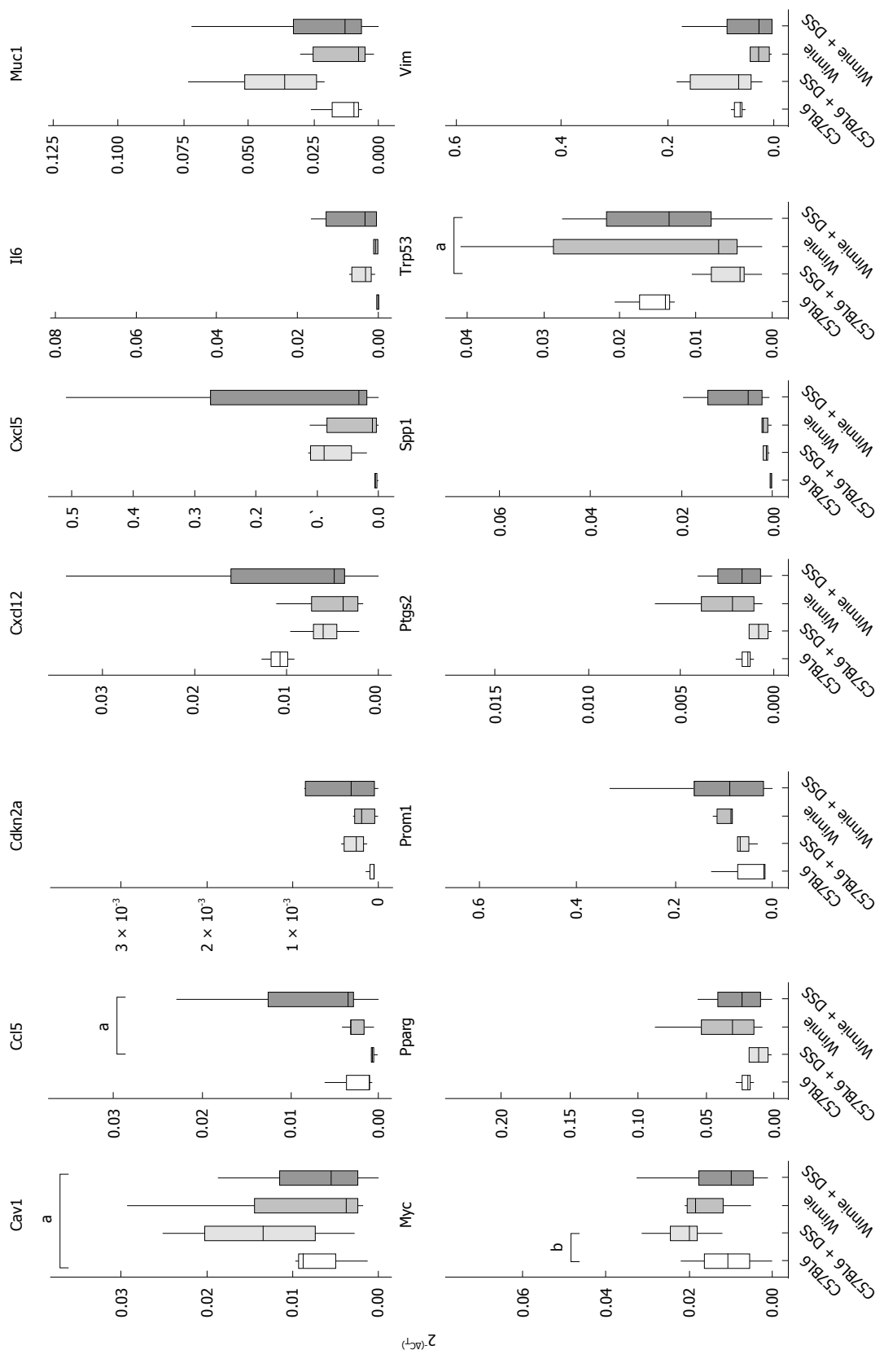

Figure 7 Relative transcript abundance of genes regulating inflammation and cell proliferation. Transcript abundance measured from 25 ng of template based on the linearised 2-ΔCT method, where each gene of interest was normalised to the relative abundance of a reference gene (Gapdh). Box plot depicts interquartile range (IQR) either side of the median for each combination of treatment and genotype (3-11 mice per group). Whiskers represent 1.5 IQR approximately equivalent to 5%-95% confidence intervals. $^{a}P < 0.05$, $^{b}P < 0.01$.

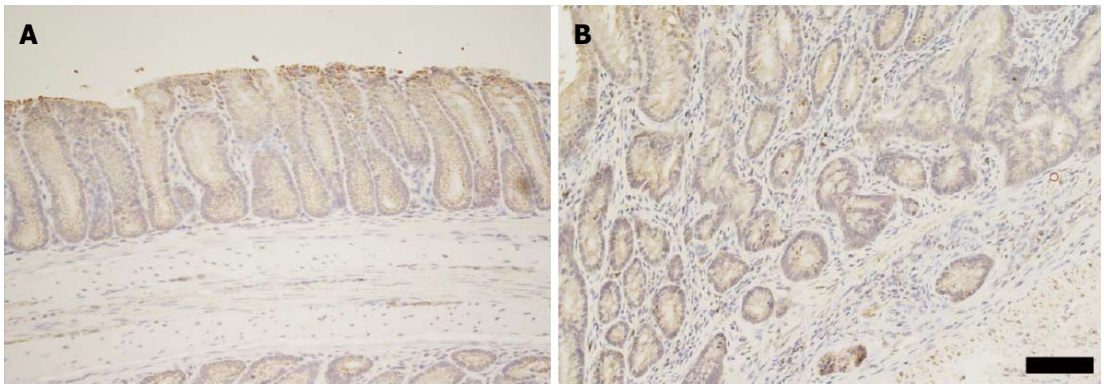

Figure 8 Immunohistochemical detection of Cxcl5 in Winnie distal colonic mucosa. A: Representative distal colon from Winnie without DSS exposure (*n* = 6). Cxcl5 labelling displayed a diffuse cytoplasmic pattern in the colonic epithelium; B: Representative distal mucosa of Winnie exposed to DSS (*n* = 11). Dysplastic glands displaying focal reduction in epithelial Cxcl5. Weak Cxcl5 immunolabelling is present within the cytoplasm of the epithelial cells lining dysplastic glands. Note that submucosal epithelium penetrating the muscularis mucosae retains Cxcl5 expression. Scale bar represents 50 μm.

simulate this aspect of UC pathology[3,29]. Mice with a homozygous deletion of *Muc2* demonstrate several key pathological differences to UC. Muc2-deficient mice display relatively normal goblet cell numbers and a compensatory increase in other mucins such as Muc6, features which are not observed in UC[10]. Increasing evidence also points to possible intrinsic defects in protein synthesis and prolonged ER stress in the colonic epithelium affected by UC[8,9]. Since Winnie mice display spontaneous chronic colitis with an impaired mucus barrier and concomitant epithelial ER stress, they may provide an informative model for the pathogenesis of UC-associated inflammation and neoplasia.

Based on the Muc2 mutant mouse, the expected colonic tumours in Winnie mice could only be infrequent even at twelve months of age, limiting its utility as an experimental model of colonic carcinogenesis. We therefore provided further insult to the colon using DSS, to further disrupt the integrity of the mucus barrier. Consistent with its ability to permeabilise the inner colonic mucus layer, Winnie mice were more sensitive to 1% DSS than Muc2 wild-type C57BL6 mice. Acute administration of DSS produced mucosal injury characterised by superficial erosion and prominent inflammatory infiltrates containing neutrophils and macrophages among other leukocytes[30]. Much of this tissue damage remained at the end of the three cycles of DSS in Winnie mice with areas of mucosal erosion interspersed between regions of hypertrophic mucosa in the distal half of the colon. Notably, Winnie mice that received DSS displayed an increased frequency of abnormal crypt foci resembling dysplasia. Pre-cancerous epithelial dysplasia has been previously reported in the distal colon of Swiss Webster mice exposed to DSS[15,31]. While DSS appeared to rapidly produce foci of non-polypoid dysplasia in the distal colon of 100% Winnie mice under our experimental conditions, the neoplastic potential of these lesions remains uncertain. The time-frame used in the present study is considerably shorter than that used by Cooper *et al*[15] and incident lesions in Winnie could be expected to be less advanced. Penetration of crypt epithelium into the submucosa was observed in 27% of Winnie mice exposed to DSS as opposed to 17% and 0% in the control Winnie group and both C57BL/6 groups respectively. Minimal fibrosis was observed surrounding the laterally spreading submucosal glands and were frequently associated with small breaches of the muscularis mucosae. Such crypts were usually cytologically bland, negative for β-catenin nuclear accumulation and contained a small population of proliferating cells. The close proximity of glands entering the submucosa to blood and lymphatic vessels perhaps suggests that infiltrate the submucosa through weak points in the muscularis mucosae through which these vessels pass. Study of later time-points post-DSS administration would provide evidence as to whether laterally spreading crypts infiltrating the submucosa underlie the rapid progression of colitis-associated neoplasms.

Given the difficulty of identifying possible malignancy in the regenerative mucosa, we subsequently examined the mucosa of the distal colon for molecular features indicative of pre-neoplastic transformation. Hyperactivation of β-catenin signalling is an event associated with the pre-cancerous polyps in 46% of sporadically occurring adenomatous dysplasia[32]. Nuclear translocation of membrane-associated β-catenin is normally limited by the ubiquitinylating activity of the APC-GSK3β-Axin-complex, thereby preventing excessive enhancement of cyclin-D1 and c-myc transcription[33-35]. Tumourigenic Apc/β-catenin mutation may result in the accumulation of active β-catenin protein in the nucleus, which are detectable immunohistochemically[36]. Apparently dysplastic lesions in Winnie lacked the cytoplasmic and nuclear redistribution of β-catenin associated with pre-neoplastic transformation. Induction of dysplasia and neoplasia in mice using DSS alone highlighted the differences in cancers arising from the flat mucosa opposed to those arising from polypoid lesions[15]. Dysplasia/carcinoma

in flat lesions in 94% of cases, displayed membranous localisation of β-catenin whereas evidence of nuclear β-catenin was detected in all polypoid lesions. Studies of DSS-induced neoplasia are concordant with those in human UC-associated neoplasms. Nuclear β-catenin in UC-associated carcinoma is infrequent compared to sporadic cases, presumably due to the low incidence of Apc or β-catenin mutations[32,37].

Gene expression in the distal colon of Winnie revealed altered expression of p53 (*Trp53*), a central regulator of various stress responses. Frequent *TP53* gene mutations in IBD-associated neoplasms and their occurrence prior to dysplasia in patients with UC indicates the importance of the gene in preventing carcinogenesis[38]. An accumulation of mutagenic reactive oxygen and nitrogen species in the mucosa may cause these initial mutations in *TP53* and potentially other genes[38]. Although the presence of mutations was not assessed, *Trp53* transcription was increased in Winnie, even without exposure to DSS, perhaps indicative of DNA damage accrued from prolonged oxidative stress. Alternatively, the differential transcription *Trp53* in Winnie may imply that the chronic ER stress and activation of the unfolded protein response (UPR) due to misfolded Muc2[39]. Since Myc is subject to transcriptional regulation via Wnt/β-catenin signalling, alterations to Myc expression are observed in colonic carcinogenesis[34]. Oncogenic alterations to Myc presumably interfere with normal Myc-dependent regulation of cell proliferation, apoptosis and cellular metabolism[24]. Up-regulation of *Myc* in wild-type mice post-DSS is consistent with reported increases in *Myc* mRNA in the mucosa of mice in patients with IBD[40,41]. It is unclear however, why a similar increase in *Myc* mRNA was absent in Winnie mice exposed to DSS. Further study into the known interactions between *Myc* and chronic activation of the PERK/eIF2α-mediated UPR pathway in Winnie may explain the different responses between genotypes[42]. Another modifier of carcinogenesis, caveolin-1 (*Cav1*), was also increased in wild-type animals post-DSS. Caveolin-1 is scaffolding protein that regulates the transduction of growth factor signalling and is thus capable of restraining external pro-proliferative signals in epithelial cells[25]. The effect of attenuated *Cav1* expression in the DSS-induced pathology of Winnie is unclear as caveolin-1 may be either under-expressed or over-expressed in colorectal cancers[43,44]. Analysis of the mutational status of *Apc* and Kras in the Winnie colonic mucosa may explain the expression level of caveolin-1[45,46].

Recruitment of leukocytes, particularly neutrophils to the colonic mucosa is a hallmark of the inflammation observed in Winnie. Consistent with the relatively large influx of leukocytes into the Winnie distal colonic mucosa and submucosa following DSS, expression of the chemokine *Ccl5* was increased. Increased Ccl5 production has been associated with ineffective lymphocytic anti-tumour responses[47]. Unlike the Gαi2$^{-/-}$ model of spontaneous colitis and colon cancer, we observed a correlation between dysplastic progression and increased Ccl5 expression[48]. Similarly, transcription of the pro-inflammatory cytokine *Spp1* also correlated with increasing severity of dysplasia.

The chemokine Cxcl5, like IL-8, contains a neutrophil-activating ELR domain and is capable of inducing neutrophil chemotaxis through the Cxcr2 chemokine receptor[49]. Unlike IL-8 however, which is produced predominantly by endothelial and myeloid-derived cells in the colon, CXCL5 is primarily produced by crypt epithelial cells. Synthesis of CXCL5 has been reported to increase in ulcerative colitis in humans, presumably prompted by direct exposure to bacterial pathogen-associated molecular patterns, or by the milieu of pro-inflammatory cytokines in the colonic mucosa[50]. Up-regulation of Cxcl5 is therefore likely important for the efficient phagocytosis of bacteria in humans and animals with an impaired colonic mucus barrier. Cxcl5 expression was observed to increase along with the progression of high-grade dysplastic lesions into invasive carcinoma. Relative to control animals, expression of Cxcl5 was considerably heterogeneous in the distal colon of Winnie mice exposed to DSS. Distribution of Cxcl5 expanded to mucosal leukocytes in the distal colon of Winnie mice after DSS exposure. Perhaps explaining the heterogeneity in Cxcl5 transcription, was the occurrence of crypt foci containing regions of epithelium expressing low levels of Cxcl5, though there was no obvious association between dysplasia and loss of Cxcl5. Despite the indications for increased Cxcl5 expression reflecting the progression of dysplasia to advanced colonic carcinoma[51,52]. In contrast, low levels of Cxcl5 expression have been associated with a more aggressive disease course in rat and human CRC cases[53]. Activation of nuclear factor-κB signalling is known to regulate the expression of both Cxcl5, therefore production of Cxcl5 could be dependent on paracrine signalling through the IL-1 receptor[50,54]. Further characterisation of cells within the abnormal glands would be required to gain an appreciation of the role of Cxcl5 in DSS-induced pathology.

In conclusion, chronic DSS exposure in mice with impaired colonic mucin secretion produced extensive mucosal hypertrophy in response to inflammation. Abnormal, dysplasia-like crypt foci were present within the hyperplastic mucosa and in several instances penetrated into the submucosa. Though no β-catenin accumulation was observed, the distal colon of Winnie mice displayed extensive epithelial proliferation and the differential expression of genes regulating epithelial cell proliferation and apoptosis (*Cav1*, *Myc* and *Trp53*) during recovery from DSS. Expression of leukocyte chemo-attractant *Ccl5* was increased in Winnie post-DSS and correlated with histological severity. Analysis of the involved pathways at further time points may yield further insight into the transition to dysplastic and cancerous colonic mucosa in the context of chronic colitis.

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COMMENTS

Background

Patients with ulcerative colitis (UC) are at increased risk of developing CRC, and are prone to more aggressive tumour progression. Chronic inflammation and subsequent regeneration of the colonic mucosa is thought to initiate the formation of dysplastic precursors through mechanisms which are yet to be fully elucidated.

Research frontiers

Much of our knowledge regarding colitis-associated neoplasia comes from experimental models utilising genotoxic carcinogens. Few studies have also explored the effect of mucin depletion and concomitant ER stress on the initiation of neoplasia.

Innovations and breakthroughs

These results suggest that altered gene expression in the distal colon of Winnie mice relative to the wild-type may correspond to the crypt abnormalities in Winnie mice.

Applications

Further characterisation of the molecular pathways involved in the repair of the Winnie mucosa may be useful targets for future studies. Manipulation of the involved pathways in experimental models of chronic colitis may provide stronger evidence for a role in the transition from inflammation to neoplasia.

Terminology

Dysplasia: In the colon, refers to histological changes in both glandular architecture and cytology, specifically those associated with pre-cancerous molecular changes. Endoplasmic reticulum stress: Impairment of the ability of the endoplasmic reticulum to facilitate protein synthesis and folding results in the engagement of various molecular pathways which may induce autophagy and apoptosis. Neoplasia: Tissue which displays a dysregulated pattern of growth and dysplastic features, commonly presenting as tumours. Neoplasms that acquire the ability to invade and/or metastasise into separate tissue compartments are considered cancerous or 'malignant' neoplasms.

Peer-review

This is an interesting manuscript on the impact of colitis in MUC2 mutation Winnie mice (decreased mucus production). The understanding of this animal model may be useful in the understanding of severe colitis leading to dysplasia and tumorigenesis in ulcerative colitis.

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ORIGINAL ARTICLE

Basic Study

Childhood chronic gastritis and duodenitis: Role of altered sensory neuromediators

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Abstract

AIM

To investigate the roles of the neuropeptides vasoactive intestinal peptide (VIP), substance P (SP), and calcitonin gene-related peptide (CGRP) in chronic gastritis and duodenitis in children.

METHODS

Biopsy samples from the gastric and duodenal mucosa of 52 patients and 30 control subjects were obtained. Samples were taken for pathological examination, immunohistochemical staining, enzyme activity measurements and quantitative measurements of tissue peptide levels.

RESULTS

We observed differential effects of the disease on peptide levels, which were somewhat different from previously reported changes in chronic gastritis in adults. Specifically, SP was increased and CGRP and VIP were decreased in patients with gastritis. The changes were more prominent at sites where gastritis was severe, but significant changes were also observed

in neighboring areas where gastritis was less severe. Furthermore, the degree of changes was correlated with the pathological grade of the disease. The expression of CD10, the enzyme primarily involved in SP hydrolysis, was also decreased in patients with duodenitis.

CONCLUSION

Based on these findings, we propose that decreased levels of VIP and CGRP and increased levels of SP contribute to pathological changes in gastric mucosa. Hence, new treatments targeting these molecules may have therapeutic and preventive effects.

Key words: Gastritis; Neuropeptides; Childhood; Neutral endopeptidase

Core tip: The etiology and pathogenesis of childhood gastritis are not entirely known. The lamina propria of the gastrointestinal tract includes sensory neuropeptides that regulate gastric blood flow, local inflammatory responses and healing processes. Vasoactive intestinal peptide (VIP), substance P (SP), and calcitonin gene-related peptide (CGRP) are such neuropeptides, and their roles in chronic childhood gastritis are not known. In this study, we investigated the changes in neuropeptides in childhood gastritis and duodenitis. Disturbances in the neuropeptide content in gastric mucosa may cause gastritis. On the basis of our findings, we propose that decreased levels of VIP and CGRP and increased levels of SP may contribute to pathological changes in gastric mucosa. New treatments targeting these molecules may have therapeutic and preventive effects.

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INTRODUCTION

Inflammation of the gastric mucosa is an important cause of chronic abdominal pain in children. Neither the gastric mucosa's protective mechanisms nor the pathogenesis of gastritis have been fully elucidated. Increases in gastric acid secretion and *Helicobacter pylori* (*H. pylori*) infections are the most important factors implicated in the pathogenesis of gastritis. Patients may not respond to conventional medical treatments, which suggests the involvement of unknown factors in the pathogenesis of gastritis[1,2].

The gastrointestinal system contains sensory neuropeptides, such as vasoactive intestinal peptide (VIP), substance P (SP), and calcitonin gene-related peptide (CGRP)[3]. SP increases the migration of immune cells and cytokine production; therefore, it has suggested that SP may regulate inflammatory processes and wound healing[4-8]. VIP has anti-inflammatory effects through (1) decreasing the secretion of proinflammatory cytokines (TNFα, IL-6, and IL-12); (2) increasing the secretion of anti-inflammatory cytokines (such as IL-10); and (3) reducing Th1 activation[9,10]. CGRP has a vasodilatory effect, and blocking the CGRP pathway causes an insufficient hyperemic response. Disturbances in neuropeptide content may result in mucosal damage, physiological stress, and mild irritation[10,11].

Neutral endopeptidase (NEP, CD10) is a cell-surface membrane-bound enzyme commonly located in the brush border of the mucosa of the small intestine. NEP has been suggested to function in the growth and differentiation of intestinal epithelial cells. NEP hydrolyzes SP and may terminate its biological activity[12]. To date, no studies have investigated changes in SP, VIP, CGRP, and NEP levels in the gastric mucosa of children. Advances in our knowledge of the underlying mechanisms will help in developing new treatment options.

The primary objective of this study was to investigate the roles of neuronal and non-neuronal peptides in the pathogenesis of chronic gastritis in children. The secondary objective was to investigate the changes in NEP expression and activity in normal and inflamed mucosa.

MATERIALS AND METHODS

This study was conducted between May 2012 and May 2013 at the Pediatric Gastroenterology Department of the Akdeniz University Faculty of Medicine. The study protocol was approved by the Ethics Committee at the Faculty of Medicine, Akdeniz University, Antalya, Turkey (protocol number: 14.05.2012/110). All patients and/or their families gave written and verbal consent to participate in the study.

Establishment of patient and control groups

Patients under the age of 18 who presented to the outpatient clinic with abdominal pain and dyspepsia (epigastric pain, discomfort, burning, and fullness, with bloating, early satiety, nausea, and vomiting) lasting longer than 3 mo and who underwent upper gastrointestinal system endoscopy were included in the study group. The control group included individuals who presented with complaints other than dyspepsia, such as weight loss, iron deficiency anemia, and suspected celiac disease, and who underwent upper gastrointestinal endoscopy.

At baseline, patients who were determined to have gastritis ± duodenitis *via* endoscopic examination were included in the study group, and patients who had normal upper endoscopic examination results were included in the control group. After the histological

examination (based on the Sydney classification and activity scores) patients were re-grouped[13]. Patients whose histological examination results were normal or who had Sydney Ⅰ gastritis were included in the control group. Patients with Sydney Ⅱ or Ⅲ gastritis were included in the study group. Patients with and without duodenitis (based on a histological assessment) were analyzed separately *via* CD10 staining.

Inclusion criteria

Pediatric patients who were scheduled to undergo endoscopy for any reason and whose families gave written and verbal consent to be included in the study; Patients who did not have any contraindications for endoscopic biopsy.

Exclusion criteria

Patients who had chronic diseases, such as malignancy, diabetes mellitus, celiac disease, hemolytic uremic syndrome, immune deficiency, and rheumatic diseases; Patients who were receiving immunosuppressive agents, anticoagulants, chemotherapeutic drugs, or any non-steroidal anti-inflammatory drug; Patients who had received a proton pump inhibitor, H_2 blocker or any antibiotics, including *H. pylori* eradication therapy, within the previous three months.

Obtaining, storing, and processing of samples

The same pediatric gastroenterologist (Islek A) performed all endoscopic examinations and biopsy procedures using a Fujinon video endoscope. The region between the oropharynx and the second part of the duodenum was examined during the endoscopic examinations, and endoscopic diagnoses were recorded.

Two biopsy samples were taken from the antrum of all patients and used for the detection of *H. pylori* using the rapid urease test. In patients with gastritis (based on the endoscopic assessment), two biopsy samples, one from where the gastric lesion was severe (S1) and one from where the gastric lesion was less severe (S2), were obtained from the antrum for pathological examination and immunohistochemical staining. The S2 biopsies were taken from tissue approximately 3-5 cm from S1. In addition, two more gastric biopsy samples from S1 and S2 were taken for quantitative measurements (ELISA) of tissue peptide levels and NEP enzyme activity.

In patients with normal appearing gastric mucosa, two biopsy samples from the antrum (C) were obtained for pathological examination and immunohistochemical staining, and two more gastric biopsy samples from the antrum were taken for quantitative measurements (ELISA) of tissue peptide levels and NEP enzyme activity.

In all patients, three biopsy samples from the second part of duodenum were obtained for histological examination and NEP staining.

Paraffin blocks were prepared from all collected tissue samples, and samples were cut into 4-μm-thick sections. Sections were then incubated at 60 ℃ for 5 min and stained with a CD10 primary antibody (anti-CD10, Clone: 2A1H5E1; Thermo Fisher Scientific Inc., United Kingdom) using a closed-system automated immunohistochemical staining device (Ventana, Roche, United States) at the Department of Pathology. The Sydney classification system was used to classify the degree of gastritis[13]. Biopsy samples for the ELISA and enzyme activity assays were snap-frozen in liquid nitrogen and kept at -80 ℃ until the analysis.

Measurement of peptide levels

We previously established a method to differentially measure the SP levels present in nerve endings and in non-neuronal tissue[14,15]. Briefly, biopsy specimens were cut into small pieces and kept in 1 mL of 2% acetic acid at 95 ℃ for 10 min. Sequential collections of the supernatant were performed in which the first 10-min extraction included predominately neuronal SP, whereas the second extraction, which was re-incubated in 1 mL of 2% acetic acid at 95 ℃ for 50 min, primarily yielded non-neuronal SP. Supernatants were dried completely and then reconstituted in 150-300 μL of sample buffer from an SP EIA kit (Cayman Chem., Catalog No. 583751). From each sample, 25 and 50 μL were used for immunoassays, which gave results with a 95% confidence interval. Tissue extractions were also used for quantifying VIP (Bachem-Pennisula Laboratories, cat. No. S1183) and CGRP (Phoenix Pharmaceuticals, cat. No. EK-015-02) *via* immunoassay. The detection limit for SP was 4-500 pg/mL, and we tested multiple dilutions of the samples to avoid very low and high concentrations because the assay is most sensitive within the 20-80 percentile (approximately 10-250 pg/mL). Under these conditions, both intra- and inter-assay variabilities were < 20%. The half maximal inhibitory concentration (IC_{50}) for VIP measurements was 200 pg/mL, and the same precautions were taken to keep intra- and inter-assay variabilities < 20%.

Measuring NEP-like activity

Dansyl-d-Ala-Gly-p-nitro-Phe-Gly, dansyl-d-Ala-Gly, and phosphoramidon, a specific NEP inhibitor, were purchased from Sigma (St. Louis, MO, United States). Measurement of NEP activity was performed as previously described, with some modifications[16]. Briefly, snap-frozen tissues were weighed and sonicated five times on ice for 15 s in ice-cold 50 mmol/L Tris-HCl (pH 7.4) buffer that included 1% Triton X-100. The homogenates were centrifuged at 10000 × *g* for 3 min to remove cellular debris and nuclei and then stored at -80 ℃ until used.

Samples (10 μL) were pre-incubated with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, to prevent the cleavage of the fluorogenic substrate (N-dansyl-d-Ala-Gly-p-nitro-Phe-Gly) by ACE, in the presence or absence of phosphoramidon. Following this preincubation, the fluorogenic substrate was

added, and samples were incubated for an additional 2 h at 37 ℃. The final concentrations were 16.5 μmol/L for enalapril, 16.5 μmol/L for phosphoramidon, and 200 μmol/L for the substrate in a reaction volume of 160 μL. After 2 h of incubation, the fluorescence absorbance of the sample was recorded using a BIOTEC FX800 Reader. The amount of product was estimated by measuring its fluorescence intensity at 562 nm, with excitation at 342 nm. Arbitrary fluorescence units for each sample were compared against a standard curve prepared using dansyl-d-Ala-Gly (cleavage product) to determine the NEP-specific activity per mg tissue. To verify the method, tissue samples from freshly frozen kidneys were included as a positive control.

Immunohistochemistry

Tissue neprilysin (NEP, CD10) levels were also examined *via* immunohistochemistry. Immunostaining was performed on formalin-fixed paraffin-embedded sections. Sections from each block were deparaffinized and heated in a microwave oven for 10 min for antigen retrieval. Endogenous peroxidase was blocked by incubating sections in 3% hydrogen peroxide in methanol for 10 min. Each sequential incubation was then followed by a thorough washing of the slides in distilled water and phosphate-buffered saline (0.001%, Sigma). After incubation with a monoclonal CD antibody (Thermo Scientific, Fremont, CA, United States) for 60 min, the sections were allowed to react with a biotinylated secondary antibody for 15 min, followed by an incubation with streptavidin for an additional 15 min. Finally, all slides were treated with DAB for color development and were then counterstained with Mayer' s hematoxylin. Negative controls were created using non-reactive TBS-1% BSA at the same concentration as the primary antibody. Serial sections from an abdominal lymph node from a patient diagnosed with diffuse large-cell lymphoma were used as positive controls for CD10 staining. Staining was evaluated separately in foveolar, glandular, and endocrine cells. The staining patterns were classified as focal or diffuse. The intensity of staining was classified as weak, moderate, or strong. The distribution of staining (cytoplasmic or membranous) was also noted.

Histopathological examination

Histopathological examinations were performed by the same experienced pathologist (OE). The evaluations were performed according to the Sydney classification system[13]. Grouping (control or study group) was performed according to the chronicity and degree of the activity score (based on mononuclear cell infiltration).

Determining the presence of H. pylori infections

H. pylori infection diagnoses were performed with rapid urease tests and histopathological examinations.

Statistical analysis

Statistical analysis were performed using SPSS software 18.0 and GraphPad InStat 3 software. Paired or unpaired *t*-tests were used for comparing parametric values in accordance with a normal distribution, and Pearson's chi-square tests were used for analyzing descriptive statistics. A P value < 0.05 was considered to be statistically significant.

RESULTS

Of the 163 consecutive patients who underwent upper endoscopic examination, 14 were excluded because of their refusal to participate in the study, 8 because of a diagnosis of celiac disease, four because of insufficient samples, and 51 because of the previous use of proton pump inhibitor, *H. pylori* eradication therapy or NSAIDs and the presence of other chronic diseases. In addition, four patients who had bile reflux during the endoscopic examination were excluded from the study because of the insufficient number of patients for a statistical analysis. After the histological examination, the study group comprised 52 patients who had grade Ⅱ or Ⅲ chronic gastritis, and the control group, who had grade Ⅰ chronic gastritis or histologically normal gastric mucosal samples, comprised 30 patients. Histologically defined gastritis was present in all patients whose endoscopic examinations indicated gastritis. After the histological examination, ten patients with grade Ⅰ gastritis were moved from the study group to the control group, and ten patients with grade Ⅱ gastritis were moved from control group to the study group (Figure 1). The demographic characteristics of the patients are shown in Table 1.

Changes in substance P levels in patients with gastritis

The S1 and S2 biopsy samples underwent a 2-step extraction, and their SP levels were measured *via* ELISA. Although the first extraction mostly contained SP from neurons, the SP in the second extraction reflected levels outside of neurons[17].

In both the first and second extractions, SP levels were significantly higher in S1 than in C samples (14980.11 ± 1950.82 pg/g *vs* 6913.20 ± 1360.56 pg/g tissue in the first extraction and 8249.51 ± 1342.45 pg/g *vs* 2977.23 ± 777.21 pg/g tissue in the second extraction, respectively). Moreover, SP levels were significantly higher in the S1 than in S2 samples in both extractions (14980.11 ± 1950.82 pg/g *vs* 6370.18 ± 1603.87 pg/g tissue in the first extraction and 8249.51 ± 1342.45 pg/g *vs* 2980.37 ± 742.35 pg/g tissue in the second extraction, respectively). However, there were no significant differences in SP levels between the S2 and C samples in either the first or second extractions (6370.18 ± 1603.87 pg/g *vs* 6913.20 ± 1360.56 pg/g tissue in the first extraction and 2980.37 ± 742.35 pg/g *vs* 2977.23 ± 777.21 pg/g tissue in the second extraction, respectively) (Figure 2A).

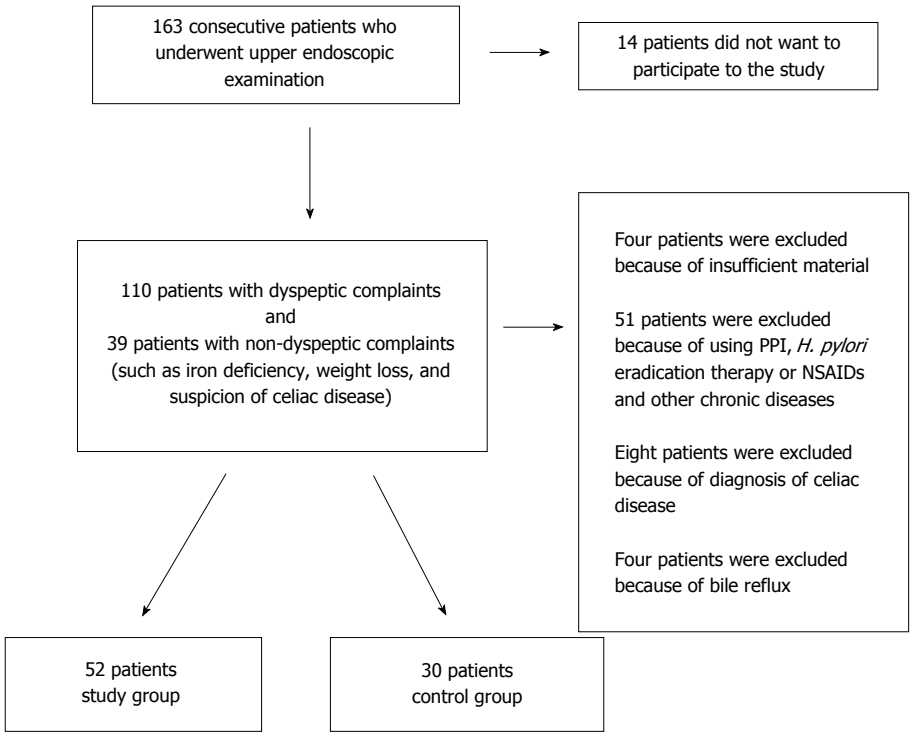

Figure 1 Patient recruitment flowchart. PPI: Proton pump inhibitor; *H. pylori*: *Helicobacter pylori*.

Table 1 Demographic characteristics of the patients

Patient characteristics	Study group	Control group	*P* value
n	52	30	
Age (yr)			
mean	12.47 ± 4.087	11.6 ± 4.065	
median	13.5	12.6	0.847
range	6-17	6-17	
Sex (F/M)	29/23	17/13	0.981
Grading of gastritis (Sydney class, activity score)	S1 S2	Normal gastric mucosa; 14 patients	-
Mild (Grade Ⅰ)	- 44		
Moderate (Grade Ⅱ)	24 8	Grade Ⅰ gastritis;	
Severe (Grade Ⅲ)	28 -	16 patients	
H. pylori	23	6	0.027

H. pylori: *Helicobacter pylori*.

Changes in SP levels in H. pylori (+) and (-) patients

H. pylori was detected in 29 of 82 individuals in the study. There were no significant differences in SP levels between *H. pylori*-positive and *H. pylori*-negative individuals (Figure 2B).

Changes in substance P levels according to gastritis grade

There were significant differences in SP levels between patients with mild (Sydney Ⅰ) and moderate/severe (Sydney Ⅱ or Sydney Ⅲ) gastritis. SP levels were higher in the patients with Sydney Ⅱ or Sydney Ⅲ gastritis than in those with Sydney I gastritis (16734.86 ± 1754.35 pg/g *vs* 6534.27 ± 986.45 pg/g tissue in the first extraction and 8790.83 ± 1326.92 pg/g *vs* 3567.23 ± 1065.21 pg/g tissue in the second extraction, respectively) (Figure 2C).

Effects of sex on tissue SP levels

We did not observe any significant difference in SP levels in the first and second extractions in the study group (S1 and S2) and control group (C) between girls and boys.

Changes in vasoactive intestinal peptide levels in gastritis patients

In the first and second extractions, VIP levels were significantly higher in the C samples than in both S2 (11633.70 ± 2596.89 pg/g *vs* 4867.64 ± 1862.84 pg/g tissue in the first extraction and 8339.03 ± 2437.23 *vs* 3435.87 ± 707.74 pg/g tissue in the second extraction, respectively) and S1 (11633.70 ± 2596.89 pg/g *vs* 288.21 ± 116.24 pg/g tissue in the first extraction and 8339.03 ± 2437.23 pg/g *vs* 1895.53 ± 655.41 pg/g tissue in the second extraction, respectively) samples. Also, VIP levels were significantly lower in S1 than in S2 samples in both the first and second extractions (288.21 ± 116.24 pg/g *vs* 4867.64 ± 1862.84 pg/g tissue in the first extraction and 1895.53 ± 655.41 pg/g *vs* 3435.87 ± 707.74 pg/g tissue in the second extraction, respectively) (Figure 3A).

Changes in VIP levels in H. pylori (+) and (-) patients

No significant differences in VIP levels was detected between *H. pylori* (+) and *H. pylori* (-) individuals (Figure 3B).

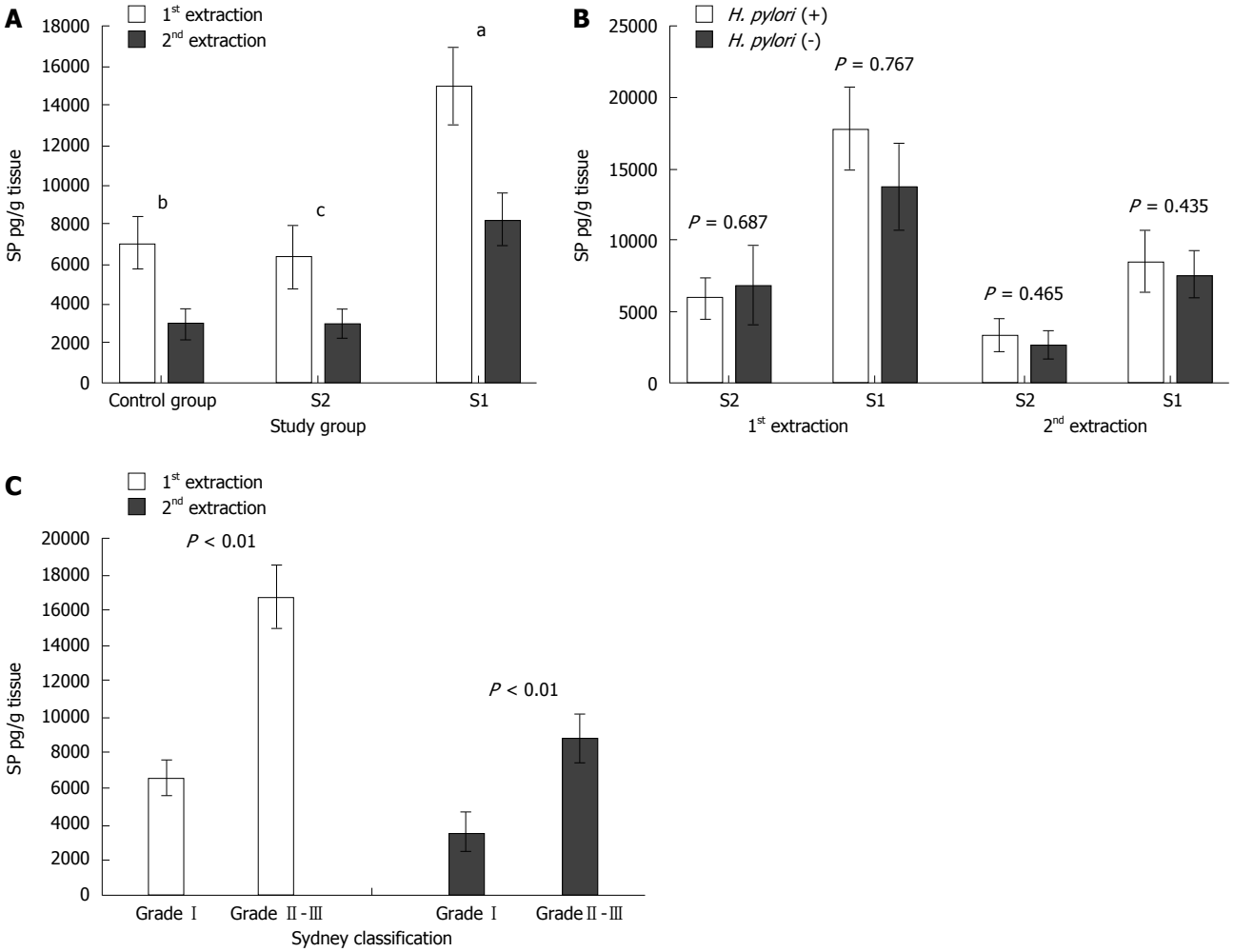

Figure 2 Substance P levels in the control (*n* = 30) and study (*n* = 52) groups. A: Substance P (SP) levels were significantly higher in the gastric mucosa from sites with severe gastritis (S1) (a) than in the control group in both the first and second extractions (b) (P = 0.001 and 0.004, respectively). SP levels were significantly higher in S1 (a) sites than in areas with less severe gastritis (S2) (c) in both extractions (P = 0.006 and 0.001, respectively). However, there were no significant differences in the SP levels between the S1 and S2 samples in both the first and second extractions (P = 0.81 and 0.99, respectively); B: There were no significant differences in the SP levels between *H. pylori* (+) and *H. pylori* (-) patients; C: SP levels were significantly higher in patients with high-grade gastritis. *H. pylori*: *Helicobacter pylori*.

Changes in VIP levels according to gastritis grade

There were significant differences in VIP levels between patients with mild (Sydney grade Ⅰ) and moderate/severe (Sydney grade Ⅱ or Ⅲ) gastritis. VIP levels were higher in patients with a mild disease than in patients with a moderate/severe disease (6876.12 ± 896.31 pg/g *vs* 256.68 ± 46.87 pg/g tissue in the first extraction and 4045.23 ± 876.21 pg/g *vs* 1042.12 ± 241.12 pg/g tissue in the second extraction, respectively) (Figure 3C).

Effects of sex on tissue VIP levels

We did not observe any significant difference in VIP levels in the first and second extractions in the study group (S1 and S2) and control group (C) between girls and boys.

Changes in calcitonin gene-related peptide levels in patients with gastritis

In the first and second extractions, CGRP levels were significantly lower in S1 than in the C samples (229.56 ± 49.63 pg/g *vs* 1184.18 ± 80.46 pg/g tissue in the first extraction and 397.80 ± 145.84 *vs* 1184.18 ± 80.46 pg/g tissue in the second extraction, respectively) and in S2 than in C samples (947.42 ± 265.65 pg/g *vs* 1184.18 ± 80.46 pg/g tissue in the first extraction and 618.02 ± 206.71 *vs* 1184.18 ± 80.46 pg/g tissue in the second extraction, respectively). Moreover, CGRP levels were lower S1 than in S2 samples (229.56 ± 49.63 pg/g *vs* 947.42 ± 265.65 pg/g tissue in the first extraction and 397.80 ± 145.84 pg/g *vs* 618.02 ± 206.71 pg/g tissue in the second extraction, respectively) (Figure 4A).

Changes in CGRP levels in H. pylori (+) and (-) patients

There was no significant difference in CGRP levels between *H. pylori* (+) and (-) individuals (Figure 4B).

Changes in CGRP levels according to gastritis grade

There were significant differences in CGRP levels

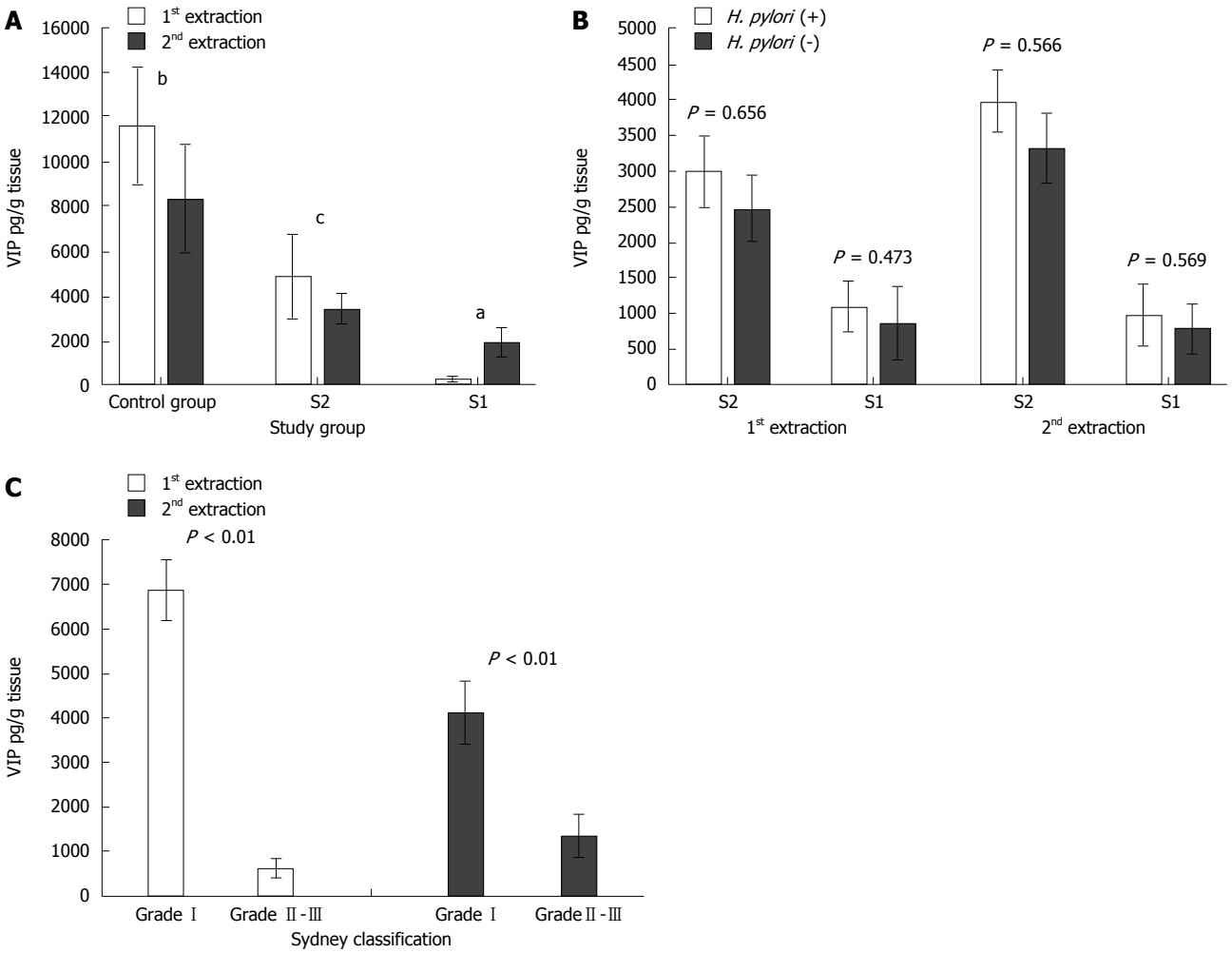

Figure 3 Vasoactive intestinal peptide levels in the control (*n* = 30) and study (*n* = 52) groups. A: Vasoactive intestinal peptide (VIP) levels were significantly lower in the gastric mucosa from sites with severe (S1) and less severe (S2) gastritis than in the control group. (P = 0.0095, P = 0.0013, first extraction, respectively (a and b); P = 0.001, P = 0.030, second extraction, respectively (a and b)]. The VIP levels were significantly lower in S1 (a) samples than in S2 (c) samples in both the first and second extractions (P = 0.017 and P = 0.030, respectively); B: There were no significant differences in VIP levels between *H. pylori* (+) and *H. pylori* (-) patients; C: VIP levels were significantly higher in patients with low-grade gastritis. *H. pylori*: *Helicobacter pylori*.

between patients with mild (Sydney grade Ⅰ) and moderate/severe (Sydney grade Ⅱ or Ⅲ) gastritis. CGRP levels were higher in patients with grade Ⅰ gastritis than in those with grade Ⅱ or Ⅲ gastritis (1063.87 ± 276.51 pg/g *vs* 312.34 ± 43.61 pg/g tissue in the first extraction and 862.85 ± 112.29 pg/g *vs* 328.13 ± 49.12 pg/g tissue in the second extraction, respectively) (Figure 4C).

Effects of sex female status on tissue CGRP levels

We did not observe any significant difference in CGRP levels in the first and second extraction in the study group (S1 and S2) and control group (C) between girls and boys.

Changes in neutral endopeptidase in patients with gastritis

The gastric mucosa samples of 45 patients from the study group and 29 patients from the control group were stained for CD10, and the staining characteristics of epithelial cells and lymphoplasmacytic cells were recorded. We also measured NEP activity in freshly frozen tissues.

In the study and control groups, no CD10 staining at the gastric epithelium was observed (Figure 5A and B). In accordance with this observation, we did not detect measurable levels of NEP-like activity in these samples.

In 38 of the 45 patients in the study group, CD10 staining was positive (focal or diffuse) in lymphoplasmacytic cells in S1 samples, whereas no staining was observed in seven patients. In 28 S1 biopsy samples from patients, diffuse staining was observed in lymphocytic cells, and focal staining was observed in 10 patient samples. In 15 S2 biopsy samples from patients, CD10 staining was diffuse, and in 23 patient samples, the staining was focal. Based on the statistical analysis of these results, S1 staining was significantly more diffuse than S2 staining (Pearson's chi-square test, P = 0.002). In the *H. pylori* (+) patients, intense staining was observed in the germinal centers of lymphoid follicles.

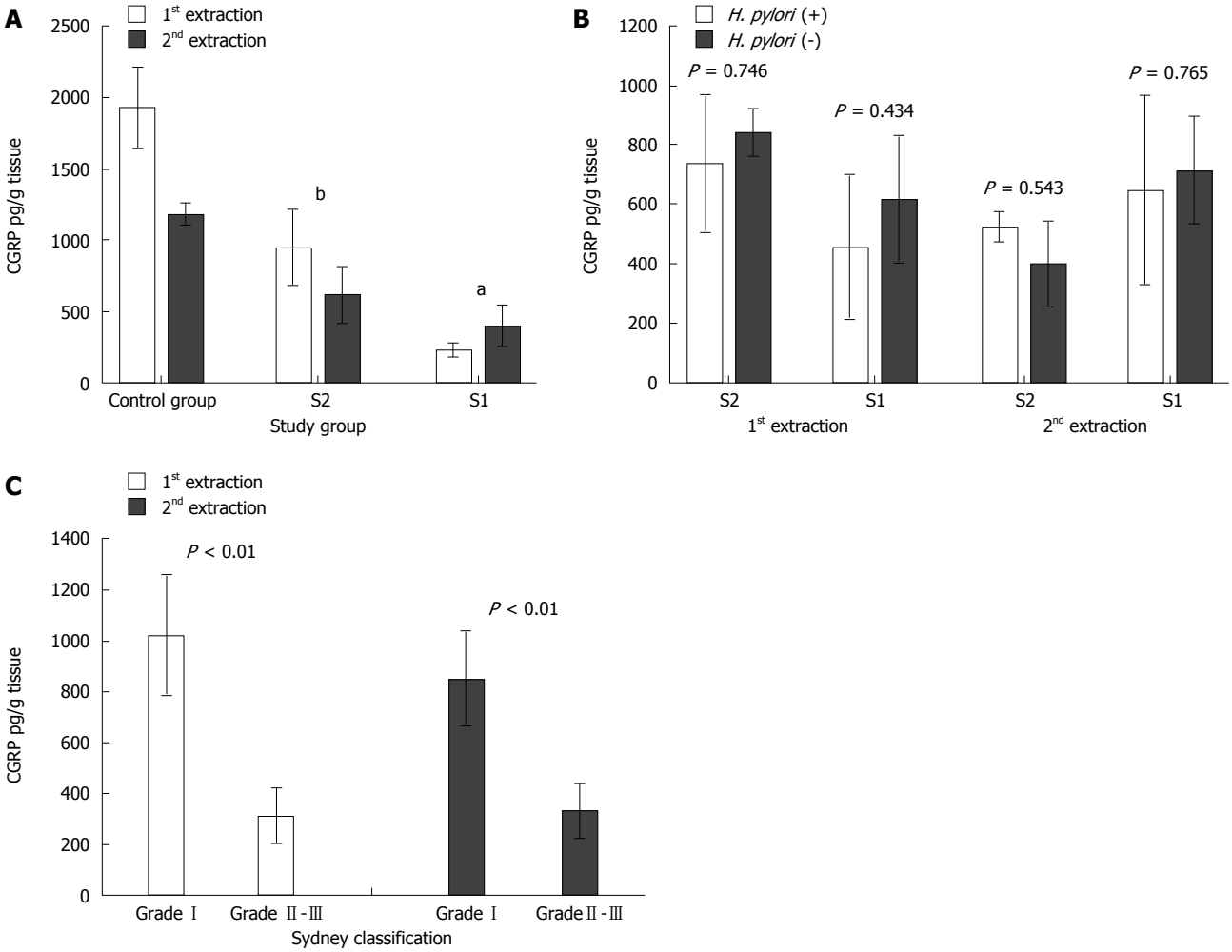

Figure 4 Calcitonin gene-related peptide measurements in the control (*n* = 30) and study (*n* = 52) groups. A: The calcitonin gene-related peptide (CGRP) levels were lower in the gastric mucosa from sites with severe (S1, b) and less severe (S2, a) gastritis than in the control group in the first and second extractions (*P* = 0.011, *P* = 0.031, first extraction, respectively; *P* = 0.002, *P* = 0.004, second extraction, respectively). Moreover, in the study group, CGRP levels were lower in S1 than in S2 samples in both the first and second extractions (*P* = 0.037 and *P* = 0.046, respectively); B: No significant differences in the CGRP levels were observed between *H. pylori* (+) and *H. pylori* (-) patients in the first and second extractions; C: CGRP levels were significantly higher in patients with low-grade gastritis. *H. pylori*: *Helicobacter pylori*.

Despite the positive staining of some samples, we could not detect measurable levels of NEP-like activity, suggesting that factors extracted during the sample preparation inhibit the enzymatic activity of NEP[17].

In 27 of the 29 patients in the control group, CD10 staining was positive in lymphoplasmacytic cells, whereas no staining was observed in samples from two patients. In eight patients, sample staining was diffuse. However, samples from 19 patients had focal staining. These results indicate that the gastric mucosa (lymphoplasmacytic cells) of the control group had less diffuse staining than did the severe and less severe areas of gastritis of the study group (*P* = 0.011 and *P* = 0.040, respectively; Pearson's χ^2 test).

Changes in CD10 in patients with duodenitis

We investigated CD10 staining in the duodenal mucosa, which reflects NEP activity. The patients without duodenitis (*n* = 30) had more pronounced diffuse and strong CD10 staining than did the patients with duodenitis (*n* = 52, *P* = 0.012) (Figure 5C). The loss of CD10 staining was more pronounced in cases of severe (based on the severity of mononuclear cell infiltration) duodenitis (33/52).

DISCUSSION

Gastritis, which is defined as an inflammation of the gastric mucosa, is a major cause of chronic abdominal pain in children[1]. In the current study, we investigated the roles of neuronal and non-neuronal peptides in the pathogenesis of chronic childhood gastritis. We observed differential effects of the disease on peptide levels. Specifically, SP was increased and CGRP and VIP were decreased in patients with severe gastritis. The changes were more prominent in S1 areas (where the gastritis was severe), but significant changes were also observed in the neighboring tissue (areas with less severe gastritis). In addition, the changes correlated with the pathological grade. The expression of CD10,

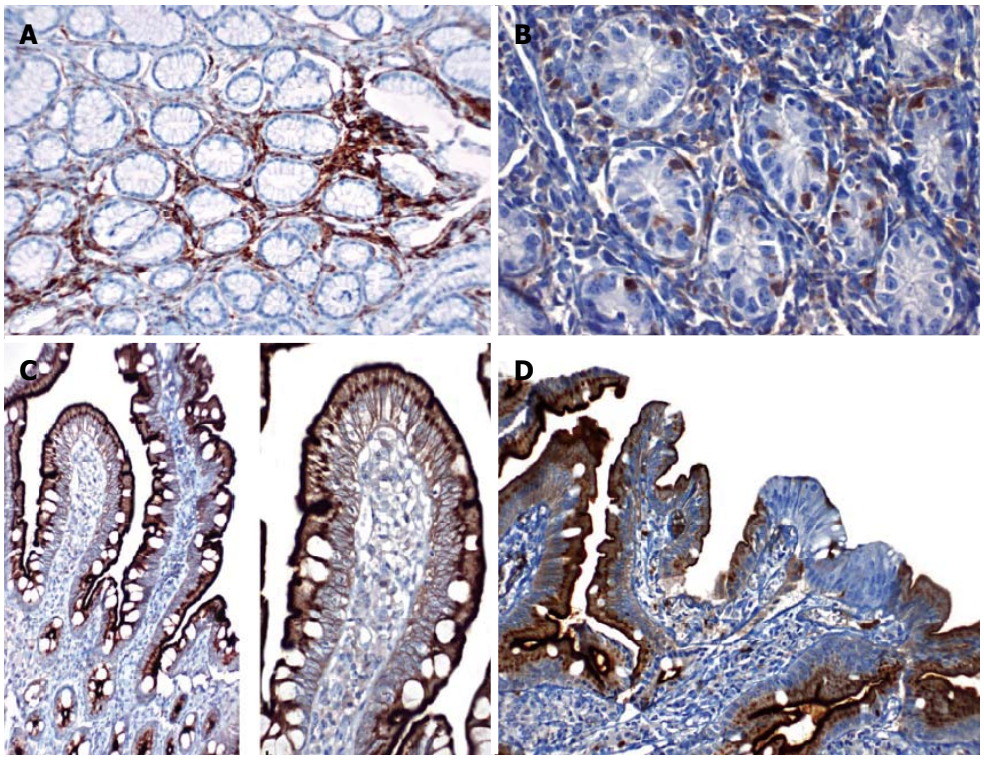

Figure 5 Gastric mucosa samples stained with CD10. A: In patients with gastritis, diffuse CD10 positivity is shown in lymphocytes under the surface epithelium. Staining was not observed in the glandular epithelium (CD10, magnification × 200); B: CD10 positivity is shown in lymphocytes inside the epithelium of patients with gastritis. Staining was not observed in the glandular epithelium (CD10, magnification × 400); C: Intense positive staining is shown in the brush borders of the surface epithelium and supranuclear cytoplasm from healthy duodenal samples (CD10, magnification × 100 and 200); D: Loss of CD10 staining on the surface epithelium of duodenal samples from patients with duodenitis.

the enzyme primarily involved in the hydrolysis of SP was also decreased in patients with duodenitis.

SP is an inflammatory peptide that is mainly released from sensory nerve endings. In the gastrointestinal tract, SP and its receptor have been reported to be extensively expressed in myenteric and submucosal nerve plexuses. In addition, SP is found in neutrophils, eosinophils, macrophages, and dendritic cells. SP plays roles in muscle contraction, epithelial ion transport, vascular permeability, and the regulation of immune function[18-23]. The inflammatory activity of SP has often been shown in *in vitro* studies and in animal experiments. Specifically, the activation of SP-containing nerve endings increases neutrophil, lymphocyte, monocyte, and fibroblast chemotaxis, suggesting a role in neurogenic inflammation[24-26]. Although experimental studies have shown that SP plays an important role in the formation of ulcers, it is not known how the levels of SP change in association with childhood gastritis. Here, we used a quantitative approach and found that SP is increased in both neuronal and non-neuronal diseased-tissue in a grade-dependent manner. Furthermore, the presence of an *H. pylori* infection did not alter SP levels; hence, SP probably exerts its inflammatory effect independent of *H. pylori* infections.

These findings correlate well with the inflammatory role of SP but differ from the results reported for adult patients in a study using a very similar approach[17]. Specifically, Erin *et al*[17] evaluated 57 adult patients with gastritis and reported two-way changes in SP levels. In patients with gastritis, SP levels in gastric mucosa with a lesion were found to be similar to the levels in healthy gastric mucosa, even though the amount of neuronal SP was significantly lower in lesion-free gastric mucosa than in healthy mucosa. The researchers suggested that neuronal SP is suppressed in lesion-free regions in patients with gastritis, which may suggest a degeneration of sensory nerve endings. However, the researchers did not compare their findings based on the Sydney classification system in that study. Also, differences between adult and pediatric patients might be due to lower physiological levels of neuronal SP in children. This possibility should be evaluated further. Our results, together with previously published data on SP, suggest that an SP antagonist may have therapeutic value in treating children with chronic gastritis.

VIP has anti-inflammatory effects by reducing the secretion of proinflammatory cytokines (TNFα, IL-6, and IL-12), increasing the secretion of anti-inflammatory cytokines (such as IL-10), and reducing Th1 activation[27]. VIP analogs have been shown to be useful in inflammatory and autoinflammatory models, such as models of endotoxic shock, Crohn's disease, ulcerative colitis, rheumatoid arthritis, Parkinson's

disease, and brain injury[28]. Information regarding the change in VIP levels in upper gastrointestinal diseases is very limited. In an experimental cold-induced stress ulcer rat model, the administration of intraperitoneal VIP led to decreased histamine and methylhistamine levels and healing of mucosal ulcers[9], and a few animal studies have demonstrated gastro-protective effects of VIP[29,30]. In a study with a limited number of adult *H. pylori* (+) gastritis patients, VIP levels were shown to be decreased in the gastric mucosa. Researchers have suggested that this decrease in VIP levels may play a role in the pathogenesis of gastritis[10]. In 1985, 8 adult patients with duodenal ulcers were reported to have increased levels of VIP in their proximal duodenal mucosa[31]. In 2012, Erin *et al*[17] evaluated 57 adult patients and reported that neuronal VIP levels were significantly higher in healthy gastric mucosa samples than in samples from patients with gastritis and ulcers. In addition, reductions in VIP levels, particularly VIP of neuronal origin, were shown to be more pronounced in patients with peptic ulcers. Therefore, the researchers suggested that reductions in VIP levels may lead to inflammatory gastrointestinal pathologies, such as gastritis and ulcers. In our study, we found that VIP levels were significantly higher in healthy gastric mucosa and in areas with mild gastritis (control group) compared with the levels in areas with moderate-severe gastritis (both S1 and S2). Moreover, we also found that VIP levels in S1 samples were significantly lower than levels in S2 samples in the first and second extractions.

In our study, we did not find any relationship between decreased VIP levels and *H. pylori* infections. VIP increases the secretion of HCO_3^- in the mucus, contributing to the protection of the underlying epithelium[32]. The decreased levels of VIP may lead to the degradation of the gastric mucus barrier and damage to gastric epithelial cells, which in turn allows the easy penetration of *H. pylori*. The results of our study suggest that reductions in VIP rather than *H. pylori* infections causes gastritis, which is consistent with the findings of the study by Erin *et al*[17] on adult patients. Our study is the first to evaluate the VIP levels in childhood gastritis and suggests that mimetics of VIP may have a therapeutic value in treating childhood gastritis.

Blocking the CGRP pathway (such as *via* the ablation of afferent nerves with capsaicin) decreases the protective hyperemic response. In experimental studies, the administration of CGRP has been shown to result in the stimulation of sensory nerves, and in various experimental gastric ulcer models, CGRP has been found to create a protective effect. CGRP also accelerates the healing of gastric mucosal lesions and gastric ulcers created by various ulcerogenic factors[10,33,34]. It has been suggested that CGRP and its receptor in the transient receptor potential vanilloid type-1 pathway might be novel targets for therapeutic agents in treating gastric mucosal injury and visceral sensitivity in functional bowel syndrome[35,36].

Sipos *et al*[10] evaluated 10 adult patients with gastritis and found that CGRP- containing immunoreactive nerve endings were slightly decreased relative to the number present in the control group. In our study, we found that CGRP levels were lower in S1 and S2 samples in the first and second extractions in children with gastritis compared with levels in the controls. Moreover, the CGRP levels were lower in S1 than in S2 samples. These results indicate that decreased levels of CGRP might be involved in the pathogenesis of gastritis. To our knowledge, this study is the first to evaluate changes in CGRP and their effects on pediatric patients with gastritis. Finally, we observed no differences in SP, CGRP, or VIP levels in the *H. pylori* (+) *vs H. pylori* (-) groups, but we found significant differences in patients with grade Ⅰ *vs* Ⅱ-Ⅲ gastritis. The reason for this is related to the fact that almost half of the patients in the study group, where severe gastritis was more common, were *H. pylori* (-). Therefore, we suggest that changes in neuropeptides can cause gastric mucosal damage independent of *H. pylori* infection.

NEP is a membrane-bound cell-surface enzyme that is densely concentrated in the brush border of the small intestinal mucosa. In healthy mucosa, linear (regular) CD10 staining was observed *via* immunohistochemical methods. Trejdosiewicz *et al*[37] reported no CD10 staining in healthy gastric mucosa in patients with celiac disease. In our study, we did not observe any NEP enzyme activity in the gastric mucosa or epithelial CD10 staining. In adults with chronic gastritis, intraepithelial neutrophil density indicates the extent of mucosal damage. Neutrophil density has been shown to be far lower in children than in adults, but lymphocyte infiltration is more prominent[38]. In our study, diffuse CD10 staining of gastric mucosal lymphoplasmacytic cells was significantly lower in the control group than in the study group. Moreover, we observed more intense CD10 staining in S1 than in S2 samples. Hematopoietic progenitor cells, called common lymphoid progenitors, have the potential to transform into T, B, and natural killer cells and express NEP. The more frequent detection of CD10-positive lymphoplasmacytic cell-inflamed regions suggests that cells expressing NEP may play a role in the onset or aggravation of inflammation.

NEP hydrolyzes SP at the Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu regions and creates SP fragments with different activities[12]. In our study, we observed elevated SP levels and an increased presence of CD10 positive cells in regions with lesions, which can be interpreted as a reactive response. The role and significance of epithelial NEP expression seem to differ from the role and significance of its expression in immune cells. Epithelial NEP expression seems to be important in the protection of the mucosa. NEP may have a role in the growth and differentiation of intestinal epithelial cells, and it has been used in

support of diagnoses of microvilli inclusion disease using light microscopy[37,39-41]. In a study of ulcerative colitis patients, a regular staining pattern was detected in healthy ileal mucosa, but no staining was observed in inflamed mucosa[42], and changes in NEP expression were reported in a patient with intestinal metaplasia accompanied by gastric adenocarcinoma[43]. Moreover, we observed a more prominent diffuse CD10 staining pattern in the duodenal mucosa of patients without duodenitis than in those with duodenitis.

Our study is the first to evaluate the level and expression of NEP in inflamed gastric mucosa and the duodenum, and our results suggests that the loss of epithelial NEP activity is involved in duodenal inflammation. The changes observed in inflammatory infiltrates most likely signify the reactive infiltration of immature immune cells associated with gastritis. In conclusion, based on these findings, we propose that decreased levels of VIP and CGRP and increased levels of SP contribute to the pathological changes in the gastric mucosa observed in patients with gastritis. Hence, new treatments targeting these molecules may have therapeutic and preventive actions.

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COMMENTS

Background

Chronic gastritis is an important childhood health problem. The disruption of balance between aggressive and protective factors of gastric mucosa has been demonstrated to lead to gastritis. And *Helicobacter pylori* (*H. pylori*) infection is the most important factor implicated in the pathogenesis of gastritis. However, neither the gastric mucosa's protective mechanisms nor the pathogenesis of gastritis have been fully elucidated. Patients may not respond to conventional medical treatments or *H. pylori* eradication therapy which suggests the involvement of unknown factors in the pathogenesis of gastritis. The gastrointestinal system contains some regulatory sensory neuropeptides that may play a role in gastritis pathogenesis. There are insufficient data regarding in this issue.

Research frontiers

It has been suggested that substance P (SP) may regulate inflammatory processes, vasoactive intestinal peptide (VIP) has anti-inflammatory effects, and calcitonin gene-related peptide (CGRP) has a vasodilatory effect. Neutral endopeptidase (NEP) hydrolyzes SP, it may terminate its biological activity and it has been suggested to function in the growth and differentiation of intestinal epithelial cells. To date, no studies have investigated changes in SP, VIP, CGRP, and NEP levels in the gastric mucosa of children. Disturbances in neuropeptide content may result in mucosal damage. Advances in current knowledge of the underlying mechanisms will help in developing new treatment options.

Innovations and breakthroughs

In a recent study with adults authors found that, neuronal SP levels decreased significantly in normally appearing mucosa of patients with gastritis while levels of non-neuronal SP increased in diseased areas of gastritis and ulcer. The content of VIP in both disease-involved and uninvolved mucosa, and expression of NEP, markedly decreased in patients with gastritis or ulcer. Since VIP, as well as SP fragments, formed following hydrolysis with NEP is recognized to have gastroprotective effects, decreased levels of VIP, SP and NEP may predispose to cellular damage. In the present study, the authors aimed to investigate the roles of neuronal and non-neuronal peptides in the pathogenesis of chronic gastritis in children.

Applications

Advances in the knowledge of the authors' underlying mechanisms will help in developing new treatment options. The use of SP antagonists, VIP analogues or targeting the CGRP pathway with novel therapeutic agents can be a good treatment option.

Terminology

Inflammation of the gastric mucosa is defined as gastritis. The gastrointestinal system contains sensory neuropeptides, such as VIP, SP, and CGRP. Disturbances in neuropeptide content may result in gastritis.

Peer-review

Authors reported that in this study decreased levels of VIP and CGRP and increased levels of SP contribute to pathological changes in gastric mucosa in children. The results suggest that new treatments targeting these molecules may have therapeutic and preventive effects.

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ORIGINAL ARTICLE

Basic Study

Increased ATG5-ATG12 in hepatitis B virus-associated hepatocellular carcinoma and their role in apoptosis

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Abstract

AIM

To investigate autophagy-related genes, particularly ATG12, in apoptosis and cell cycle in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) and non-HBV-HCC cell lines.

METHODS

The expression of autophagy-related genes in HBV-associated hepatocellular carcinoma and non-HBV-HCC cell lines and human liver tissues was examined by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) and western blotting. The silencing of target genes was used to examine the function of various genes in apoptosis and cell cycle progression.

RESULTS

The expression of autophagy related genes *ATG5*, *ATG12*, *ATG9A* and *ATG4B* expression was analyzed in HepG2.2.15 cells and compared with HepG2 and THLE cells. We found that *ATG5* and *ATG12* mRNA expression was significantly increased in HepG2.2.15 cells compared to HepG2 cells ($P < 0.005$). Moreover, ATG5-ATG12 protein levels were increased in tumor liver tissues compared to adjacent non-tumor tissues mainly from HCC patients with HBV infection. We also analyzed the function of ATG12 in cell apoptosis and cell cycle progression. The percentage of apoptotic cells increased by 11.4% in ATG12-silenced HepG2.2.15 cells ($P < 0.005$) but did not change in ATG12-silenced HepG2 cells under starvation with Earle's balanced salt solution. However, the combination blockade of Notch signaling and ATG12 decreased the apoptotic rate of HepG2.2.15 cells from 55.6% to 50.4% ($P < 0.05$).

CONCLUSION

ATG12 is important for HBV-associated apoptosis and a potential drug target for HBV-HCC. Combination inhibition of ATG12/Notch signaling had no additional effect on HepG2.2.15 apoptosis.

Key words: Autophagy; Hepatitis B virus; Hepatocellular carcinoma; ATG12; Apoptosis; Cell cycle; Notch signaling

Core tip: ATG5-ATG12 protein expression was increased in hepatitis B virus (HBV)-transfected HepG2.2.15 cells when they were compared with HepG2 cells and was increased in tumor liver tissues compared to adjacent non-tumor tissues from hepatocellular carcinoma (HCC) patients with HBV infection. We showed that silencing of ATG12 increased cell apoptosis of HepG2.2.15 cells but not HepG2 cells under starvation conditions. These results suggest that ATG5-ATG12 proteins are important for the survival of HBV-associated HCC during states of limited tumor nutrients. The inhibition of ATG12 might be a good target for HBV-associated HCC treatment.

Kunanopparat A, Kimkong I, Palaga T, Tangkijvanich P, Sirichindakul B, Hirankarn N. Increased ATG5-ATG12 in hepatitis B virus-associated hepatocellular carcinoma and their role in apoptosis. *World J Gastroenterol* 2016; 22(37): 8361-8374 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i37/8361.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i37.8361

INTRODUCTION

Previous studies have reported that HBV expression is correlated with autophagy induction[1-3]. HBV enhances and uses autophagy for its replication *via* the HBx protein, which binds and activates phosphatidylinositol-3-kinase class 3 (PIK3C3), an enzyme important for the initiation of autophagy. Autophagy inhibitors or the silencing of enzymes essential for the formation of autophagosomes suppresses HBV DNA synthesis with a minimal effect on the HBV mRNA levels[2]. The role of autophagy in the production of HBV virions was demonstrated in HBV transgenic mice with a liver-specific deficiency of Atg5[4]. We recently confirmed that ATG12 knock down reduced HBV DNA levels in HepG2.2.15 cells and induced the interferon signaling pathway, suggesting that autophagy machinery may aid HBV survival by reducing antiviral innate immunity[5].

Many studies have provided evidence to support the role of autophagy in human cancer. Beclin-1 was the first mammalian autophagy gene to be identified. The monoallelic deletion of Beclin-1 at chromosome 17q21 is sporadically observed in approximately 75% of ovarian cancers[6,7], 50% of breast cancers[8], and 40% of prostate cancers[9]. Other mutations in autophagy genes such as Atg5, Atg12, Atg9B are frequent in gastric and colon cancers[10]. UVRAG, a Beclin1-interacting protein[10,11] and Atg4C were also shown to suppress tumor gene activity[12]. Liver-specific Beclin-1 knockout heterozygous mice showed increased rates of hepatocellular carcinoma in old aged mice[13,14]. Furthermore, mosaic $Atg5^{-/-}$ mice developed benign liver tumors at 6-mo of age[15] and Atg7 hepatocyte-specific knockout mice also developed liver tumors later in life[15]. These data support the idea that autophagy defects contribute to tumorigenesis.

However, autophagy deficient cells can occur *via* cellular damage caused by dysfunctional mitochondria, oxidative stress, endoplasmic reticulum stress, necrosis and p62 accumulation[16]. The accumulation of cell damage can lead to chromosome instability[17] and inflammatory responses[18], resulting in tumor development. Although, autophagy functions as a tumor suppressor in primary cells, it is important for cancer cell survival. Interestingly, spontaneously occurring liver tumors did not progress in chimeric mice with Atg5 or Atg7 loss[15]. This finding implies that autophagy is required for tumor progression. Additionally, autophagy is required for cancer progression of other types of cancers. For example, an Atg3 deletion in hematopoietic cells prevents BCR-Abl-mediated leukemia[19]. Some

Table 1 Sequences of primers used in this study

Gene names	Primer sequences (5'-3')	
Beclin-1	BECN1-F	GGATCAGGAGGAAGC
	BECN1-R	GATGTGGAAGGTTGC
ATG5	ATG5-F	GCTTCGAGATGTGTGGTTTGG
	ATG5-R	ACTTTGTCAGTTACCAACGTCA
ATG12	ATG12-F	TTGTGGCCTCAGAACAGTTG
	ATG12-R	GAGAGTTCCAACTTCTTGGTCTG
ATG9A	ATG9A-F	CGTGTGGGAAGGACAG
	ATG9A-R	GGCGCTTTCTCCACTC
ATG4B	ATG4B-F	TCCATAGGCCAGTGGTACG
	ATG4B-R	TGCACAACCTTCTGATTTCC
B-actin	B-actin-F	ACCAACTGGGACGACATGGAGAA
	B-actin-R	GTGGTGGTGAAGCTGTAGCC

tumor cells are susceptible to growth inhibition or death when autophagy is inhibited. Guo *et al*[20], found that Ras-driven tumors required autophagy for tumor cell survival upon starvation. Therefore, autophagy has a dual-function in cancer. It functions as a tumor suppressor during cancer initiation, but also functions to promote tumor progression and metastasis later in the development process.

Because HBV infection is associated with hepatocellular carcinoma (HCC) and requires the induction of autophagy for its survival, we investigated the involvement of autophagic genes in cancer cell survival using HBV-associated HCC and non HBV-HCC cell lines and liver tissues.

MATERIALS AND METHODS

Cell lines

The immortalized human liver epithelial cell line (THLE-2; ATCC® CRL-2706™, Manassas, VA, United States) was cultured in BEGM medium (CC3170 Bullet Kit; Lonza, Walkersville, MD, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 ng/mL epidermal growth factor and 70 ng/mL phosphoethanolamine and maintained at 37 ℃ in 5% CO_2. The flasks were precoated with a mixture of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin dissolved in BEBM medium. The human hepatocellular carcinoma (HepG2) and HBV-transfected HepG2.2.15 cell lines were obtained from Professor Antonio Bertoletti (Singapore Institute for Clinical Sciences, A*Star). These two cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, United States) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and streptomycin at 37 ℃ in 5% CO_2. For HepG2.2.15 cells, the medium was supplemented with 150 μg/mL of G418 (Geneticin; Gibco, United States) to maintain HBV plasmids.

Autophagy induction

To induce starvation conditions, the cells were incubated in serum-free Earle's balanced salt solution (EBSS; starvation medium; Invitrogen, United States) for the indicated number of hours (between 4-8 h).

Human HCC samples

Human liver tissues were obtained from King Chulalongkorn Memorial Hospital. The use of tissue biopsy was approved by the Institutional Review Board (IRB No. 396/55), Faculty of Medicine, Chulalongkorn University. The sections of tumor tissue and adjacent non-tumor tissue were performed at the Department of Pathology, Chulalongkorn University. The tissue samples were divided into two groups: HBV-infected and non-HBV infected groups. The diagnosis of HBV infection from HCC patients was established by seropositivity for HBsAg, HBV viral DNA, anti-HBcAg and seronegativity to anti-HBsAg. All fresh tissue samples were snap frozen in liquid nitrogen and stored at -80 ℃ prior to use.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted using Real Genomics Total RNA Extraction kit (RBC Bioscience, Taiwan). The quantity of RNA was measured using a spectrophotometer. RNA concentrations in a solution were read and a 260 nm absorbance reading of 1.0 was equivalent to about 40 μg/mL of RNA and the ratio of absorbance at 260 and 280 nm was used to assess the RNA purity of RNA preparations. Then 1 μg of total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, United States).

Analysis of mRNA expression using real-time quantitative PCR

PCR reactions were performed in a 20 μL reaction volume in a 96-well plate (Applied Biosystems, United States). The PCR mixture contained 2 μL of cDNA template, 10 μL of commercial (2 ×) Power SYBR® Green PCR Master Mix (Applied Biosystems), and each primer at a final concentration of 0.5 μmol/L. Real-time PCR runs were carried out using an ABI Thermal cycler 7500 real-time PCR instrument (Applied Biosystems). The conditions for thermal cycling were as follows: initial denaturation at 95 ℃ for 10 min, followed by 40 amplification cycles at 95 ℃ for 15 s and then 60 ℃ for 1 min. For each PCR run, a negative (no-template) control was used to test for false-positive results or contamination. The absence of nonspecific amplification was confirmed by generating a melt curve using the Applied Biosystems real-time PCR system software. The primers utilized in this study are summarized in Table 1.

Quantifying relative gene expression

To analyze the relative gene expression data, we used real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method[21]. The C_T values were obtained from real-time PCR instrumentation. The quantity of mRNA relative to a reference gene was calculated using the formula $2^{-\Delta C_T}$,

where ΔC_T = (C_T target RNA - C_T reference RNA). Comparison of gene expression was based on a comparative C_T method ($\Delta\Delta C_T$), and the relative RNA expression was quantified according to the formula of $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = (C_T target RNA (experimental group) - C_T reference RNA (experimental group)) - (C_T target RNA (control group) - C_T reference RNA (control group)). Genes with high expression levels were selected for the functional study.

Western blot analysis

All cell lines and human liver tissues samples were lysed with RIPA buffer (25 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). After sonication, protein concentrations of cell lysates were measured using BCA assay (Thermo Scientific, United States). Then 20 μg of protein from each cell line was loaded onto a 12% polyacrylamide gel and run at 130 V for 80 min. The proteins were transferred onto nitrocellulose membranes and were immunoblotted with primary mAb against Atg9a, Atg12, cNotch1, GAPDH (dilution, 1:1000; rabbit mAb; Cell Signaling Technologies, United States) and Atg4b (dilution, 1:1000; mouse mAb; Abcam, United States). After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (dilution 1:2000) and were analyzed with enhanced chemiluminescence (ECL) substrate (SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific). Images were quantified using a Licor-odyssey image system (LI-COR®, United States).

Determination of HBx gene effect on autophagic gene expression

HepG2 cells were grown in DMEM supplemented with 10% FBS, antibiotic-free at 37 ℃ in 5% CO_2 for 24 h. HepG2 cells were transfected with 500-1000 ng of pGFP-HBx plasmids (Addgene, MA, United States) or pGFP (negative control) using 1-3 μL of Lipofectamine® 2000 Transfection Reagent (Invitrogen, United States). Opti-MEM®I Reduced Serum Medium (Invitrogen) was used to dilute Lipofectamine® 2000 and plasmids, which were then gently mixed at a 1:1 ratio, incubated for 40 min at room temperature. HepG2 cells were diluted in complete growth medium without antibiotics to a final concentration of 4 × 10^5 cells/mL, then the cells were added to culture plates with plasmid-Lipofectamine® 2000 complexes and gently mixed by rocking the plate. The cells were incubated for 24-72 h at 37 ℃ in a 5% CO_2 incubator. The relative autophagic gene expression was detected by real-time quantitative PCR.

RNA interference

HepG2 and HepG2.2.15 cells were transiently transfected with 500 ng of short hairpin RNA (shRNA) plasmid specific to ATG12 or a negative control (SureSilencing shRNA Plasmid; Qiagen, United States). The shATG12 plasmids contained following sequence: 5′-GCAAATCCTCTATGCCTTCTT-3′. Negative controls were inserted with a mock target sequence as follows: 5′-GGAATCTCATTCGATGCATAC-3′. Transfection of shRNA plasmids was carried out using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions. At 72 h post-transfection, cells were collected and the silencing efficiency of the shRNA was detected by western blotting.

Flow cytometric analysis

Specific autophagic silenced cells (shATG12) were assayed for apoptosis using PE-labeled annexin V staining (BioLegend, San Diego, CA, United States) and Fixable Viability Dye eFluor® 780 staining (eBioscience, CA, United States). Total apoptotic cells and early apoptotic cells were defined as annexin V-positive and Fixable Viability Dye eFluor® 780-negative and late apoptotic cells were defined as annexin V-positive and Fixable Viability Dye eFluor® 780-positive. For cell cycle analysis, cells were fixed with 70% ice-cold ethanol for at least 2 h on ice. The supernatant was removed, and the pellet was washed and treated with RNaseA to resuspend cells. Cells were then stained with propidium iodide (PI) solution at room temperature in the dark for 30 min. Cells were acquired on a FACScan (BD Biosciences, United States) and analyzed with FlowJo 10.0 software (FlowJo LLC., United States).

Statistical analysis

Significant differences were determined by unpaired *t* test with GraphPad Prism software, version 5.0 (San Diego, CA, United States). Statistical significance was set at a P value of < 0.05.

RESULTS

Expression profile of autophagic genes in cell lines and HCC biopsy tissue

To investigate the involvement of autophagy in HCC, we used Beclin-1 as a marker for HBV-induced autophagy under starvation conditions. We analyzed Beclin-1 mRNA expression in HBV-transfected HepG2.2.15 cells and the parental cell line, HepG2. At 4 h post-starvation, Beclin-1 was up regulated in both cell lines and was then down regulated at 8 h (data not shown). Thus, we selected autophagy induction with starvation in EBSS for 4 h for all subsequent experiments.

Autophagy related genes that represent each major part of the autophagy machinery including (1) induction step (Beclin1); (2) the first ubiquitin-like conjugation molecules (ATG5 and ATG12); (3) the cysteine protease that catalyzes the second ubiquitin-like conjugation molecules (ATG4B); and (4) the transporter membrane required for autophagosome

formation (ATG9A) were tested. The mRNA expression levels of these selected molecules were determined at baseline and at 4 h post-culture in EBSS. There were no significant differences at baseline (data not shown). At 4 h post starvation, Beclin1 mRNA was down regulated in HepG2.2.15 compared to THLE2 and HepG2 (P = 0.0213 and P = 0.0236, respectively) (Figure 1A). No significant difference was observed for ATG4B expression between HBV-transfected HepG2.2.15 and HepG2, but it was significantly increased in HepG2 and HepG2.2.15 compared with THLE-2 (P = 0.0002 and P = 0.0007, respectively) (Figure 1E). ATG9A mRNA expression was higher in HepG2 and HepG2.2.15 compared to THLE2 but significantly down regulated in HBV-transfected HepG2.2.15 compared with HepG2 (P = 0.0013) (Figure 1D). Only the mRNA expression of ATG5 and ATG12 was significantly increased in HBV-transfected HepG2.2.15 compared with HepG2 cells (P = 0.0027 (Figure 1B) and P = 0.0039 (Figure 1C), respectively). The results of mRNA levels were confirmed by western blot analysis. ATG5-ATG12 proteins were up regulated in HBV-transfected HepG2.2.15 whereas ATG9A were down regulated compared to HepG2 cells. There was no difference in ATG4B protein expression between HBV-transfected HepG2.2.15 and HepG2 (Figure 1F).

Next, we analyzed ATG5-ATG12 levels in tumor tissues compared to adjacent non-tumor tissues from HCC patients with or without HBV infection. In HBV-associated HCC tissues, ATG5-ATG12 protein levels were increased in the majority of tumor liver tissues compared to adjacent non-tumor tissues (9/10 sample pairs) (Figure 2A and C). In non-HBV HCC, ATG5-ATG12 protein levels were only increased in 3/8 tumor liver tissue samples (Figure 2B and C). These results suggested that ATG5-ATG12 proteins might have a selective advantage in HBV-associated HCC compared to non-HBV HCC.

Effect of HBx gene on autophagic gene expression

The X protein was shown to induce HBV replication by increasing Beclin-1 transcription leading to the induction of autophagy[1]. In this study, we analyzed the effect of HBx on Beclin-1 and on ATG12 by transfection of HepG2 cells with the pGFP-HBx plasmid. Then, we analyzed Beclin-1 and ATG12 mRNA expressions using quantitative real-time RT-PCR. We found that Beclin-1 (P = 0.0027) (Figure 3A) and ATG12 (P = 0.0139) (Figure 3B) mRNA expression were significantly increased in HepG2-GFP-HBx compared with HepG2-GFP after 48 h of transfection. These results suggested that HBX plays a role in ATG12 induction, either directly or indirectly through Beclin1.

ATG12 gene silencing in HepG2 and HepG2.2.15

Next, we studied the functional role of the ATG12 autophagic gene by monitoring the biological effect after knockdown of its expression. Cells containing plasmids with the greatest silencing efficiency were subjected to western blot analysis for protein expression. The transfection efficiency was estimated by monitoring GFP expression under inverted fluorescence microscope (data not shown). The protein level of ATG12 in transfected HepG2 and HepG2.2.15 cells was decreased compared with mock controls (Figure 4A).

Effect of ATG12 knock down on cell apoptosis and cell cycle progression

We investigated apoptosis and cell cycle progression in HepG2.2.15 cells compare to HepG2 cells after the silencing of ATG12. The percentage of apoptotic cells was slightly increased (8.3%) in ATG12-silenced HepG2 but unchanged in HepG2.2.15 under normal conditions, which is similar to what we have previously reported[5]. Under starvation conditions with EBSS, the percentage of apoptotic cells increased 11.4% in ATG12-silenced HepG2.2.15 but did not change in ATG12-silenced HepG2 (Figure 4B). No significant changes in cell cycle progression were observed in either cell line (Figure 5). These results suggest that ATG5-ATG12 proteins are important for the survival of HBV-associated HCC during states of limited tumor nutrients.

Analysis of combination treatment using ATG12-silencing and Notch inhibition

We recently reported that Notch signaling played a role in cell cycle progression and apoptosis, particularly in HBV-associated HCC[22]. Because ATG12 was up regulated in HepG2.2.15 compared to HepG2, and plays a role in cell apoptosis, we assessed the effect of ATG12 silencing in combination with a Notch inhibitor in the HepG2.2.15 cell line. Gamma-secretase inhibitors, also known as N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DAPT, is used to block the Notch pathway[23]. When using DAPT combined with gene silencing of ATG12 under starvation conditions, HepG2 cell apoptosis was increased from 38.5% to 48.3%, compared to DAPT treatment alone, although there was no synergistic effect (Figure 6). Interestingly, the combination blockade of DAPT and ATG12 gave the opposite result by decreasing the apoptosis rate in HepG2.2.15 from 55.6% to 50.4% (Figure 6). No significant changes in cell cycle progression were observed for the combination treatment of ATG12 siRNA and DAPT (Figure 7). These results suggested that autophagy and Notch signaling are independent from each other in HBV-expressing cells compared to non-HBV HCC.

Finally, we analyzed ATG12 expression after treatment with DAPT and detected cleaved Notch1 expression after silencing *ATG12* genes at baseline. ATG12 mRNA was decreased by DAPT treatment in HepG2.2.15 (Figure 8A). Moreover, cleaved Notch1 was diminished in ATG12-silenced HepG2.2.15 under

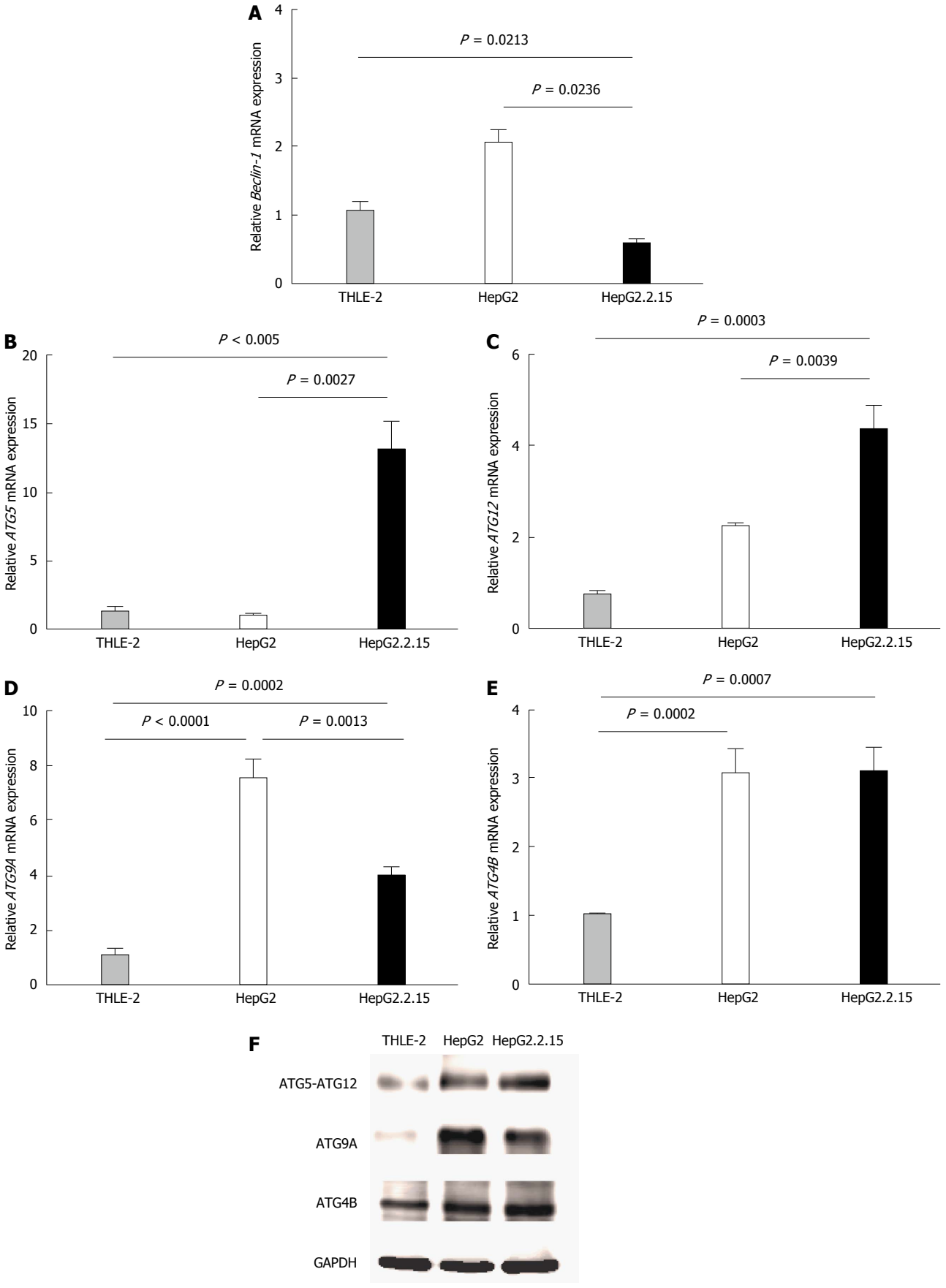

Figure 1 Quantitative real-time reverse transcriptase-polymerase chain reaction analysis of Beclin-1 (A), ATG5 (B), ATG12 (C), ATG9A (D) and ATG4B (E) mRNA in THLE-2, HepG2 and HepG2.2.15 cell lines at 4 h post-culture in EBSS. β-actin was used as an internal control. Data represent the mean and SE from five independent experiments. Western blotting with specific antibodies was used to analyze ATG12, ATG9A and ATG4B protein expression in THLE-2, HepG2 and HepG2.2.15 cells at 4 h post-culture in EBSS (F). GAPDH was used as a protein loading control.

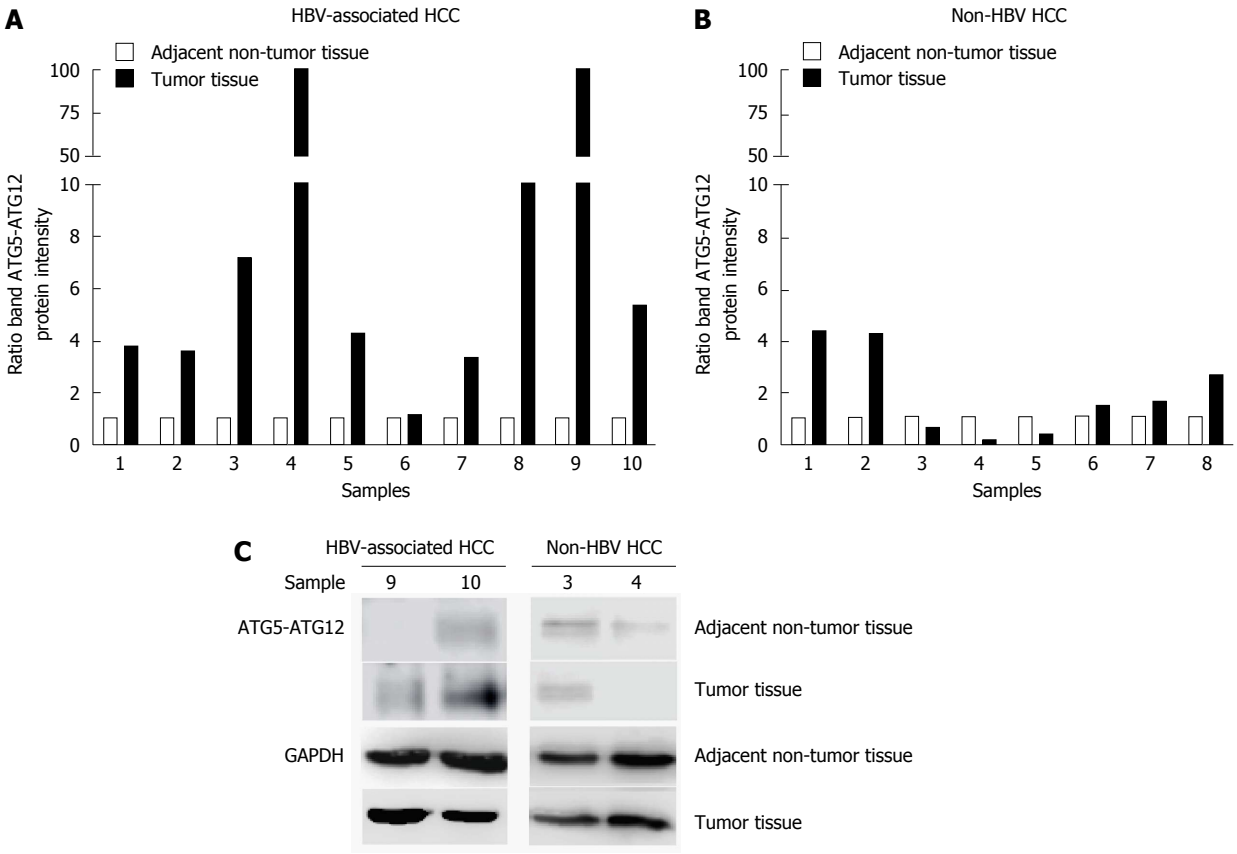

Figure 2 Quantification of ATG5-ATG12 protein levels from hepatitis B virus-infected hepatocellular carcinoma patients and non-hepatitis B virus hepatocellular carcinoma patients. Western blotting with specific antibodies was used to analyze ATG12 protein expression in HBV-associated HCC and non-HBV HCC. GAPDH was used as a protein loading control. Graphs showing the intensity band ratio (tumor tissue/adjacent non-tumor tissue) quantified using the LI-COR® image system from western blot analysis were shown in A and B. Representative western blot results were shown in C. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

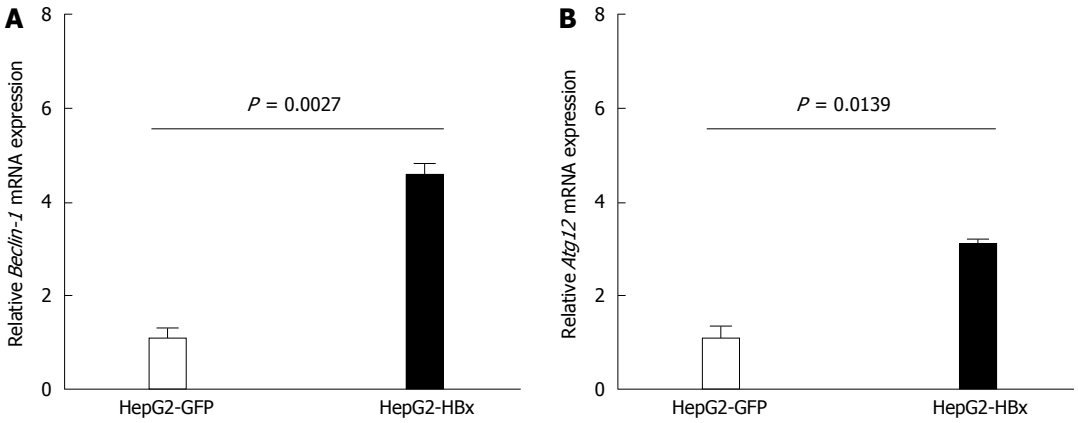

Figure 3 Quantitative real-time reverse transcriptase-polymerase chain reaction analysis of *Beclin-1* (A) and *ATG12* (B) mRNA expression in HepG2-GFP and HepG2-HBx transfected cell lines. β-actin was used as an internal control. Data represent the mean and SE from three independent experiments.

normal conditions (Figure 8B).

DISCUSSION

Autophagy has been reported to be associated with HBV and HCC[13,15,24]. However, the mechanism is still poorly understood. Moreover, recent evidence suggests that specific ATG genes might contribute differently to viral infection and cancer development[25-28]. Some ATG genes have autophagy independent functions[29-32]. In this study, we focused on the ATG5-ATG12 complex that was up regulated in HepG2.2.15 and were highly expressed in HBV-associated HCC compared to non-HBV cell lines and HCC.

HBX appears to play a role in ATG12 induction, probably indirectly through the stimulation of Beclin-1 and PIK3C3[1,2]. We showed that silencing of ATG12, which represented the elimination of the ATG5-ATG12

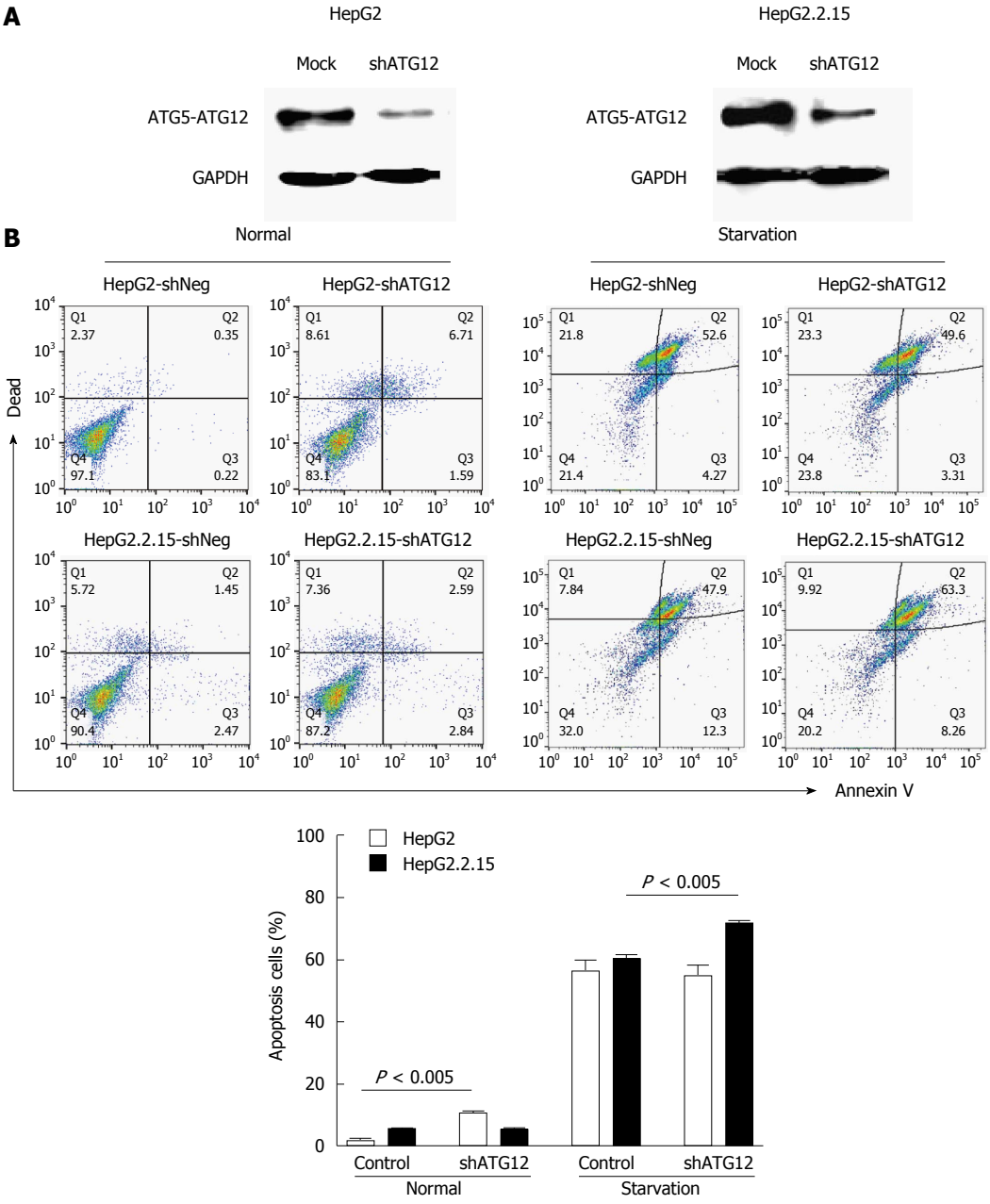

Figure 4 Apoptosis assays of HepG2 and HepG2.2.15 cells transfected with shATG12 or shNeg (control). A: Suppression of ATG12 expression was done by RNA interference. Western blotting was used to analyze ATG12 proteins from mock treatment (control) and ATG12 knockdown (shATG12) in HepG2 and HepG2.2.15 cells; B: GAPDH was used as a protein loading control. Cells were transiently transfected with shRNA plasmids for 72 h (normal) and then cultured in EBSS for 4 h (starvation). Bar graphs show the percentage of total apoptotic cells detected by Annexin V binding. Data represent the mean and SE from three independent experiments.

complex, increased cell apoptosis under starvation conditions in HepG2.2.15 but not in HepG2. These results suggested that ATG5-ATG12 is important for the survival of HBV-associated HCC during states of limited tumor nutrients. A previous study by Mao *et al*[33] showed that HepG2.2.15 cells have a higher survival rate than HepG2 cells after culturing in a starvation medium for 48 h. Cell death was reduced through activation of the autophagy pathway. However, our study did not show any significant difference between the rate of cell death for HepG2 and HepG2.2.15 after starvation for 4 h. This might be explained by our shorter starvation time. The inhibition of autophagy-induced liver cancer cell apoptosis under starvation conditions was reported by Chang *et al*[34] They showed that HepG2 and HuH6 induced autophagy (Beclin-1, ATG5 and LC3B expression) for cell survival under starvation conditions with serum-free medium or chemotherapy. The inhibition of autophagy *via* the silencing of autophagic genes (Beclin-1 and ATG5) or 3-MA treatment induced HuH6 cell apoptosis under starvation conditions[34]. A study by Liu *et al*[35] demonstrated that starvation with serum-free medium for 48 h induced autophagy

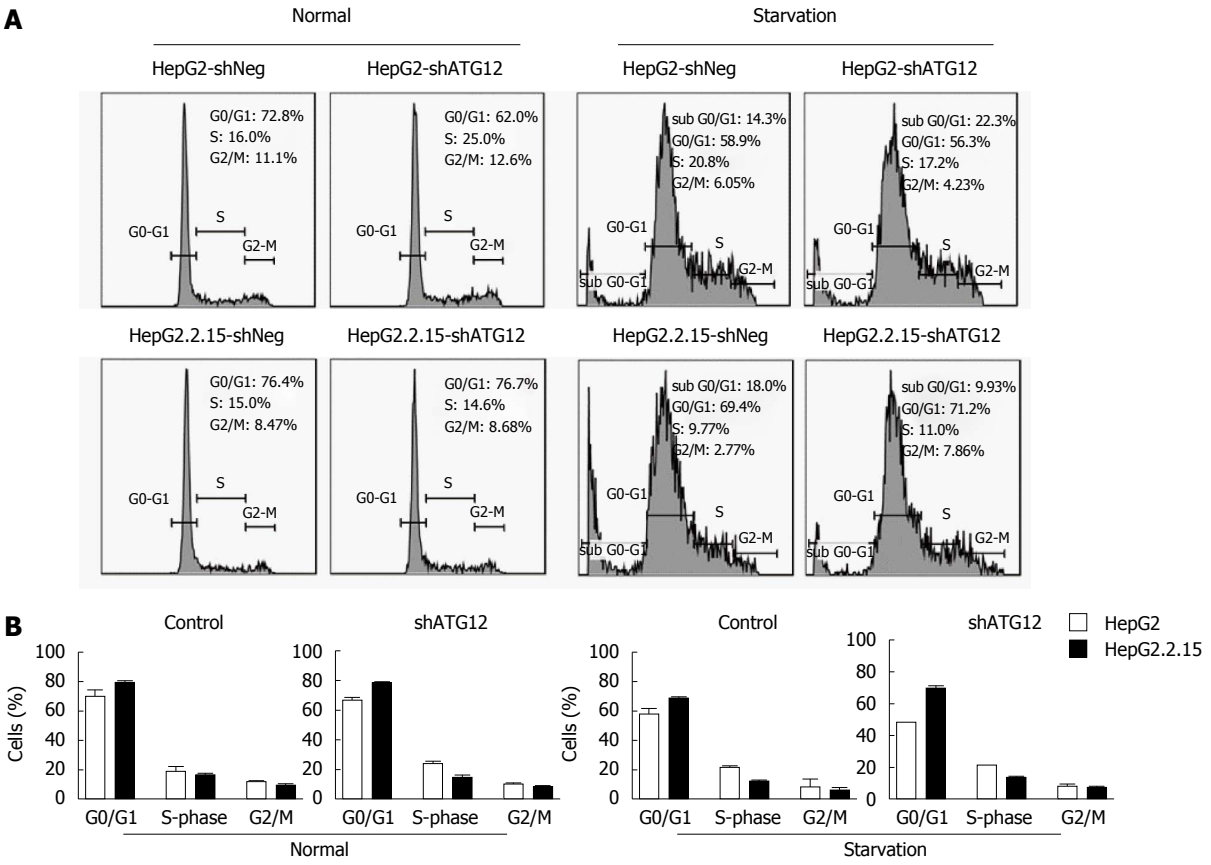

Figure 5 Cell cycle analysis of HepG2 and HepG2.2.15 cells transfected with shATG12 or shNeg (control). A: Cells were transiently transfected with shRNA plasmids for 72 h (normal) and then cultured in EBSS for 4 h (starvation); B: Bar graphs show the percentage of cells in each phase. Data represent the mean and SE from three independent experiments.

and apoptosis in 7702, HepG2, Hep3B and Huh7 cell lines; however, the inhibition of autophagy *via* Bafilomycin A1 was not affected in HepG2 apoptosis under starvation conditions similar to our result. These results imply that targeting the inhibition of autophagy might have different effects depending on the cell type and various metabolic stress conditions present.

Although ATG12 has been demonstrated to have pro-apoptotic activity through the ATG12-ATG3 conjugate[36], ATG12 also has anti-apoptotic activity. In mammalian HeLa cells, disruption of autophagy by silencing ATG12 promotes cells to die *via* apoptosis under starvation conditions[37]. ATG12 and other autophagic proteins (LC3 and ATG5) are associated with mitochondrial quality control of human umbilical vein endothelial cells. ATG12, ATG5 and LC3B were up-regulated after mitochondrial damage, leading to increased anti-apoptotic effects and increased life span in an *in vitro* aging model[38]. The combination of trastuzumab with silencing of ATG12 reduces cell viability and tumor growth in nude mice[39]. Therefore, we suggest that the inhibition of ATG12 might be a good target for the treatment of HBV-associated HCC.

Many cancer drug treatments increase tumor cell autophagy to protect cancer cells from apoptosis. Autophagy inhibitors in combination with chemotherapy are designed to induce apoptosis in human cancers. For example, autophagy inhibition was previously shown to enhance the growth inhibitory effects of sorafenib[40] as well as the combination of vorinostat with sorafenib in HCC cell lines[41]. In this study, we examined apoptosis cell death after the inhibition of ATG12 in combination with Notch signaling in HepG2 and HepG2.2.15 cell lines. The combination treatment increased cell apoptosis in HepG2 cells. However, when we blocked both Notch signaling and ATG12 under starvation conditions, cell apoptosis did not increase. There appears to be some interaction between ATG12 and Notch signaling, specifically in HepG2.2.12, because the Notch inhibitor DAPT caused the down-regulation of ATG12 mRNA but this was not observed in HepG2. In addition, the inhibition of ATG12 impaired Notch activation in HepG2.2.15 but increased Notch activation in HepG2.

This observation regarding the regulation between autophagy and notch signaling in HepG2.2.15 was unexpected. Both autophagy and Notch signaling are highly conserved signaling pathways in eukaryotic cells. The core Notch signaling pathway is through the binding of Notch ligand to Notch receptor which induces two proteolytic cleavages, metalloprotease and γ-secretase, to release the Notch intracellular

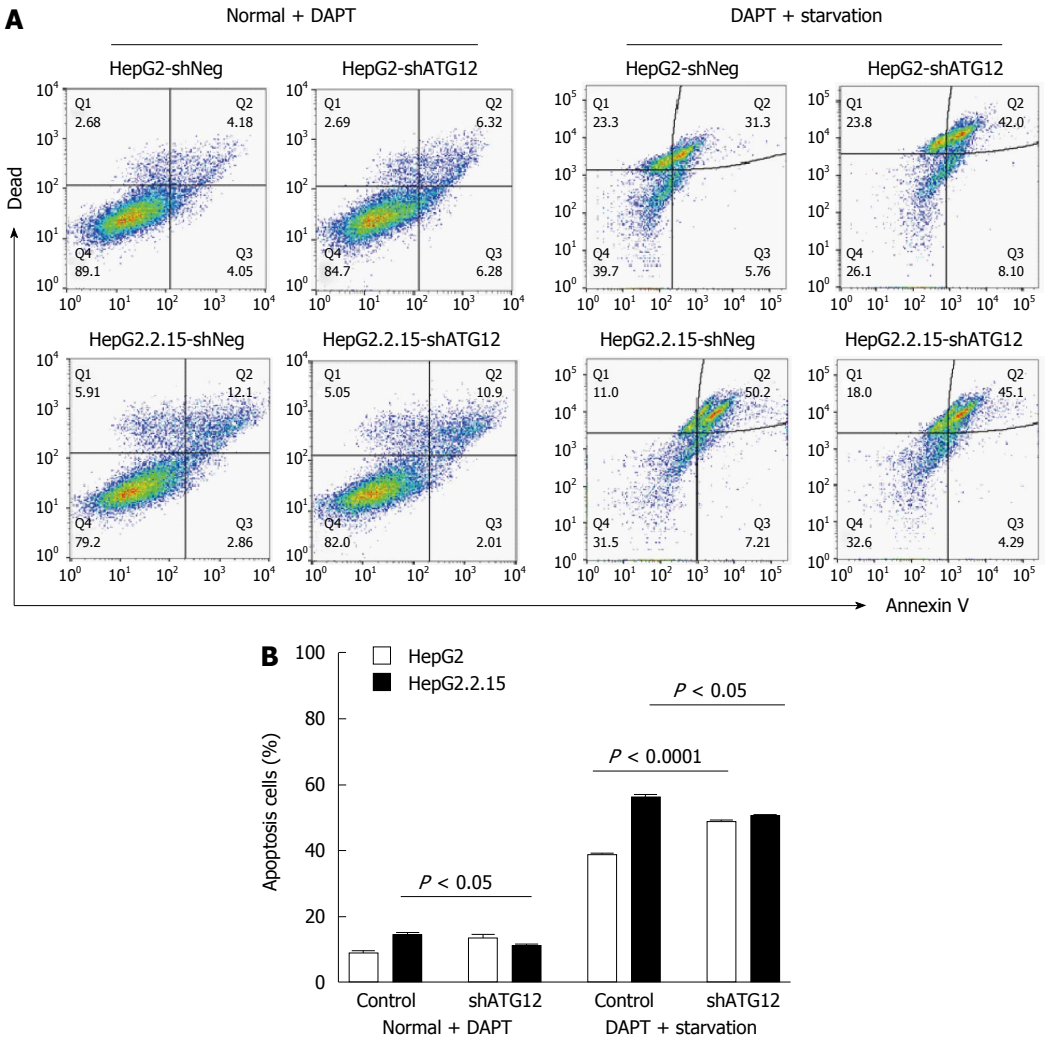

Figure 6 Apoptosis assays of HepG2 and HepG2.2.15 cells transfected with shATG12 or shNeg (control) pretreated with DAPT. A: Cells were transiently transfected with shRNA plasmids for 24 h, and then pretreated with DAPT (50 μmol/L, 48 h) and cultured in EBSS for 4 h (starvation); B: Bar graphs show the percentage of total apoptotic cells detected by Annexin V binding. Data represent the mean and SE from three independent experiments.

domain, which translocates to the nucleus and binds to DNA to regulate the transcription of target genes[42]. The Notch pathway is associated with both tumor suppression and tumorigenesis in HCC[43,44]. The connection of autophagy and Notch signaling was demonstrated in Drosophila where the ATG4 mutation enhanced the notched-wing phenotype resulting in defective Notch signaling[45]. In contrast, the overexpression of ATG1 enhanced the notched-wing phenotype[46], suggesting that some autophagic proteins have additional functions independent of autophagy that are related to the enhancement or suppression of Notch signaling.

Mammalian target of rapamycin (mTOR) is a positive regulator of Notch signaling[47] and a negative regulator of autophagy[48]. Previous reports have shown that silencing of Notch1 activated phosphorylated Akt and decreased mTOR to induce glioblastoma cell apoptosis[49]. The inhibition of Notch signaling induced autophagy *via* PTEN-PI3K/Akt/mTOR pathway to promote the adipogenic differentiation of BM-MSCs[50]. Other studies have demonstrated that autophagy regulates Notch signaling by reducing the impact of Notch1 on stem cell differentiation. The silencing of autophagy (ATG7 or ATG16L1) induced Notch1 and cleaved Notch1 in HEK cells[51].

The unexpected relationship between autophagy and Notch signaling in HepG2.2.15 was different from HepG2 and might be explained by the HBV genome. The HBV X protein activates the NF-κB pathway (p65 and p50) *via* Notch signaling through a specific ligand and receptor to promote cell survival[52]. After treatment with DAPT, NF-κB expression was decreased[53]. Thus, the activation of NF-κB can either stimulate or inhibit autophagy *via* the upregulation of Beclin-1 in T-cells[54], whereas prolonged NF-κB activation suppresses ATG5 and Beclin-1 expression in macrophages[55]. However, the connection between HBV and Notch related autophagy needs further study.

In conclusion, our previous study demonstrated the role of autophagy machinery in HBV replication. We found that ATG12-knockdown reduced HBV DNA

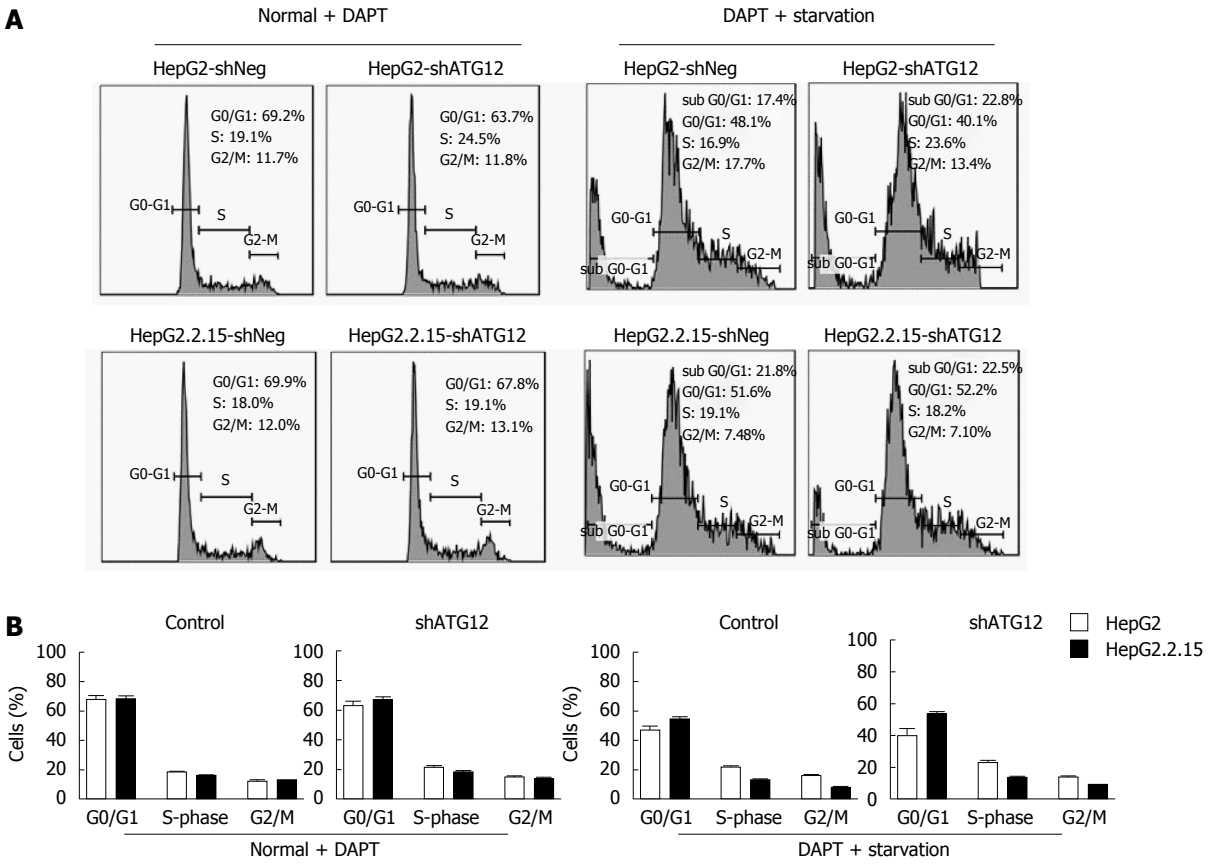

Figure 7 Cell cycle analysis of HepG2 and HepG2.2.15 cells transfected with shATG12, ATG9A or shNeg (control) pretreated with DAPT. A: Cells were transfected with shRNA plasmids for 24 h, and then pretreated with DAPT (50 μmol/L, 48 h), and cultured in EBSS for 4 h (starvation); B: Bar graphs show the percentage of cells in each phase. Data represent the mean and SE from three independent experiments.

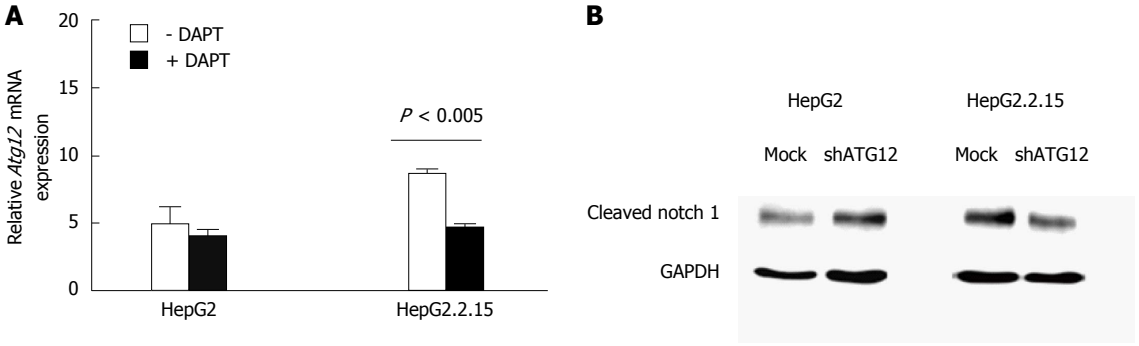

Figure 8 Quantitative real-time reverse transcriptase-polymerase chain reaction analysis of ATG12 mRNA in HepG2 and HepG2.2.15 cells with DAPT treatment for 48 h. A: Data represent the mean and SE from three independent experiments; B: Notch activation was detected by cleaved Notch1 protein expression in HepG2 and HepG2.2.15 cells after silencing ATG12 for 72 h. GAPDH was used as a protein loading control.

levels in HepG2.2.15 and induced the interferon signaling pathway. Thus, the ATG12 machinery of autophagy may aid HBV survival by reducing antiviral innate immunity[5]. Moreover, this study demonstrated that autophagy is related to HBV-associated cell death responses. Atg12 silenced HepG2.2.15 showed increased apoptosis under starvation conditions. The function of Atg12 seems to be dominated in HBV-associated HCC. These results suggested that ATG5-ATG12 is important for the survival of HBV-associated HCC during states of limited tumor nutrients. However, as mentioned previously that autophagy has a dual-function in cancer. Despite its role as tumor promotion in the later phase, it can act as a tumor suppressor during cancer initiation. Therefore, the therapeutic intervention that target autophagy has to take this information into consideration too. Moreover, our result showed that the use of autophagy inhibition in combination with other anti-tumor therapies such as Notch inhibitor, might not be a good strategy. Further characterization of HBV and Notch related autophagy is needed.

COMMENTS

Background

Hepatitis B virus (HBV) infection is associated with hepatocellular carcinoma (HCC) and has been proved to induce autophagy for its replication and survival. The involvement of some autophagic genes on cancer cell survival is still unknown. Therefore, we investigated the role of ATG genes in HBV-associated HCC and non HBV-HCC in this study.

Research frontiers

Autophagy is a catabolic process for cell survival under nutrient limitation or stress conditions. However, autophagy can act as either an oncogene or tumor suppressor gene. A recent report showed that HBV used the autophagic pathway for its own benefit; however the molecular mechanism is still unclear.

Innovations and breakthroughs

ATG5-ATG12 protein was up regulated in HepG2.2.15 and expressed in a high percentage of HBV-associated HCC compared to non-HBV cell lines and HCC. The Atg12 silenced HepG2.2.15 cell line had increased apoptosis under starvation conditions. This is the first study to show a function of Atg12 in apoptosis in HBV-associated HCC.

Applications

These findings raise the possibility of targeting the autophagic pathway for the treatment of HBV-associated HCC patients.

Peer-review

This paper reported that ATG5-ATG12 protein expression was increased in HBV-transfected HepG2.2.15 cells compared to HepG2 cells and was increased in tumor liver tissues compared to adjacent non-tumor tissues from HCC patients with HBV infection. The silencing of ATG12 increased cell apoptosis under starvation conditions in HepG2.2.15 cells but not in HepG2 cells. Their results suggest that ATG5-ATG12 is important for the survival of HBV-associated HCC in the state of tumor nutrient limitation. The inhibition of ATG12 might be a good target for HBV-associated HCC.

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ORIGINAL ARTICLE

Basic Study

Percutaneous transgastric endoscopic tube ileostomy in a porcine survival model

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Abstract

AIM

To introduce natural orifice transgastric endoscopic surgery (NOTES) tube ileostomy using pelvis-directed submucosal tunneling endoscopic gastrostomy and endoscopic tube ileostomy.

METHODS

Six live pigs (three each in the non-survival and survival groups) were used. A double-channeled therapeutic endoscope was introduced perorally into the stomach. A gastrostomy was made using a 2-cm

transversal mucosal incision following the creation of a 5-cm longitudinal pelvis-directed submucosal tunnel. The pneumoperitoneum was established *via* the endoscope. In the initial three operations of the series, a laparoscope was transumbilically inserted for guiding the tunnel direction, intraperitoneal spatial orientation and distal ileum identification. Endoscopic tube ileostomy was conducted by adopting an introducer method and using a Percutaneous Endoscopic Gastrostomy Catheter Kit equipped with the Loop Fixture. The distal tip of the 15 Fr catheter was placed toward the proximal limb of the ileum to optimize intestinal content drainage. Finally, the tunnel entrance of the gastrostomy was closed using nylon endoloops with the aid of a twin grasper. The gross and histopathological integrity of gastrostomy closure and the abdominal wall-ileum stoma tract formation were assessed 1 wk after the operation.

RESULTS

Transgastric endoscopic tube ileostomy was successful in all six pigs, without major bleeding. The mean operating time was 71 min (range: 60-110 min). There were no intraoperative complications or hemodynamic instability. The post-mortem, which was conducted 1-wk postoperatively, showed complete healing of the gastrostomy and adequate stoma tract formation of ileostomy.

CONCLUSION

Transgastric endoscopic tube ileostomy is technically feasible and reproducible in an animal model, and this technique is worthy of further improvement.

Key words: Natural orifice transluminal endoscopic surgery; Tube ileostomy; Endoloop; Pigs; Submucosal tunneling

Core tip: A novel technique, natural orifice transgastric endoscopic surgery tube ileostomy, may be successfully performed in a porcine survival model using pelvis-directed submucosal tunneling endoscopic gastrostomy, followed by endoscopic tube ileostomy using an Introducer Kit containing a loop fixture.

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INTRODUCTION

Anastomotic leakage is one of the major complications that occurs after anterior resection of the rectum for rectal cancer with a low extraperitoneal colorectal anastomosis[1,2]. With the development of surgery, a defunctioning stoma, colostomy or ileostomy has become a mainstay of proximal fecal diversion to protect against primary colorectal anastomosis, and to reduce the risk and lessen the severity of the sequelae of anastomotic leakage, such as fecal peritonitis and sepsis. However, established conventional defunctioning stomas, including loop colostomy and loop ileostomy, require a second surgical reversal[3], which may cause morbidity and even mortality. Percutaneous tube ileostomy, which is achieved by inserting a tube into the distal ileum through the abdominal wall, has been reported as an alternative that can achieve similar temporary fecal diversion, ensuring greater comfort for the patient and easier management, as well as avoiding the additional surgical procedure required for the closure of a conventional loop-ostomy[4].

Percutaneous tube ileostomy for anastomotic protection is clinically performed by laparotomy or laparoscopy. Natural orifice transluminal endoscopic surgery (NOTES)[5-8] may be an alternative for the execution of the tube ileostomy procedure, as this method is less invasive and is associated with less postoperative pain compared with the two former methods. To date, there is no literature regarding NOTES tube ileostomy. The aim of our study was to demonstrate the feasibility and reproducibility of a novel technique of percutaneous transgastric endoscopic tube ileostomy using an Introducer Kit in a porcine survival model.

MATERIALS AND METHODS

Animal model

Our study was performed on six healthy female domestic pigs weighing between 15 and 20 kg, of which three were enrolled in a non-survival group and three in a 1-wk survival group. All animals were fasted for 24 h prior to surgery. Induction of anesthesia was achieved by intramuscular injection of 100 mg ketamine, 10 mg droperidol and 1 mg atropine, and maintenance of anesthesia was achieved by an intravenous drip of propofol at a dose of 10 mL/h, with endotracheal intubation. The heart rate and oxygen saturation of each animal were monitored during the operation. Animals were maintained in a supine position to allow for optimal access and peritoneal exploration. This study was approved by the Institutional Animal Use and Care Committee of Fujian Provincial Tumor Hospital, Teaching Hospital of Fujian Medical University, Fuzhou, China.

NOTES tube ileostomy

Creation of the gastrostomy by directed submucosal tunneling: A 2-cm transversal mucosal incision was created near the gastroesophageal junction with a dual knife (KD650L; Olympus, Tokyo,

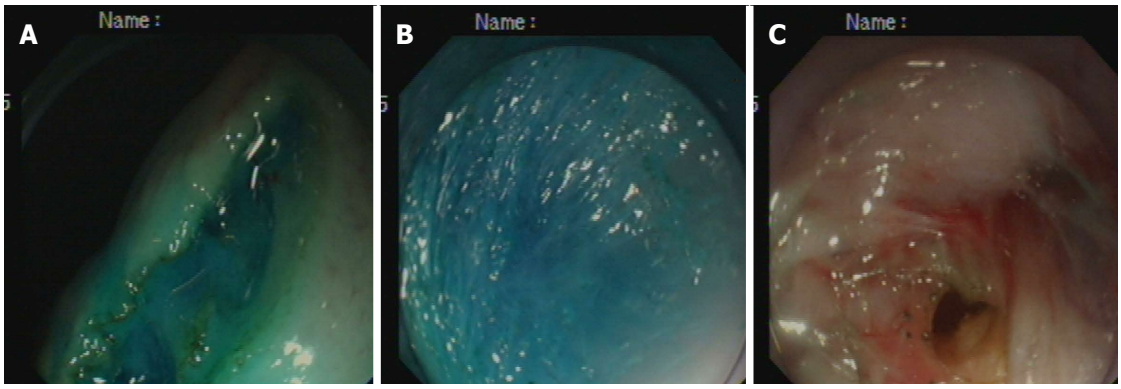

Figure 1 Step-by-step procedure of pelvis-directed submucosal tunneling endoscopic gastrostomy. A: Gastric mucosal incision; B: Submucosal tunneling; C: Seromuscular incision.

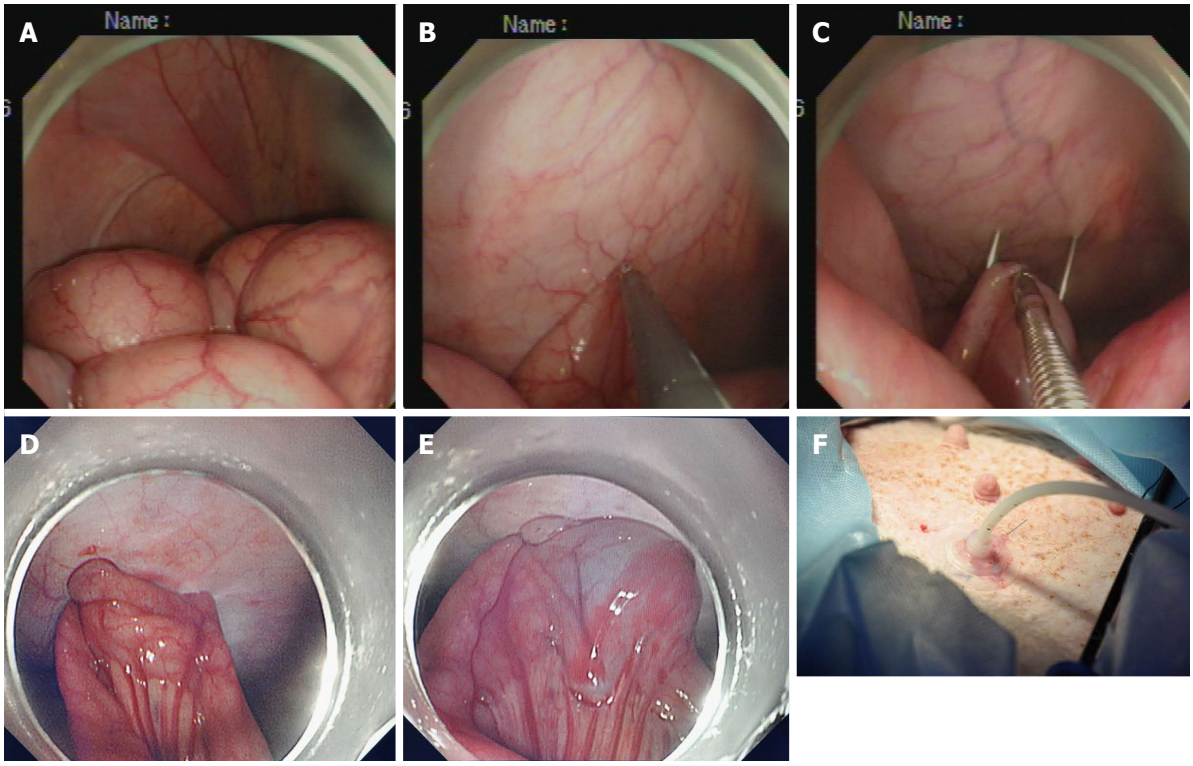

Figure 2 Step-by-step procedure of endoscopic tube ileostomy. A: Endoscopic identification of the distal ileum in the left lower quadrant; B: Endoscopic view of a loop of the distal ileum on its anti-mesenteric side grasped and held to the anterior abdominal wall; C: Percutaneous terminal ileum puncture; D: Endoscopic view of the distal ileum sutured to the abdominal wall with two stitches; E: Endoscopic view of the inflated balloon in the ileal lumen; F: Accomplished tube ileostomy.

Japan), followed by the creation of a 5-cm longitudinal submucosal pelvis-directed tunnel[9,10] (Figure 1A-C). The tunnel ended with a seromuscular incision, and the exit site was selected at the greater curvature of the stomach. A dual-channel therapeutic endoscope (GIF2TQ260M; Olympus) was advanced into the peritoneal cavity through the gastrostomy site. A pelvis-directed tunnel (left lower quadrant abdomen) was considered successful if the exit was in line with the urinary bladder, or the body or left horn of the uterus. The pneumoperitoneum was established *via* the endoscope. In the initial three operations in the non-survival group, transumbilical laparoscopic visualization was used to guide tunnel direction assessment, intraperitoneal spatial orientation, and subsequent terminal ileum identification.

Creation of the ileostomy using the introducer method: A 15 Fr rubber-coated silicone balloon catheter (Cliny PEG Kit; Create Medic, Yokohama, Japan) was used as the tube stoma (Figure 2A-F). The optimal ileostomy site was determined by depressing the left lower abdominal wall with a finger until the tip of the finger was visible intraperitoneally under endoscopic visualization. The pneumoperitoneum was decompressed to bring the distal ileum closer to the parietal peritoneum. After being grasped and held toward the needle, which had penetrated through the

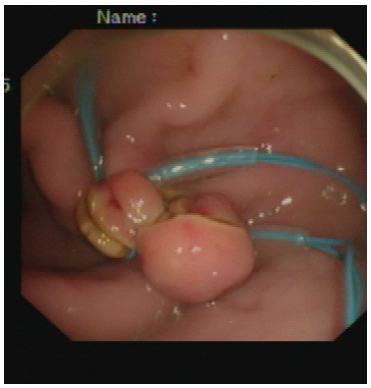

Figure 3 An endoscopic view of gastrostomy closure with endoloop ligation.

anterior abdominal wall, the antimesenteric side of the distal ileum was punctured using a double-lumen loop fixture device under laparoscopic or endoscopic guidance. Two stitches were placed about 2-3 cm apart. An abdominal wall incision was made between the two suture points, and a trocar with a plastic "T" peel-away sheath was introduced into the ileum through the incision site. After removal of the trocar, a 15 Fr catheter was introduced into the plastic sheath. The distal tip of the 15 Fr catheter was placed toward the proximal limb of the ileum to optimize intestinal content drainage. The balloon at the tip of the catheter was inflated with 5 mL sterile water, and the catheter was pulled until the appropriate approximation of the balloon to the abdominal wall was achieved. The peel-away sheath was then removed, and the retaining plate was positioned.

Closure of the gastrostomy: The pneumoperitoneum was evacuated endoscopically, and the dual-channel therapeutic endoscope was withdrawn into the stomach through the gastrostomy site (Figure 3). An endoloop, followed by the twin grasper, was inserted into the gastric cavity through one of the two channels and placed at the gastrostomy side. The gastric tissues adjacent to the mucosal defect were clamped with the twin grasper and dragged into the transparent cap previously mounted on the tip of the endoscope, followed by fully aspirated into the transparent cap, and the bilateral gastric mucosal layers were approximated by tightening the endoloop. If the defect closure was incomplete, more endoloops were used to close the remaining portions. Two or three endoloops were placed to secure the closure.

Postoperative care

At 48 h after the procedure, the animals were fed a liquid oral diet for 2 d, followed by a semi-fluid diet for 3 d. Irrigation was performed once daily to keep the tube patent. The animals were monitored for food intake, defecation, infection and other postoperative complications for 1 wk.

Euthanasia and necropsy

Euthanasia was performed immediately in the non-survival group and 1 wk after the procedure in the survival group. Necropsy results, including injury to adjacent organs, vascular bleeding, gross and histopathological evaluation of gastrostomy closure and stoma tract formation of ileostomy, were recorded. Both the healing and formation processes were classified into three phases: inflammatory, proliferative, and remodeling. The inflammatory phase (first) was characterized by accumulation of polymorphonuclear neutrophils and vasodilation; the proliferation phase (second) was characterized by accumulation of macrophages and fibroblasts, angiogenesis, and collagen deposition; and the remodeling phase (third) was characterized by rearrangement of collagen fibers. The microscopic factors of the aforementioned phases were assessed using a semi-quantitative scoring method of 0-3 points, where 0 = absence, 1 = a little, 2 = moderate, and 3 = abundant[11].

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. All animals were killed by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

RESULTS

Transgastric endoscopic tube ileostomy was successfully performed in all six pigs, without major bleeding. The mean operating time was 71 min (range: 60-110 min). There were no intraoperative complications or hemodynamic instability.

In the non-survival group, the post-mortem showed secure closure of the gastrostomy created by submucosal tunneling and immediate patency of the ileostomy. Neither gastrostomy nor ileostomy leakage were identified on methylene blue evaluation.

In the survival group, emission of both trans-ileostomy and trans-anal feces were observed. At necropsy, all three gastrostomy sites had completely healed (Figure 4A and B), the tubes were kept patent, and their corresponding ileostomy sites were intact, without evidence of serious infection or significant leakage, as determined by gross assessment. Formation of granulation tissue was observed around the central stoma tract (Figure 5A and B). On postoperative day 7, both the gastrostomy and ileostomy were classified as being in the proliferation phase (Table 1).

DISCUSSION

To the best of our knowledge, this is the first study addressing NOTES tube ileostomy in a porcine survival model. Safe peritoneal access and secure closure of the access site during NOTES remain significant concerns and must be addressed before

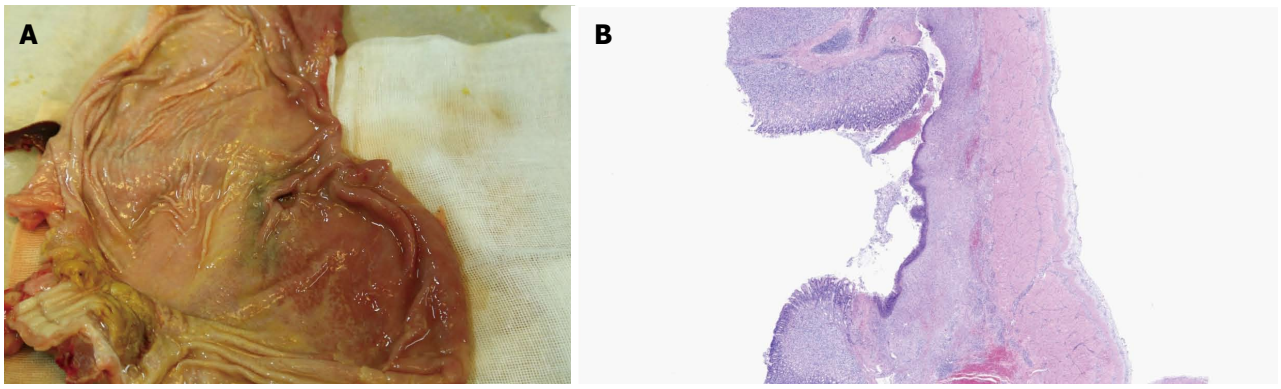

Figure 4 Gross and histopathological evaluation of gastrostomy closure 1 wk after natural orifice transgastric endoscopic surgery. A: Gross examination showing complete healing of gastrostomy; B: Histopathological examination showing complete healing of gastrostomy.

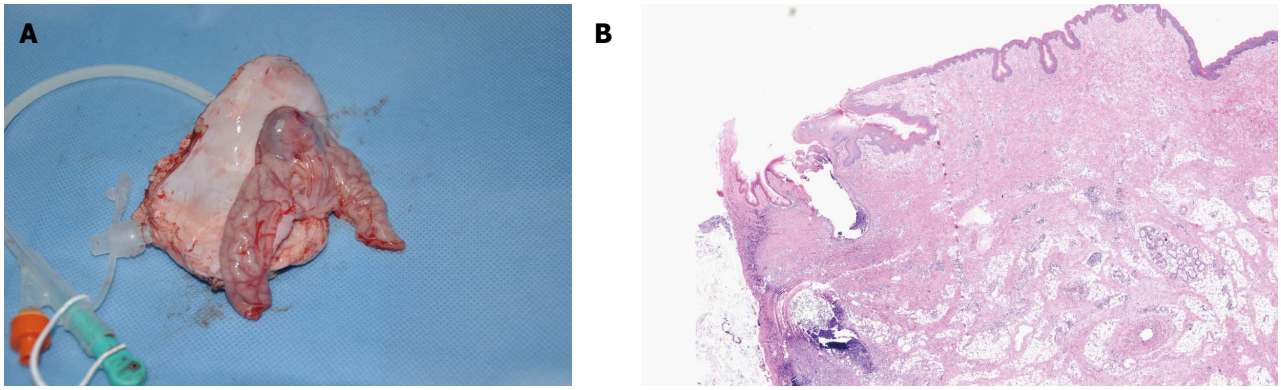

Figure 5 Gross and histopathological evaluation of stoma tract formation of the ileostomy 1 wk after natural orifice transgastric endoscopic surgery. A: Gross examination showing adequate stoma tract formation of ileostomy; B: Histopathological examination showing adequate stoma tract formation of ileostomy.

Table 1 Summary of procedures and outcomes following creation of natural orifice transgastric endoscopic surgery tube ileostomy in three surviving pigs

Observation parameters	Pig 1	Pig 2	Pig 3
Time required to create gastrostomy (min)	26	21	24
Time required to create tube ileostomy (min)	33	31	29
Time required to close gastrostomy (min)	5	11	7
Complications			
Major bleeding	-	-	-
Anastomotic leak	-	-	-
Necropsy on postoperative day 7			
Integrity of gastrostomy closure	The proliferation phase		
Stoma tract formation of the ileostomy	The proliferation phase		

its introduction into clinical practice. In our study, the pelvis-directed submucosal tunneling technique was applied to achieve transgastric peritoneal access. Under direct visualization, blood vessels can be clearly identified and precisely coagulated in the submucosal tunnel. Moreover, compared with a direct full-thickness incision, a seromuscular incision may minimize inadvertent injury because the adjacent organs can be visualized through the thin seromuscular layer of the stomach. Furthermore, a designed submucosal tunnel enables in-line endoscope positioning toward predetermined left lower abdominal target locations, without the need for endoscopic retroflexion[10].

As has been reported, the closure of the gastric mucosal incision site with conventional endoscopic clips was easy and secure[9,10]. In the current study, a novel technique of endoloop ligation[12,13], in combination with the dedicated twin grasper, was introduced to facilitate endoscopic closure of the tunnel entrance to the stomach. The Ovesco twin grasper has two jaws that can open and close separately to approximate defect edges[14,15]. Histopathological examination showed satisfactory healing of the submucosal tunnel, thus proving that gastric closure with endoloops is both simple and reliable.

With regards to using tube ileostomy as a diversion procedure, there has been a re-emergence of interest in the use of tube ileostomy to de-function a distal colorectal anastomosis. A systematic review and pooled analysis of seven studies comparing tube with loop ileostomy showed no significant difference in anastomotic leak rate[1]. Tube ileostomy is an alternate diversion procedure that has few complications and is easy to construct and manage compared to conventional loop ileostomy. This procedure can divert the bowel contents and avoid the need for a second operation and its related complications[16-22].

Clinically, there are two methods for placing the

tube. One is the transcecal approach, which after anastomosis, an appendectomy or cecostomy is performed, allowing the introduction of the tube into the cecal cavity and then into the terminal ileum *via* the ileocecal valve. The other method is to place the tube directly into the terminal ileum. In our study, the introducer method *via* the distal ileum approach was similar to gastropexy[23-29]. The only difference was that, in our study, the antimesenteric side of the distal ileum was sutured to the lower anterior abdominal wall, while gastropexy was achieved by suturing the anterior gastric wall to the anterior abdominal wall before PEG-catheter insertion. Gastropexy may prevent stomal leakage and subsequent peritonitis secondary to early inadvertent percutaneous gastrostomy dislodgement. Similarly, suturing the ileum to the abdominal wall may reduce the risk of peristomal cellulitis before stoma tract maturation. Adhesion of the ileum to the abdominal wall was achieved by placement of two stitches using a double-lumen loop fixture device around a target abdominal stoma site, which has not been previously reported.

The maturation of the stoma tract is critical to percutaneous gastrostomy, and this principle also applies to percutaneous tube ileostomy. Our study showed that the time required for stoma tract formation was about 1 wk after ileostomy, thus providing a safe timeframe for tube removal. Similar results were obtained in human studies, in which the exact timing of tube removal ranged from 7 d to > 3 wk postoperatively[1,30].

In conclusion, this novel technique for performing tube ileostomy exclusively by NOTES is technically feasible and reproducible in an animal model and is worthy of further improvement.

COMMENTS

Background

Established conventional defunctioning stomas, loop colostomy and loop ileostomy have become a mainstay of proximal fecal diversion to protect against primary colorectal anastomosis and to reduce the risk and lessen the severity of the sequelae of anastomotic leakage, such as fecal peritonitis and sepsis. Percutaneous tube ileostomy has been reported as an alternative that can achieve similar temporary fecal diversion, ensuring greater comfort for the patient and easier management, as well as avoiding the additional surgical procedure required for the closure of a conventional loop-ostomy.

Research frontiers

Percutaneous tube ileostomy for anastomotic protection is clinically performed by laparotomy or laparoscopy. To date, there is no literature regarding the natural orifice transgastric endoscopic surgery (NOTES) tube ileostomy.

Innovations and breakthroughs

A novel technique of percutaneous transgastric endoscopic tube ileostomy may be successfully performed in a porcine survival model, using pelvis-directed submucosal tunneling endoscopic gastrostomy and endoscopic tube ileostomy using an Introducer Kit containing a loop fixture.

Applications

This study demonstrates the potential application of percutaneous transgastric endoscopic tube ileostomy using an Introducer Kit for temporary fecal diversion.

Terminology

A novel NOTES tube ileostomy is performed using pelvis-directed submucosal tunneling endoscopic gastrostomy, following by endoscopic tube ileostomy using the introducer method.

Peer-review

This is an interesting study about the percutaneous transgastric endoscopic tube ileostomy using an Introducer Kit in a porcine survival model. The study is well designed and the results are good discussed.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy and safety of emergency endoscopic retrograde cholangiopancreatography for acute cholangitis in the elderly

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Author contributions: Tohda G wrote the manuscript; Dochin M collected the data; and Ohtani M reviewed the manuscript.

Institutional review board statement: This study was conducted in accordance with the principles of the Declaration of Helsinki, and was reviewed ethically and approved by the Fukui Kosei Hospital Institutional Review Board.

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Abstract

AIM

To investigate the efficacy and safety of emergency endoscopic retrograde cholangiopancreatography (ERCP) in elderly patients with acute cholangitis.

METHODS

From June 2008 to May 2016, emergency ERCPs were performed in 207 cases of acute cholangitis at our institution. Patients were classified as elderly if they were aged 80 years and older (n = 102); controls were under the age of 80 years (n = 105). The patients' medical records were retrospectively reviewed for comorbidities, laboratory data, etiology of cholangitis (presence of biliary stones, biliary stricture and malignancy), details of the ERCP (therapeutic approaches, technical success rates, procedure duration), ERCP-related complications and mortality.

RESULTS

The frequency of comorbidities was higher in the elderly group than the control group (91.2% *vs* 67.6%). Periampullary diverticulum was observed in the elderly group at a higher frequency than the control group (24.5% *vs* 13.3%). Between the groups, there was no significant difference in the technical success rates (95.1% *vs* 95.2%) or endoscopic

procedure durations. With regard to the frequency of ERCP-related complications, there was no significant difference between the two groups (6.9% *vs* 6.7%), except for a lower rate of post-ERCP pancreatitis in the elderly group than in the control group (1.0% *vs* 3.8%). Neither angiographic nor surgical intervention was required in any of the cases with ERCP-related complications. There was no mortality during the observational periods.

CONCLUSION
Emergency ERCP for acute cholangitis can be performed safely even in elderly patients aged 80 years and older.

Key words: Acute cholangitis; Endoscopic retrograde cholangiopancreatography; Complication; Comorbidity; Elderly

Core tip: We retrospectively evaluated the efficacy and safety of emergency endoscopic retrograde cholangiopancreatography (ERCP) in elderly patients with acute cholangitis. Patients who have undergone emergency ERCPs were classified as elderly group aged 80 years and older (n = 102) or controls under the age of 80 years (n = 105). The frequency of comorbidities was higher in the elderly group than the control group. However, there was no significant difference in the technical success rates, endoscopic procedure durations and ERCP-related complications between the two groups. There was no mortality during the observational periods. Emergency ERCP for acute cholangitis can be performed safely even in elderly patients.

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INTRODUCTION

With the increase in life expectancy, pancreatic and biliary diseases have become common problems in the elderly. Endoscopic retrograde cholangiopancreatography (ERCP) has been established as an alternative treatment to surgery for patients with high operative risk[1]. However, ERCP also has higher rates of adverse events compared with the other types of gastrointestinal endoscopic procedures[2]. Complications of ERCP include pancreatitis, hemorrhage, perforation, cholangitis and cardiorespiratory problems[3]. Although previous studies have shown that ERCP can be safe and well tolerated even in the elderly[4-24], the management of elderly patients with acute cholangitis presents certain risks for endoscopists. These risks are attributable not only to acute cholangitis itself but also to the risks associated with the overall health of the patient and their concomitant medical disorders. The general state of a patient's health plays an important role in determining the outcome of any invasive treatments. However, in cases of acute cholangitis, interventional ERCP is often required as an emergency procedure, so it is difficult to evaluate the patient's overall clinical condition before the procedure. Because of various risks, there is a tendency for patients, family members and even physicians to adopt a conservative approach and avoid therapeutic ERCP for these cases. Because few studies have focused on the outcomes of emergency ERCP in elderly patients with acute cholangitis[22], we retrospectively evaluated the safety and efficacy of emergency ERCP for patients aged 80 years and older who presented with acute cholangitis.

MATERIALS AND METHODS

Study design

This was a retrospective review of patients with acute cholangitis at a single hospital. From June 2008 to May 2016, 207 cases of emergency ERCP were performed for acute cholangitis at our institution. Of these, 102 patients who were 80 years of age or above were classified into the elderly group, while 105 patients who were below the age of 80 years were classified as the control group. After obtaining ethical approval from the Institutional Review Board at our hospital, we conducted a retrospective review of medical records of patients who underwent emergency ERCP for acute cholangitis. Demographic characteristics, medical history (specifically comorbidities), clinical features, laboratory data, ERCP findings (*i.e.*, periampullary diverticulum, presence of biliary stones, benign biliary stricture and malignant obstruction), details of ERCP procedures (*i.e.*, therapeutic approaches, technical success rates, procedure duration), ERCP-related complications and mortality were evaluated. All of the patients involved in this study underwent emergency ERCP within 24 h of admission.

Definition

Acute cholangitis was diagnosed as clinical symptoms characterized by a fever, jaundice, and abdominal pain that thought to be a result of cholestasis and bacterial infection in the biliary tract. Laboratory data indicative of the presence of inflammation (*e.g.*, leukocytosis), biliary obstruction (*e.g.*, hyperbilirubinemia, elevation of biliary and liver enzymes), and imaging findings supporting the evidence of inflammation and biliary obstruction were also used for a more accurate diagnosis of acute cholangitis. Severity of acute cholangitis can range from mild to serious life-threatening levels. We classified acute cholangitis

Table 1 Clinical characteristics and laboratory data of enrolled patients

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
Age (yr)	85 (81-92)	67 (58-75)	< 0.05
Sex			
Male	41 (40.2)	62 (59.0)	< 0.05
Female	61 (59.8)	43 (41.0)	
Fever (centigrade)	39.0 (38.5-39.3)	38.9 (38.7-39.4)	NS
WBC (10^3/L)	12.8 (10.4-15.2)	12.5 (10.7-14.6)	NS
Platelet (10^3/L)	132 (105-180)	171 (130-231)	< 0.05
Albumin (g/dL)	2.9 (2.6-3.3)	3.6 (3.4-4.0)	< 0.05
Bilirubin (mg/dL)	4.3 (3.1-6.0)	4.2 (2.8-6.7)	NS
AST (U/L)	229 (150-418)	182 (141-256)	< 0.05
ALT (U/L)	195 (128-277)	197 (144-303)	NS
ALP (U/L)	798 (576-1154)	811 (510-1175)	NS
γ-GTP (U/L)	336 (256-451)	350 (249-548)	NS
ASA			
Class 2	34 (33.3)	59 (56.2)	< 0.05
Class 3	67 (65.7)	45 (42.9)	
Class 4	1 (1.0)	1 (1.0)	

Continuous variables are expressed as median (interquartile range; IQR), categorical variables are expressed as *n* (%). WBC: White blood cells; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; ASA: American Society of Anesthesiology; NS: Not significant.

into three grades; mild (grade Ⅰ), moderate (grade Ⅱ), and severe (grade Ⅲ), in accordance with the Tokyo Guidelines[25], which have since been widely used all over the world as the diagnostic criteria and a severity assessment of acute cholangitis. We performed emergency endoscopic biliary drainage in cases of severe acute cholangitis with at least one of the following conditions: either (1) shock; (2) mental confusion; or (3) a coagulation defect that was not attributable to the use of anticoagulants.

The presence of comorbidity was defined as the presence of one or more of the following conditions; hypertension, ischemic heart disease, chronic heart failure, arrhythmia, cerebrovascular disease, diabetes mellitus, chronic liver disease, chronic pulmonary disease, chronic renal failure, or malignancy. ERCP-related complications were defined according to the previously published criteria[3]. Bleeding after endoscopic sphincterotomy (EST) was classified into two types: (1) minor bleeding, defined as controllable by endoscopic hemostasis and no need for blood transfusion; or (2) major bleeding, defined as bleeding requiring a blood transfusion and/or angiographic or surgical intervention to control the hemorrhage. Post-ERCP pancreatitis was defined as abdominal pain with a concurrent rise in the serum amylase level after the endoscopic procedure. The primary aim of this study was to evaluate the safety and efficacy of emergency ERCP in patients with acute cholangitis. Therefore, we reviewed the data on these patients over a period of 30 d post-admission. Technical success of interventional ERCP was defined as the achievement of endoscopic biliary drainage with or without stone removal.

Endoscopic procedure

Before performing ERCP, informed consent was obtained from each patient and/or caregiver. All endoscopic procedures were performed by experienced endoscopists who had performed more than 500 cases of therapeutic ERCP with more than 15 years of experience. Moderate sedation was administered by gastroenterologists by giving intravenous injections of midazolam and pethidine hydrochloride. All of the patients underwent continuous monitoring by electrocardiogram and pulse oximetry and received 2 L/min of oxygen through a nasal cannula throughout the endoscopic procedure. During the procedure, the following events were considered cardiorespiratory suppression associated with sedation: (1) a decline in SpO_2 to less than 90%; (2) heart rate less than 45 beats per min; or (3) systolic blood pressure below 80 mmHg. In case of EST, an electrosurgical generator with an automatic controlled cutout system (Endocut mode) was used. Plastic type biliary stents (7 Fr diameter) were routinely used for biliary drainage during the initial ERCP. To remove residual biliary stones, follow up ERCP was performed depending on each patient's specific needs and medical condition.

Statistical analysis

We have confirmed that the sample size of each group in this study is sufficient in size to make a definite conclusion using power calculations. Various parameters were compared between the elderly and control groups. Continuous variables with normal distributions were compared by the two-sample *t*-test. The Mann-Whitney *U* test was used for the comparison of continuous variables with skewed distributions. The χ^2 test or Fisher's exact test was used for categorical variables as appropriate. *P* values of 0.05 or less were considered statistically significant. All statistical analyses were performed using the EZR[26] (Saitama Medical Center, Jichi Medical University, Saitama, Japan, version 1.32), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander that was designed to add statistical functions frequently used in biostatistics.

RESULTS

The clinical data of the patients involved in this study are presented in Table 1. Between the elderly and control groups, patients in the elderly group showed a significantly lower level of serum albumin and platelet, a higher level of aspartate transaminase (AST), and a higher stage of American Society of Anesthesiology (ASA) classification. With regard to the presence of comorbidities (Table 2), the elderly group had a significantly higher prevalence of hypertension, ischemic heart disease, cerebrovascular disease, dementia, chronic obstructive pulmonary disease, and malignancy other than a primary biliary and

Table 2 Comorbidities of patients with acute cholangitis *n* (%)

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
Comorbidity	93 (91.2)	71 (67.6)	< 0.05
Hypertension	80 (78.4)	53 (50.5)	< 0.05
Ischemic heart disease	40 (39.2)	18 (17.1)	< 0.05
Chronic heart failure	8 (7.8)	6 (5.7)	NS
Arrhythmia	17 (16.7)	14 (13.3)	NS
Cerebrovascular disease	30 (29.4)	10 (9.5)	< 0.05
Dementia	23 (22.5)	4 (3.8)	< 0.05
Diabetes mellitus	21 (20.6)	19 (18.1)	NS
Chronic liver disease	9 (8.8)	8 (7.6)	NS
Liver cirrhosis	3 (2.9)	3 (2.9)	NS
COPD/Asthma	15 (14.7)	7 (6.7)	< 0.05
Chronic renal failure	5 (4.9)	4 (3.8)	NS
Malignancy[1]	8 (7.8)	3 (2.9)	< 0.05
Periampullary diverticulum	25 (24.5)	14 (13.3)	< 0.05

[1]Malignancy other than a primary biliary and pancreatic lesion. COPD: Chronic obstructive pulmonary disease; NS: Not significant.

Table 3 Etiology of acute cholangitis *n* (%)

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
CBD stone	65 (63.7)	72 (68.6)	NS
Gallstone cholecystitis	2 (2.0)	4 (3.8)	NS
Pancreatitis	1 (1.0)	2 (1.9)	NS
Malignant obstruction	31 (30.4)	23 (21.9)	< 0.05
Pancreatic cancer	4 (3.9)	5 (4.8)	NS
Biliary cancer	21 (20.6)	11 (10.5)	< 0.05
GB cancer	3 (2.9)	4 (3.8)	NS
Other cause[1]	3 (2.9)	3 (2.9)	NS
Benign stricture	3 (2.9)	4 (3.8)	NS

[1]Biliary obstruction caused by metastatic lymph node(s). CBD: Common bile duct; GB: Gall bladder; NS: Not significant.

pancreatic lesion. Periampullary diverticulum was also highly observed in the elderly group. The etiology of each case of acute cholangitis is shown in Table 3. In both groups, common bile stones were the most frequent etiology of acute cholangitis. The frequency of malignant obstruction was significantly higher in the elderly group than the control group.

Procedural details of the therapeutic ERCPs are described in Table 4. Endoscopic biliary drainage by insertion of a biliary stent was successful in 95.1% of elderly patients and 95.2% of control patients. Between the groups, there was no significant difference in the endoscopic procedure durations. Second ERCPs were performed approximately 1 wk after the initial ERCP in 87.3% (89 out of 102 cases) of elderly patients and 97.1% (102 out of 105 cases) of control patients. EST was carried out in 67.6% of the elderly group and 82.9% of the control group. For cases with a prior intake of Warfarin, vitamin K was given to the patients before EST. Complete clearance of biliary stones was achieved in 81.5% of the elderly group and in 93.1% of the control group.

The frequency of ERCP-related complications was

Table 4 Procedural details of endoscopic retrograde cholangiopancreatography *n* (%)

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
Details of the procedure			
Biliary stent insertion	97 (95.1)	100 (95.2)	NS
Endoscopic sphincterotomy	69 (67.6)	87 (82.9)	< 0.05
Complete stone removal	53/65[1] (81.5)	67/72[2] (93.1)	< 0.05
Technical success[3]	97 (95.1)	100 (95.2)	NS
Procedures time (min), mean ± SD	37.6 ± 8.1	40.1 ± 7.7	NS
Failed cannulation	5 (4.9)	5 (4.8)	NS

Number of patients with biliary stones in the elderly[1] and in the control[2] groups, [3]technical success is defined as an endoscopic biliary drainage at an initial ERCP. ERCP: Endoscopic retrograde cholangiopancreatography; NS: Not significant.

Table 5 Endoscopic retrograde cholangiopancreatography-related complications *n* (%)

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
Total	7 (6.9)	7 (6.7)	NS
Bleeding after EST	3 (2.9)	2 (1.9)	NS
Post-ERCP pancreatitis	1 (1.0)	4 (3.8)	< 0.05
Aggravation of cholangitis	2 (2.0)	1 (1.0)	NS
Aspiration pneumonia	1 (1.0)	1 (1.0)	NS
Mortality	0	0	NA

ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; NS: Not significant; NA: Not available.

6.9% in the elderly group, while it was 6.7% in the control group (Table 5). There was no significant difference between the two groups, except for a lower rate of post-ERCP pancreatitis in the elderly group. All cases of bleeding from the EST site were mild, and neither blood transfusion nor angiographic/surgical intervention was required in any of the reviewed cases. In this study, all of the ERCP-related complications were mild and none of the patients required surgical intervention. During the procedures, a few cases of cardiorespiratory suppression related to sedation were observed (Table 6). All patients with hypoxemia responded immediately to an increase in concentration of administered oxygen (5 L/min). Hypotension and bradycardia were also corrected immediately using intravenous saline solution or atropine injections. Finally, no patient required mask ventilation, endotracheal intubation, or any resuscitative treatment. The frequency of cardiorespiratory suppression during the procedures was similar between the elderly and control groups.

Transient aggravation of cholangitis was observed in 3 cases of patients who underwent endoscopic biliary drainage. However, these patients gradually recovered after the endoscopic procedure. Ten patients with unsuccessful endoscopic biliary drainage received

Table 6 Cardiorespiratory suppression during endoscopic retrograde cholangiopancreatography under moderate sedation *n* (%)

	Age ⩾ 80 yr (*n* = 102)	Age < 80 yr (*n* = 105)	*P* value
Total	9 (8.8)	8 (7.6)	NS
Hypoxemia	2 (2.0)	2 (1.9)	NS
Bradycardia	4 (3.9)	4 (3.8)	NS
Hypotension	5 (4.9)	4 (3.8)	NS

Hypoxemia: Peripheral oxygen saturation (SpO2) below 90%; Bradycardia: Heart rate less than 45 beats per minutes; Hypotension: Systolic blood pressure below 80 mmHg; ERCP: Endoscopic retrograde cholangiopancreatography; NS: Not significant.

percutaneous transhepatic biliary drainage (PTCD) as an alternative treatment. Finally, all cases with septic condition by severe acute cholangitis achieved source control *via* endoscopic biliary drainage (*n* = 197) or PTCD (*n* = 10), and procedure-related mortality was not observed in either group. The median duration of hospitalization periods was significantly longer in the elderly group than in the control group (20 d *vs* 15 d) because many elderly patients required rehabilitation periods for improvement of their overall health and other conditions.

DISCUSSION

With the progressive increase in the elderly population, the frequency of pancreatic and biliary diseases has increased. Among this population, bile duct stones often cause clinical problems, such as acute cholangitis. Because of the increasing degree of morbidity that accompanies the aging process, urgent treatments are required for elderly patients with severe infectious conditions. Elderly patients with acute cholangitis are sometimes critically ill, so emergency interventions are necessary. However, physicians and patients' family members tend to be reluctant to consent to surgical treatments because elderly patients can be at a higher risk of developing complications from surgical procedures. ERCP is an established procedure for pancreatic and biliary diseases. With a rise in life expectancy, the demand for ERCP in the elderly has been increasing. It has been reported that the rates of ERCP-related complications or mortality in the elderly are comparable with those of younger patients[4-24], but there are few reports of emergency ERCP for elderly patients with acute cholangitis[22]. The primary aim of this study was to evaluate the outcomes of emergency ERCP in the elderly with acute cholangitis.

In this study, we performed aggressive endoscopic approaches for elderly patients with acute cholangitis. They received urgent biliary drainage by an endoscopic biliary stent insertion, and the removal of bile duct stones with EST was considered in accordance with the patients' general conditions. Biliary drainage with complete stone removal at the time of the first procedure is desirable for patients. However, such an interventional approach is not feasible in all cases, often due to severe biliary infection and/or coagulopathy. In this study, biliary drainage by stent insertion with or without EST was performed as an initial treatment, and repeat ERCPs for the extraction of residual biliary stones were considered as indicated when the patients' clinical conditions were improved. As a result, the ERCP-related complication rate in our study was comparable with that of previous studies, even in elderly patients with serious condition by acute cholangitis.

Several studies have shown that there is no relationship between coexisting medical conditions and ERCP-related complications[27], except for cases with underlying liver cirrhosis[28]. Other studies have reported that an elderly age with concomitant medical illness is associated with a higher mortality in cases of acute cholangitis[29]. In this study, complication rates of ERCP in the elderly group was similar to those of previous reports[4-24], and there was no relationship between comorbidities and ERCP-related complications. It has also been reported that the risk of sedation-related adverse events during ERCP increases in the elderly[6,7]. However, our study showed that there was no difference in the frequency of cardiorespiratory suppression between the elderly and control groups because we use fewer sedative agents with lower initial and cumulative doses in the elderly groups.

In our institution, all cases of emergency ERCP were carried out by two endoscopists who had previously performed more than 500 cases of therapeutic ERCPs. During the study period, which spanned 8 years, endoscopists used different types of ERCP devices. However, we believe that a bias caused by endoscopic accessories, including a catheter and guidewire, would be negligibly small. Before reviewing the data retrospectively, we estimated that technical success rate of ERCP in the elderly population would be less than that in younger patients due to anatomical factors, such as the presence of periampullary diverticulum and/or difficult bile duct stones with impaction. However, there was no statistically differences in the technical success rate of ERCP between the elderly and control groups.

In conclusion, emergency ERCP for acute cholangitis is a safe and effective procedure in elderly patients over 80 years of age. Advanced age is not a contraindication to ERCP, even in cases with acute cholangitis. To perform urgent ERCP safely in these cases, informed consent, adequate monitoring during the procedure, prompt detection and management of ERCP-related complications are crucial. We believe that our study can be informative in deciding whether ERCP is the best treatment in elderly patients with acute cholangitis. These results can be utilized in discussions with patients and their families through the informed consent process. The primary aim of this study was to evaluate the outcomes of emergency ERCP for elderly

patients with acute cholangitis. Therefore, all patient data were reviewed for short observational periods of 30 d. Due to this limitation, the low complication rates observed in this study may not hold true because patients could have had more late complications that were not captured in our study. Prospective studies with long-term follow-up periods will be required to confirm the efficacy and safety of emergency ERCP in elderly patients with acute cholangitis.

COMMENTS

Background

Previous studies have shown that endoscopic retrograde cholangiopancreatography (ERCP) can be safe and well tolerated, even in the elderly. However, the management of elderly patients with acute cholangitis presents certain risks, which are attributable not only to acute cholangitis itself but also to the risks associated with patients' overall health conditions. Therefore, there is a limited data on the outcome of emergency ERCP in elderly patients with acute cholangitis.

Research frontiers

Prior reports described interventional ERCPs as a useful tool for the treatment of biliary diseases. However, only few prior reports evaluated the emergency ERCP for acute cholangitis in the elderly because of high-risks. This study contributes to clarify the efficacy and safety of emergency ERCP for severe acute cholangitis especially in the elderly.

Innovations and breakthroughs

Severe acute cholangitis is a potentially life-threatening condition, and biliary drainage should be considered as soon as possible to improve the general condition of patients. In this study, emergency ERCP for acute cholangitis could be performed safely even in the elderly, and all cases receiving interventional ERCPs were improved. The results presented emphasize the efficacy and safety of emergency ERCP for severe acute cholangitis even in the elderly patients.

Applications

This study suggests that emergency interventional ERCP could be a less-invasive and effective treatment for acute cholangitis even in elderly patients in spite of their high frequency of concomitant medical disorders.

Terminology

Interventional ERCP: endoscopic treatments such as a biliary drainage by stent insertion, endoscopic sphincterotomy with or without stone removal.

Peer-review

The author of this paper evaluated that emergency ERCP could be performed safely for severe acute cholangitis even in the elderly. Further clinical trials in a large population will be valuable.

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ORIGINAL ARTICLE

Retrospective Study

Programmed death ligand-1 expression and its prognostic role in esophageal squamous cell carcinoma

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Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at bhumsuk@snu.ac.kr. Gaining informed consent for publication of the dataset from participants is not possible because of the retrospective design of this study. However, publication of this data does not compromise anonymity or confidentiality or breach local data protection laws, for the dataset to be considered for publication.

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Abstract

AIM

To investigate the expression and prognostic role of programmed death ligand-1 (PD-L1) in locally advanced esophageal squamous cell carcinoma (ESCC).

METHODS

A total of 200 patients with ESCC who underwent radical esophagectomy with standard lymphadenectomy as the initial definitive treatment in Seoul National University Hospital from December 2000 to April 2013 were eligible for this analysis. Tissue microarrays were constructed by collecting tissue cores from surgical specimens, and immunostained with antibodies directed against PD-L1, p16, and c-Met. Medical records were reviewed retrospectively to assess clinical outcomes. Patients were divided into two groups by PD-L1 status, and significant differences in clinicopathologic characteristics between the two groups were assessed.

RESULTS

Tumor tissues from 67 ESCC patients (33.5%) were PD-L1-positive. Positive p16 expression was observed in 21 specimens (10.5%). The H-score for c-Met expression was $\geqslant$ 50 in 42 specimens (21.0%). Although PD-L1-positivity was not significantly correlated with any clinical characteristics including age, sex, smoking/alcoholic history, stage, or differentiation, H-scores for c-Met expression were significantly associated with PD-L1-positivity (OR = 2.34, 95%CI: 1.16-4.72, P = 0.017). PD-L1 expression was not significantly associated with a change in overall survival (P = 0.656). In contrast, the locoregional relapse rate tended to increase (P = 0.134), and the distant metastasis rate was significantly increased (HR = 1.72, 95%CI: 1.01-2.79, P = 0.028) in patients with PD-L1-positive ESCC compared to those with PD-L1-negative ESCC.

CONCLUSION

PD-L1 expression is positively correlated with c-Met expression in ESCC. PD-L1 may play a critical role in distant failure and progression of ESCC.

Key words: Esophageal neoplasm; Programmed death ligand-1 protein; c-Met protein; Prognosis; p16INK4A protein

Core tip: The clinical significance of expression of programmed death ligand-1 (PD-L1) in esophageal squamous cell carcinoma (ESCC) has not yet been fully established. We analyzed tissue microarrays of surgical specimen of 200 ESCC patients by immunohistochemistry with antibodies directed against PD-L1, p16, and c-Met. Our results suggest that tumors from approximately one-third of the ESCC patients are positive for PD-L1 expression, and PD-L1 expression is positively correlated with c-Met expression. Although PD-L1 positivity was not found to be associated with survival of ESCC patients, we show that it may play a critical role in distant failure and progression of ESCC.

Kim R, Keam B, Kwon D, Ock CY, Kim M, Kim TM, Kim HJ, Jeon YK, Park IK, Kang CH, Kim DW, Kim YT, Heo DS. Programmed death ligand-1 expression and its prognostic role in esophageal squamous cell carcinoma. *World J Gastroenterol* 2016; 22(37): 8389-8397 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i37/8389.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i37.8389

INTRODUCTION

Esophageal cancer is one of the most common gastrointestinal malignancies and is highly prevalent in Asia. Esophageal squamous cell carcinoma (ESCC) represents the most common histological type of esophageal cancer, accounting for more than 90% of all cases in Asian patients[1]. Although the 5-year survival rate for patients with ESCC has improved in recent decades, the prognosis remains unfavorable due to the invasive nature of the disease and the frequency of late diagnosis[2].

An improved understanding of immunobiology has uncovered the interaction between programmed death ligand-1 (PD-L1) and programmed death 1 (PD-1) as one of mechanisms by which cancer cells evade immune surveillance. PD-1 is a negative co-stimulatory factor that inhibits T cell activation when activated by PD-L1 or one of its other ligands[3,4], PD-L1 is a cell surface glycoprotein that belongs to the B7 family and is expressed not only on normal cells, such as T cells, B cells, monocytes, macrophages, and dendritic cells, but also on cancer cells[4-7]. The PD-L1/PD-1 interaction has been found to be associated with poor prognosis and clinical outcomes in various cancers such as non-small cell lung cancer, breast cancer, gastric cancer, soft tissue sarcomas and meningioma[8]; however, its prognostic value is still controversial. Immune checkpoint-blocking agents directed at this interaction have been clinically successful and have been shown to produce a durable clinical response in esophageal cancer patients[9].

Considering the clinical importance of PD-L1, there is great interest in understanding the mechanisms that regulate its expression. PD-L1 upregulation has been reported in human papilloma virus (HPV)-associated malignancies, including uterine, cervical, and head and neck cancers[10-12]. PD-L1 expression may be associated with HPV infection, which represents one of the potential causes of ESCC[13]. Furthermore, c-Met, a receptor tyrosine kinase that is aberrantly activated

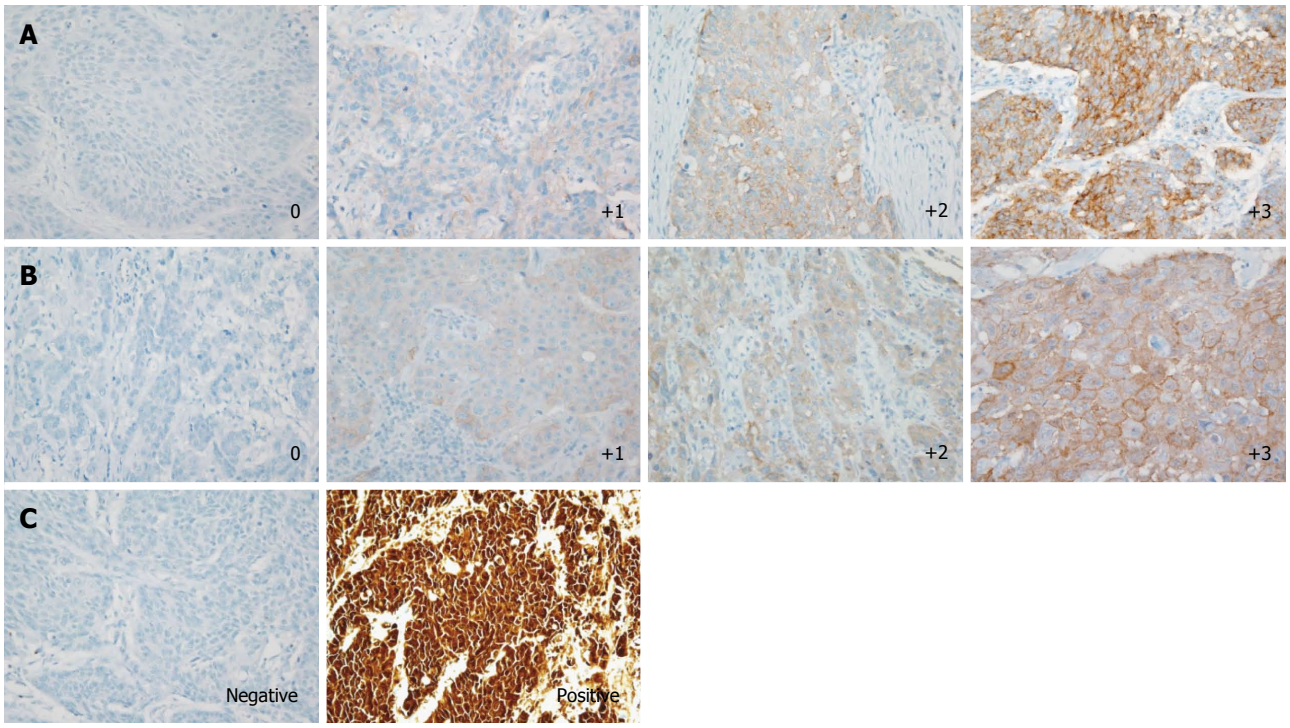

Figure 1 Representative immunohistochemical images of programmed death ligand-1, c-Met, and p16 expression in esophageal squamous cell carcinoma tissues. A: PD-L1 expression was scored from 0 to 3+. Cases with scores of 0 were considered PD-L1-negative; B: c-Met intensity was scored from 0 to 3+. The H-score was calculated for each case. Cases with H-scores ⩾ 50 were considered positive for c-Met expression; C: p16 expression was scored as negative or positive (Original magnification, × 400). PD-L1: Programmed death ligand-1.

in numerous human cancers[14], has been shown to promote PD-L1 overexpression in renal cell and pulmonary squamous cell carcinoma[15,16]. However, a comprehensive analysis of the correlation between PD-L1 and c-Met expression in ESCC has not yet been reported.

In this study, we aimed to investigate the expression of PD-L1 in ESCC and explore the correlation between PD-L1 expression and c-Met, as well as p16, a surrogate marker for HPV infection[17]. Additionally, we evaluated the potential for a prognostic role for PD-L1, p16, and c-Met expression in ESCC.

MATERIALS AND METHODS

Patients and samples

Patients with ESCC who underwent radical esophagectomy with standard lymphadenectomy (two-field or three-field lymphadenectomy) as the initial definitive treatment in Seoul National University Hospital from December 2000 to April 2013 were eligible for this retrospective analysis. All tumor tissues were confirmed to be ESCC through hematoxylin and eosin staining after surgical resection. Tissue microarrays (TMAs; 2 mm in diameter) were constructed by collecting tissue cores from representative intratumoral areas from surgical specimens. The pathologic tumor-node-metastasis (TNM) stage was characterized according to the 7th American Joint Committee on Cancer Guidelines[18].

Treatment strategies

Treatment decisions were made via a multidisciplinary team-based approach. All patients without clinical evidence of metastatic disease were treated with radial esophagectomy. The type of esophageal resection was dictated by the size, stage, and location of the primary tumor. According to the clinician's judgment, selected patients were offered neoadjuvant or adjuvant chemotherapy with or without radiation therapy. The chemotherapy regimen was fluoropyrimidine-/taxene-based and was selected based on the performance statuses, comorbidities, and toxicity profiles of individual patients.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using the Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, United States) to estimate the expression of PD-L1, p16, and c-Met. A rabbit anti-PD-L1 (E1L3N) XP® monoclonal antibody (mAb) (Cell Signaling Technology, Danvers, MA, United States), a mouse anti-p16 (E6H4) mAb (Ventana), and a rabbit anti-c-Met (SP44) mAb (Ventana) were used for staining. PD-L1 IHC was evaluated based on the intensity and proportion of membranous staining and/or cytoplasmic staining in tumor cells and was scored as 0 (no or any staining less than 10% of cells), 1+ (weak), 2+ (moderate), or 3+ (strong staining in more than 10% of tumor cells) (Figure 1A). Cases

Table 1 Clinicopathologic characteristics of esophageal squamous cell carcinoma patients *n* (%)

Characteristics	Total (*n* = 200)	PD-L1 status		*P* value
		Negative (*n* = 133)	Positive (*n* = 67)	
Age, median years (range)	65 (41-83)	65 (50-83)	64 (41-82)	0.519[1]
Sex				
Male	188 (94.0)	125 (94.0)	63 (94.0)	
Female	12 (6.0)	8 (6.0)	4 (6.0)	1.000
Smoking history	168 (84.9)	110 (84.0)	58 (86.6)	0.630
Alcoholic intake	166 (84.3)	110 (84.0)	56 (84.9)	0.873
Stage				
Ⅰ	66 (33.0)	47 (35.3)	19 (28.4)	
Ⅱ	59 (29.5)	41 (30.8)	18 (26.9)	
Ⅲ	71 (35.5)	44 (33.1)	27 (40.3)	
Ⅳ	4 (2.0)	1 (0.8)	3 (4.5)	0.200
Differentiation				
W/D	41 (23.0)	36 (27.1)	10 (14.9)	
M/D	131 (65.5)	83 (62.4)	48 (71.6)	
P/D	23 (11.5)	14 (10.5)	9 (13.4)	0.152
Treatment				
Surgery alone	122 (61.0)	83 (62.5)	39 (58.2)	
Surgery → Adj.	58 (29.0)	38 (28.6)	20 (29.9)	
Neoadj. → Surgery	14 (7.0)	9 (6.8)	5 (7.5)	
Neoadj. → Surgery → Adj.	6 (3.0)	3 (2.3)	3 (4.5)	0.927
Surgery results				
R0 resection	176 (88.0)	121 (91.0)	55 (82.1)	
R1, R2 resection	24 (12.0)	12 (9.0)	12 (17.9)	0.068
p16				
Negative	179 (89.5)	121 (91.0)	58 (86.6)	
Positive	21 (10.5)	12 (9.0)	9 (13.4)	0.616
H-score				
< 50	158 (79.0)	112 (84.2)	46 (68.7)	
⩾ 50, < 100	31 (15.5)	16 (12.0)	15 (22.4)	
⩾ 100, < 200	11 (5.5)	5 (3.8)	6 (9.0)	0.036
Follow-up duration, median months (range)	33.2 (0.6-178.7)	33.9 (0.6-176.7)	31.7 (2.3-178.7)	0.790[1]

[1]Estimated by Mann-Whitney test. N: Number; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated; Adj.: Adjuvant chemotherapy; Neoadj.: Neoadjuvant chemotherapy.

with scores of 1+, 2+, or 3+ were considered to be PD-L1-positive. c-Met expression was analyzed by the membranous and/or cytoplasmic staining pattern and the positivity was evaluated by H-score. The c-Met staining intensity was scored as 0 (none or staining in less than 10% tumor cells), 1 (weak), 2 (moderate), or 3 (strong) based on membranous and/or cytoplasmic staining as previously reported[19,20] (Figure 1B), and each score multiplied by the percentage of cells (0%-100%). Therefore, H-score was ranged from 0 to 300. The median value 50 of c-Met H-score among samples with positive c-Met IHC staining was arbitrarily defined as the cutoff value for c-Met positivity. Samples were considered positive for p16 staining if diffuse strong nuclear and cytoplasmic immunostaining was observed in more than 50% of the tumor cells (Figure 1C)[21]. All slides were blinded with respect to clinical characteristics and outcome and were reviewed and scored by two experienced pathologists (Kwon D and Jeon YK).

Outcome measurements

The primary objectives of this study were to evaluate PD-L1 expression in ESCC and to evaluate the correlations between PD-L1 expression and other clinicopathologic features, including p16 and c-Met expression. The secondary objective was to assess the prognostic value of PD-L1, p16, and c-Met expression for overall survival (OS) and progression-free survival (PFS). OS was defined as the time from the date of diagnosis until either death due to any cause or the last follow-up date. PFS was defined as the time from the first day of definitive treatment until locoregional/distant relapse or progression, death, or last follow-up. Locoregional relapse refers to regional lymph node metastasis or tumor recurrence at the primary site. OS of patients who received palliative chemotherapy and/or radiotherapy (OS_{pall}) was measured from the date of relapse or surgery (if R0 resection was not achieved) until either death due to any cause or the last follow-up date.

Statistical analysis

Patients were divided into two groups by PD-L1 status. Significant differences in clinicopathologic characteristics between the two groups were assessed using the Mann-Whitney test for continuous variables and the χ^2 (or Fisher's exact test, if appropriate) for categorical variables. Significant correlations between clinicopatholoic factors and PD-L1-positivity were assessed by logistic regression analysis. Survival analyses were performed using the Kaplan-Meier method and were compared using a log-rank test. Univariate and multivariate analyses using the Cox proportional hazard regression model were applied to determine the hazard ratio (HR) for specific variables with respect to OS and PFS. For all statistical analyses, two-sided *P* values < 0.05 were considered statistically significant. All statistical analyses were carried out using STATA version 12 (StataCorp LP, College Station, TX, United States).

RESULTS

Patient characteristics

A total of 200 ESCC patients were included in our analysis. The clinicopathologic characteristics of the patients are summarized in Table 1. Most of the patients (94.0%) were males who ranged in age from 41 to 83 years (median age, 65 years). A majority of the patients were ex/current-smokers (84.9%) or alcohol drinkers (84.3%). All patients underwent radical esophagectomy as an initial definitive treatment, and R0 resection was achieved in 176 patients (88.0%). Twenty patients (10.0%) received neoadjuvant chemotherapy prior to surgery, and 64 patients (32.0%) received adjuvant chemotherapy.

Table 2 Univariate and multivariate logistic regression analysis for clinicopathologic factors affecting programmed death ligand-1 expression

Factors	Ref.	OR (95%CI)	*P* value
Univariate analysis			
Age[1]		0.98 (0.94-1.02)	0.323
Sex	Male *vs* Female	1.01 (0.29-3.48)	0.99
Smoking	Yes *vs* No	1.23 (0.53-2.86)	0.63
Alcohol	Yes *vs* No	1.07 (0.47-2.42)	0.873
CEA[1]		0.86 (0.66-1.11)	0.234
TNM stage	Ⅲ/Ⅳ *vs* Ⅰ/Ⅱ	1.59 (0.87-2.89)	0.133
Differentiation	M/D or P/D *vs* W/D	2.12 (0.98-4.58)	0.058
Neoadj.	Yes *vs* No	1.37 (0.53-3.53)	0.517
p16	Positive *vs* Negative	1.56 (0.62-3.92)	0.34
c-Met H-score	≥ 50 *vs* < 50	2.43 (1.21-4.88)	0.012
Multivariate analysis			
Differentiation	M/D or P/D *vs* W/D	2.01 (0.92-4.40)	0.08
c-Met H-score	≥ 50 *vs* < 50	2.34 (1.16-4.72)	0.017

[1]Treated as continuous variables. OR: Odds ratio; CEA: Carcinoembryonic antigen; TNM: Tumor-node-metastasis; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated; Neoadj.: Neoadjuvant chemotherapy.

Table 3 Univariate and multivariate Cox proportional hazard regression analysis for clinicopathologic factors overall survival

Factors	Ref.	HR (95%CI)	*P* value
Univariate analysis			
Age[1]		1.03 (1.00-1.05)	0.023
Sex	Male *vs* Female	6.29 (1.55-25.4)	0.010
Smoking	Yes *vs* No	1.36 (0.80-2.33)	0.261
Alcohol	Yes *vs* No	1.30 (0.80-2.13)	0.286
CEA[1]		1.07 (0.95-1.20)	0.269
TNM stage	Ⅲ/Ⅳ *vs* Ⅰ/Ⅱ	2.77 (1.97-3.90)	< 0.001
Differentiation	M/D or P/D *vs* W/D	1.23 (0.82-1.85)	0.308
Neoadj.	Yes *vs* No	1.70 (1.04-2.78)	0.032
Adj.	Yes *vs* No	1.73 (1.23-2.45)	0.002
Operation result	R1/R2 *vs* R0	3.53 (2.25-5.52)	< 0.001
p16	Positive *vs* Negative	0.49 (0.24-1.01)	0.053
c-Met H-score	≥ 50 *vs* < 50	1.12 (0.73-1.72)	0.601
Multivariate analysis			
Age[1]		1.03 (1.01-1.06)	0.001
Sex	Male *vs* Female	4.31 (1.06-17.6)	0.042
TNM stage	Ⅲ/Ⅳ *vs* Ⅰ/Ⅱ	2.52 (1.64-3.87)	< 0.001
Neoadj.	Yes *vs* No	1.26 (0.73-2.19)	0.405
Adj.	Yes *vs* No	0.91 (0.58-1.44)	0.685
Operation result	R1/R2 *vs* R0	2.53 (1.48-4.32)	0.001
p16	Positive *vs* Negative	0.51 (0.25-1.05)	0.069

[1]Treated as continuous variables. CEA: Carcinoembryonic antigen; TNM: Tumor-node-metastasis; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated; Neoadj.: Neoadjuvant chemotherapy; Adj.: Adjuvant chemotherapy.

Correlation of PD-L1 expression with p16 and c-Met expression in ESCC

IHC was performed to assess PD-L1, p16, and c-Met expression in surgical specimens collected from a total of 200 ESCC patients (Table 1). Tumor tissues from 67 patients (33.5%) were PD-L1-positive, and the remaining specimens (133 patients, 66.5%) were PD-L1-negative. PD-L1-positivity was not significantly correlated with any clinical characteristics, including age, sex, smoking/alcoholic history, stage, or differentiation (Table 1). A total of 21 samples were positive for p16 expression (10.5%), 12 of which were PD-L1-negative and 9 of which were PD-L1-positive. The c-Met H-scores were ≥ 50 in 42 of 200 samples (21.0%). Of these cases, 21 were PD-L1-negative, and the remaining 21 were PD-L1-positive.

The factors associated with PD-L1 expression were investigated by univariate and multivariate analyses using a logistic regression model (Table 2). Most clinical characteristics, including age, sex, smoking/alcoholic history, carcinoembryonic antigen (CEA) level, TNM stage and neoadjuvant chemotherapy were not significantly associated with PD-L1 expression. Moderately or poorly differentiated ESCC tended to be PD-L1-positive compared to well-differentiated ESCC in both univariate (P = 0.058) and multivariate analysis (P = 0.080). PD-L1 expression was not significantly associated with p16 expression (P = 0.340), but elevated c-Met expression (H-score ≥ 50) was significantly associated with PD-L1-positivity compared to lower c-Met expression (H-score < 50) (OR = 2.34, 95%CI: 1.16-4.72, P = 0.017 in multivariate analysis).

Prognostic implications of PD-L1, p16, and c-Met expression for ESCC

In our cohort of ESCC patients, there was no significant difference in the OS (P = 0.656) according to PD-L1 expression (Figure 2A). Modifying the threshold for PD-L1-positivity by IHC score (*e.g.*, 1+, 2+ or 3+) also did not yield a significant difference (data not shown). However, the locoregional relapse rate tended to increase (P = 0.134; Figure 2B), and the distant metastasis rate was significantly increased in patients with PD-L1-positive ESCC compared to those with PD-L1-negative ESCC (HR = 1.72, 95%CI: 1.06-2.79, P = 0.028; Figure 2C). To investigate the prognostic factors for OS in ESCC, univariate and multivariate Cox regression analyses were carried out (Table 3). There was no significant difference in OS according to c-Met expression (P = 0.601; Figure 3A). However, there was a trend toward improved OS in patients with p16-positive ESCC compared to those with p16-negative ESCC in univariate analysis (HR = 0.49; 95%CI: 0.24-1.01, P = 0.053; Figure 3B) and multivariate analysis (HR = 0.51, 95%CI: 0.25-1.05, P = 0.069). This tendency became statistically significant in PD-L1-positive ESCC patients (HR = 0.23, 95%CI: 0.06-0.96, P = 0.043; Figure 3C), but not in PD-L1-negative ESCC patients (P = 0.932; Figure 3D). Interestingly, the OS_{pall} was significantly better in patients with PD-L1-positive ESCC compared to those with PD-L1-negatvie ESCC (HR = 0.59, 95%CI: 0.36-0.96, P = 0.034; Figure 4).

DISCUSSION

In this study, we report that approximately one-third of

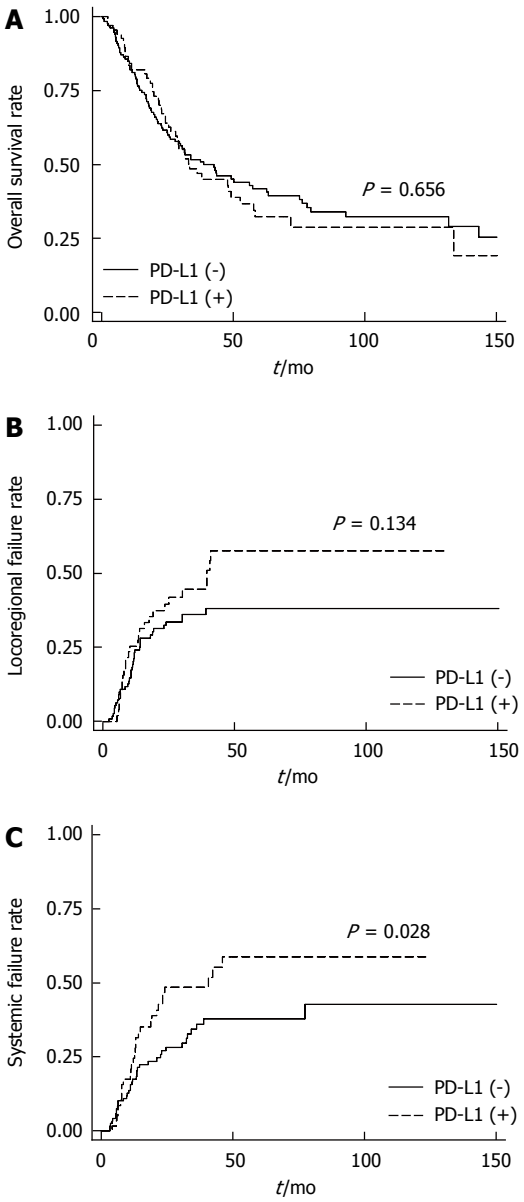

Figure 2 Kaplan-Meier plots for esophageal squamous cell carcinoma patients stratified by programmed death ligand-1 expression. A: Overall surviva; B: Locoregional relapse rate; C: Distant metastasis rate.

ESCC cases were positive for expression of PD-L1, and that PD-L1 expression was positively correlated with c-Met-positivity. Although PD-L1-positivity appeared to have no prognostic value for OS, it was associated with increased rate of distant failure. Expression of p16, a marker for HPV infection, was not correlated with the PD-L1 expression.

Because the PD-L1/PD-1 interaction has a negative regulatory function in T cell activation, cancer patients with elevated PD-L1 expression often exhibit a poor prognosis and clinical outcomes[8]. With respect to ESCC, Ohigashi *et al*[22] analyzed data from 41 patients with ESCC and showed that PD-L1 can serve as a prognostic biomarker for ESCC. Similarly, Chen *et al*[23] reported that PD-L1 expression was significantly associated with patient survival by analyzing 99 patients with ESCC. In contrast, we found no significant difference in survival of ESCC patients according to PD-L1 expression. The discrepancies among these studies can be partially explained by variations in the antibodies used for detection, as well as differing IHC cut-off definitions for PD-L1-positivity. ESCC specimens with any positive immunohistochemial staining using the rabbit anti-PD-L1 (E1L3N) XP® mAb were considered positive in our analysis. However, Ohigashi *et al*[22] performed immunostaining with a mouse anti-PD-L1 immunoglobulin G1 mAb (MIH1) and considered specimens with ⩾ 10% PD-L1-positive tumor cells to be positive. In contrast, Chen *et al*[23] used the H-score method to assess PD-L1 immunostaining with a rabbit anti-human PD-L1 mAb (NBP1-03220). In addition to these variations in technique, differences in the multidisciplinary approach to treatment, such as use of palliative chemotherapy, radiotherapy, and supportive care might have also influenced OS. Furthermore, differences in tissue preparation and processing variability could have confounded results regarding the use of PD-L1 IHC as a prognostic marker for ESCC.

c-Met is one of the most important cancer-associated receptor tyrosine kinases and is activated through binding to its specific ligand, HGF[24]. c-Met has been reported to be involved in the development of a number of human primary tumors, such as gastric, breast, colorectal, liver, and rectal cancers[25]. Activation of the receptor enhances oncogenesis through a wide range of mechanisms, including the promotion of tumor cell invasiveness, angiogenesis, and the epithelial-to-mesenchymal transition[24,26]. Based on its many roles in regulation of pro-oncogenic pathways, we hypothesized that c-Met modulates PD-L1 expression in ESCC. Consistent with this hypothesis, c-Met-induced signaling has been reported to lead to PD-L1 overexpression in renal cancer cells[15]. Our analysis demonstrated that c-Met expression is significantly correlated with PD-L1-positivity in ESCC. However, in agreement with a previous report[27], c-Met overexpression had no prognostic value for OS[27]. Thus, the biological impact of the correlation between PD-L1 and c-Met expression should be further investigated in future studies.

HPV infection is one of the risk factors associated with esophageal cancer, oropharyngeal squamous cell carcinoma, and cervical cancer[13,28-30]. Previous reports have shown that HPV-positive tumors exhibit high PD-L1 expression compared to HPV-negative tumors[10-12]. With respect to ESCC, we did not observe any significant correlation between expression of PD-L1 and that of p16, a surrogate marker for HPV infection[17]. However, we did find that p16 expression has potential prognostic value for ESCC. This result was similar to that observed in patients with oropharyngeal squamous cell carcinoma[30,31]. Interestingly, the prognostic value of p16 expression was more significant in patients with PD-L1-positive ESCC.

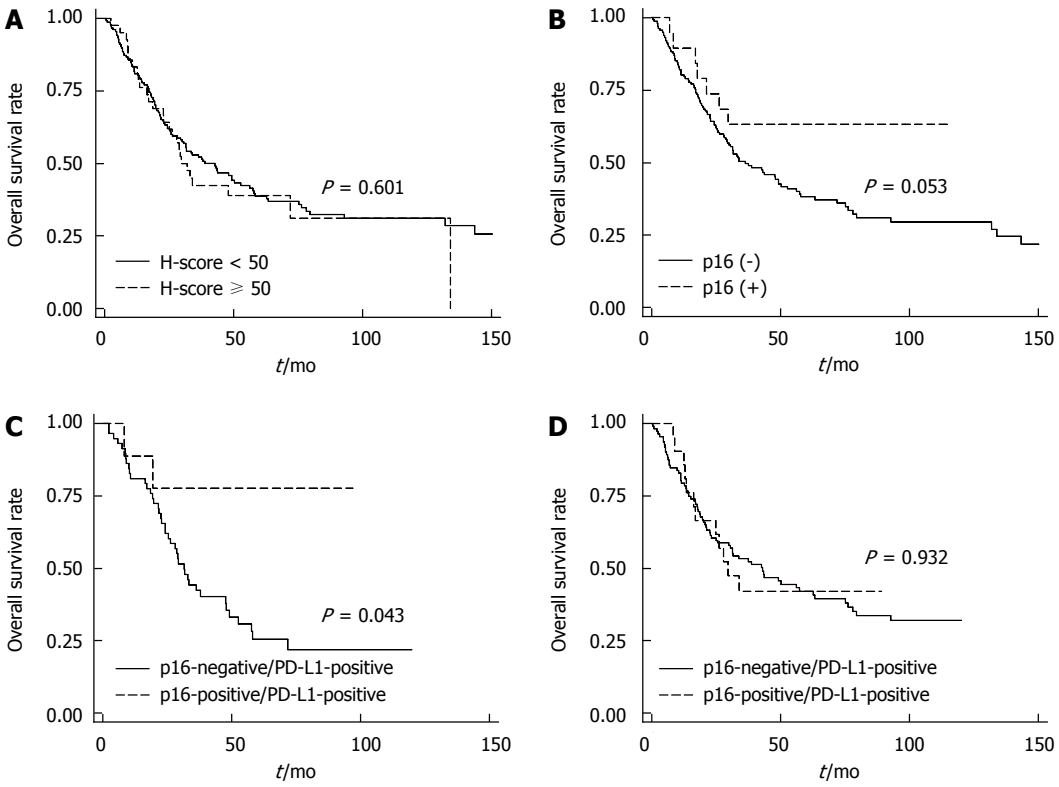

Figure 3 Overall survival according to c-Met, p16 expression. Kaplan-Meier plots of overall survival for all patients stratified by (A) c-Met expression and (B) p16 expression; Kaplan-Meier plots of overall survival stratified by p16 expression (C) for patients with PD-L1-positive esophageal squamous cell carcinoma; and (D) for patients with PD-L1-negative esophageal squamous cell carcinoma.

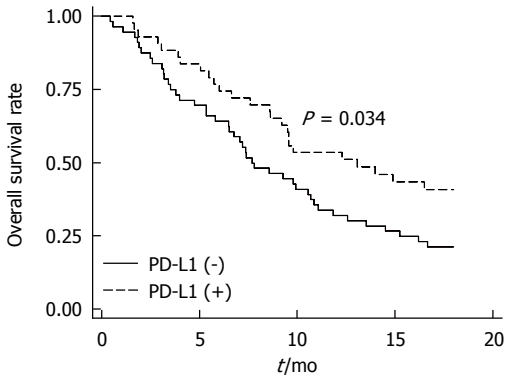

Figure 4 Kaplan-Meier plot of overall survival for esophageal squamous cell carcinoma patients who received palliative treatment. Overall survival was measured from the date of relapse or surgery (if R0 resection was not achieved) until either death by any cause or the last follow-up date.

There are several limitations that must be considered regarding the findings of this study. First, the retrospective design could bias the results. Second, there is no standard IHC threshold definition for PD-L1, p16 and c-Met positivity, and our definition was arbitrary. Therefore, one should be cautious when generalizing the results of our analysis. Third, we used p16 IHC as a surrogate marker instead of detecting HPV DNA to assess HPV infection. Fourth, although we identified a positive correlation between PD-L1 and c-Met expression, the biological meaning of the correlation was not investigated in this study. Therefore, further follow-up studies including external validation are needed. Nevertheless, the present study represents the largest retrospective study to date with sufficient statistical power to assess PD-L1 expression and its prognostic implications in ESCC patients. Furthermore, the correlation between PD-L1 and c-Met expression in ESCC identified in this study is a novel finding.

In conclusion, approximately one-third of the ESCC patient samples analyzed were positive for PD-L1 expression, and PD-L1 expression was positively correlated with c-Met expression. Although PD-L1-positivity was not found to be associated with the prognosis of ESCC patients, it may play a critical role in distant metastasis and progression of ESCC. Because most cancer-related deaths are associated with distant metastasis, our findings provide a rationale for immunotherapy targeting PD-L1 for ESCC.

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COMMENTS

Background

Recently, an improved understanding of immunobiology has uncovered the interaction between programmed death ligand-1 (PD-L1) and programmed

death 1 (PD-1) as one of mechanisms by which cancer cells evade immune surveillance. The prognostic role of this interaction is controversial, and the mechanisms that regulate the expression of PD-L1 are obscure. There has been relatively scarce study examining the clinical meaning of PD-L1/PD-1 interaction in esophageal cancer, which is one of the most common gastrointestinal malignancies and is highly prevalent in Asia.

Research frontiers

PD-L1 upregulation has been reported in human papilloma virus-associated malignancies such as esophageal squamous cell carcinoma (ESCC), and c-Met has been shown to promote PD-L1 overexpression in some type of cancers. However, a comprehensive analysis of the correlation between PD-L1 and c-Met expression in ESCC has not yet been reported.

Innovations and breakthroughs

This study revealed that PD-L1 play a critical role in distant failure and progression of ESCC. In addition, this study found that PD-L1 expression is positively correlated with c-Met expression, which is a novel finding.

Applications

PD-L1-positivity may play a critical role in distant metastasis and progression of ESCC. Because most cancer-related deaths are associated with distant metastasis, this finding provides a rationale for immunotherapy targeting PD-L1 for ESCC.

Terminology

PD-1 is a negative co-stimulatory factor that inhibits T cell activation when activated by PD-L1 or one of its other ligands. PD-L1 is a cell surface glycoprotein that belongs to the B7 family and is expressed not only on normal cells, such as T cells, B cells, monocytes, macrophages, and dendritic cells, but also on cancer cells.

Peer-review

The article focuses on the role of PD-L1 and another two markers, the authors are looking for new and different correlations.

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ORIGINAL ARTICLE

Retrospective Study

Application of side-to-side anastomosis of the lesser curvature of stomach and jejunum in gastric bypass

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Abstract

AIM

To evaluate the feasibility of side-to-side anastomosis of the lesser curvature of stomach and jejunum in laparoscopic Roux-en-Y gastric bypass (LRYGB).

METHODS

Seventy-seven patients received side-to-side anastomosis of the lesser curvature of stomach and jejunum by utilization of linear stapler in LRYGB from April 2012 to July 2015 were retrospectively analyzed.

RESULTS

All patients were successfully completed laparoscopic gastric bypass with the side-to-side anastomosis of the lesser curvature of stomach and jejunum. No patient was switched to laparotomy during operation. No early complications including gastrointestinal anastomotic bleeding, fistula, obstruction, deep vein thrombosis, incision infections, intra-abdominal hernia complications were found. One patient complicated with stricture of gastrojejunal anastomosis (1.3%) and six patients complicated with incomplete intestinal obstruction (7.8%). BMI and HbA1c determined at 3, 6, 12, 24 mo during follow up period were significantly reduced compared with preoperative baselines respectively. The percentage of patients who maintain HbA1c (%) < 6.5% without taking antidiabetic drugs reached to 61.0%, 63.6%, 75.0%, and 63.6% respectively. The outcome parameters of concomitant diseases were significantly improved too.

CONCLUSION
Present surgery is a safety and feasibility procedure. It is effective to lighten the body weight of patients and improve type 2 diabetes and related complications.

Key words: Laparoscopic Roux-en-Y gastric bypass; Gastric bypass; Gastrojejunostomy; Metabolic surgery; Bariatric surgery; Type 2 diabetes mellitus

Core tip: Laparoscopic Roux-en-Y gastric bypass (LRYGB) has been widely applied in the treatment of obesity patient with type 2 diabetes mellitus. Gastrojejunostomy is one of the most important procedures in LRYGB. However, the surgical mode has not been standardized. We have proved in this study that the modified side to side anastomosis of the lesser curvature of stomach and jejunum applied with linear cutting closer in LRYGB is a safe, feasible, and effective therapeutic option in the treatment of obesity patients with type 2 diabetes and complications, which could make gastrojejunostomy standardized easily in LRYGB.

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INTRODUCTION

With the increasing incidence of obesity and type 2 diabetes mellitus (T2DM), bariatric surgery has becoming more and more popular as a therapeutic option. At present time, laparoscopic Roux-en-Y gastric bypass (LRYGB) is generally applied in the treatment of obesity patient with T2DM. Following 20 years clinical practice, the advantages of this surgery in aspects of curative effectiveness, weight loss, diabetes control and safety are gradually recognized worldwide and has become a gold standard mode for patients indicated[1]. LRYGB procedure mainly contains 3 processes including gastric pouch formation, gastrojejunostomy and jejunojejunostomy. Wherein, gastrojejunostomy is the most critical step because it needs proficient and skilled surgeon to control the size of anastomosis, which is usually associated with the occurrence of complications. Currently, the diverse surgical techniques applied for anastomosis in LRYGB are circular-stapled gastrojejunostomy (43%), linear-stapled gastrojejunostomy (41%), and full hand-sewn (21%) gastrojejunostomy individually[2].

The circular-stapled gastrojejunostomy was first described by Wittgrove in 1994. It possesses the characteristic of easy size control of anastomosis. However, this surgical mode demands prolonged length of operation time because it is difficult to insert a stapling anvil block inside the gastric pouch and hardly find the active bleeding spot at anastomotic site[3]. Although the technique of full hand-sewn gastrojejunostomy was first reported by Higa *et al*[4], it was not expediently accepted by surgeons due to the requirement of longer learning curve and stronger laparoscopic suture skill. Schauer *et al*[5] first introduced the linear-stapled gastrojejunostomy in LRYGB, which triumphed over the complexity of stapling anvil block placement in circular-stapled gastrojejunostomy and improved the management of anastomosis bleeding.

Since irregular and unsmooth anastomotic suture will produce the stricture of anastomosis, it requires more professional and skillful surgeon to perform the surgery. However, no matter what mode of anastomosis is selected, certain rate of complications related to gastrointestinal anastomosis will still happen after surgery, for instance, the rate of fistula is 0.93%-6%, stenosis is 1.6%- 6.41%, and bleeding is 0%-1.75%[3-16].

Torres *et al*[17] considered that the anastomosis of Roux-en-Y gastrojejunostomy from the lesser curvature could bring on the food continuous through the anastomostic stoma and effectively moderate the gastric pouch expansion after surgery. We, therefore, take the linear stapler instead of Torres' circular stapler to improve the gastrojejunostomy in lesser curvature of stomach and close the anastomosis.

The purpose of this study is to investigate the safety and feasibility of modified LRYGB and its impact on perioperative complications and the resolutions of T2DM.

MATERIALS AND METHODS

Patient characteristics

All data of consective 82 obese patients with T2DM (diagnosed according to the China Diabetes Association guidelines[18]) undergoing LRYGB at the general surgery department of Beijing Tian Tan Hospital from April 2012 to July 2015 were retrospectively reviewed. Among them, 5 patients were excluded because of incomplete data collection. Thus, 77 patients in total were qualified to enter the study finally. The patients included 46 women and 31 men with a mean age of 46 years (range 19-67), a mean body mass index (BMI) 32.1 kg/m^2 (range 27.7-51.4), a mean glycosylated hemoglobin (HbA1c) 8.3% (range 5.5%-13.3%) and a mean T2DM duration 7 years (range 1-25), respectively. In addition, 29 patients showed hypertension, 31 patients showed fatty liver and 38 patients showed abnormal lipid metabolism (Table 1). The average follow-up period was 16.5 ± 9.4 mo (range 6-39 mo). The number of patients who completed postoperative follow-up at 12, 24, 36 mo was 56 cases (72.7%), 33 cases (42.9%) and 6 cases (7.8%) correspondingly.

Table 1 Patient demographics

Characteristic	
Gender (*n*)	
Men	46
Women	31
Age (yr)	46 ± 11 (19-67)
BMI (kg/m^2)	32.1 ± 4.9 (27.7-51.4)
HbA1c (%)	8.3 ± 1.6 (5.5-13.3)
Mean T2DM duration(yr)	7 ± 5 (1-25)
Concomitant disorder (*n*)	
Hyperlipidemia	38
Fatty liver	31
Hypertension	29
Follow-upduration (*n*)	
6 mo	77
12 mo	56
24 mo	33
36 mo	6

BMI: Body mass index; HbA1c: Glycosylated hemoglobin; T2DM: Type 2 diabetes mellitus.

Surgical procedure

The patient was placed in a dorsal elevated position on surgical table and the legs were separated to allow the surgeon standing between them. A 10-mm trocar was inserted through a periumbilical port for developing a pneumoperitoneum and laparoscopic guidance. Two pieces of 13-mm trocar were inserted separately at the left/right points above umbilicus. Two pieces of 5-mm trocars were inserted at subxiphoid (for liver retractor) and left subcostal position, seperately. The distance between each trocar should be over 6-7 cm for avoiding interference each other. The intra-abdominal pressure should be maintained constantly at 13 mmHg.

The liver was retracted by a laparoscopic forceps through subxiphoid port. The esophagus was exposed and the cardia-esophageal junction and angle of His were identified.

The isolating dissection began at the lesser curvature of stomach by harmonic scalpel from 9 to 4 cm below the cardia approximately. The stomach was elevated and an aperture was created adjacent to the posterior gastric wall and around the lesser curvature. The dissection started at the lesser curvature from 9 cm below the cardia approximately. The LLC Echelon 60 endopath stapler -1.5 mm (ETHICON ENDO-SURGERY) was then applied initially in an inclined direction (near 45° angle of lesser curvature). The dissection was continued from the lesser curvature about 2.5-3.0 cm and the direction of the transaction line was turned vertically, aiming to the angle of His. The pouch should be oriented vertically and the width was near 2.5-3.0 cm. Four or five times repeated of the Echelon 60 endopath stapler -1.5 mm were required to completely divide the stomach.

The harmonic scalpel was implemented for tissue separation along with the intestinal wall distal to the ligament of Trietz about 100 cm, range from approximately 5 cm distally. The distal jejunum was then grasped by atraumatic laparoscopic forceps and brought up to the gastric pouch in front of the colon.

A stab wound was made on the end of lesser curvature of the gastric pouch and on the wall of the jejunum contra of the mesentery with laparoscopic coagulation hook separately (Figure 1A). A blade of the Echelon 60 endopath stapler -1.5 mm was inserted through the stab wound of the distal jejunum. The other blade of the linear stapler was placed into the gastric pouch, and the lesser curvature of the gastric pouch is anastomosed to the jejunum side-to-side with the stapler (Figure 1B). The staple lines were inspected for bleeding under laparoscopic vision. The common open of stomach and jejunal loop were clamped together with the linear staplers (Echelon 60 endopath stapler -1.2 mm and Echelon 60 endopath stapler -1.5 mm) after staunching the bleeding of the staple line, insuring the width of gastrointestinal anastomosis attaining 2.5 cm or so (nearly 25 mm circular stapler) (Figure 1C). The side-to-side anastomosis between the gastric pouch and jejunum was then completed.

A stab wound made with laparoscopic coagulation hook on the wall of the jejunum contra of the mesentery, near 100 cm distal to the gastrojejunostomy (alimentary limb were varied in length depend on preoperative BMI of the patient (such as 100 cm length for patient BMI $\leqslant$ 50 kg/m^2, 120 cm length for patient BMI > 50 kg/m^2), the proximal loop of the jejunum brought down to the site of the stab wound, the proximal loop and distal segment of the jejunum was anastomosed side-to-side with the Echelon 60 endopath stapler -1.2 mm. Another Echelon 60 endopath stapler -1.2 mm was used to close the opening of the jejunum after staunching the bleeding of the staple line to complete the Roux-en-Y anastomosis surgery (Figure 1D). A drain tube was placed on the left side of cardia and washed the abdominal cavity by saline without the bleeding of gastrointestinal and intestinal anastomosis.

Postoperative management

No patient was employed the nasogastric suction postoperatively. All patients were taken the gastrointestinal contrast radiograph with ioversol (iodic contrast agent) on the third day morning after surgery. Figure 2 showed a typical appearance of gastric pouch. Patients were allowed to ingest the liquid food to ensure the anastomotic stoma without leakage. Most patients were ready for discharge on the 4th-6th postoperative day.

Patients were prescribed to take proton pump inhibitors orally, and instructed to supplement consistently of multivitamins and trace elements for a long period. The follow up visits for patients were scheduled at 1st, 3rd, 6th and 12th mo respectively after surgery in the first year, and twice a year thereafter.

Complication is defined as a major event happening and requiring intervention during or after procedure. T2DM remission is defined by the laboratory test of

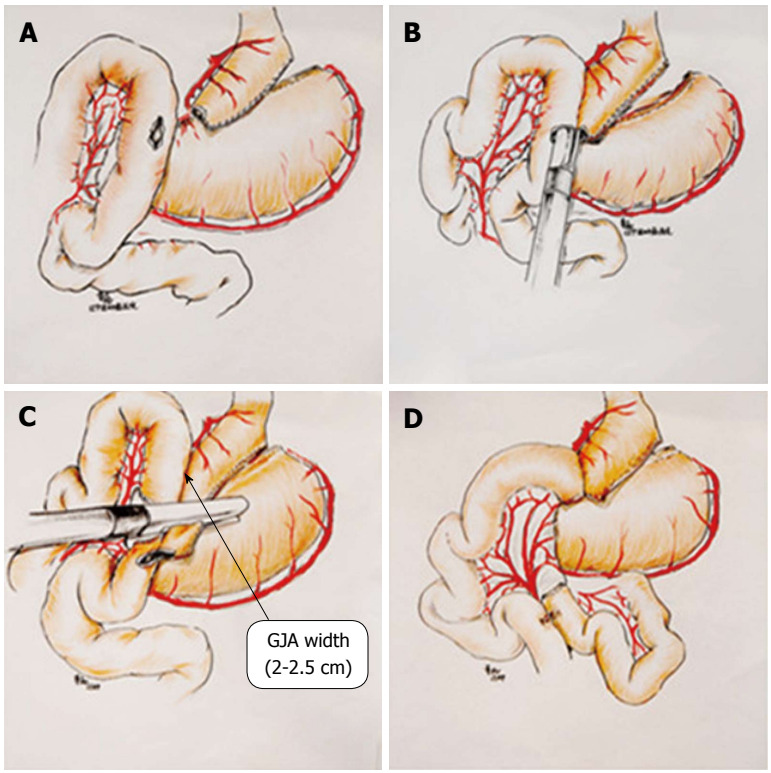

Figure 1 Surgical procedures of laparoscopic Roux-en-Y gastric bypass. A: Making gastric pouch; B: Completing the side-to-side anastomosis between the gastric pouch and jejunum; C: Clamping the common open of stomach and jejunal loop; D: Completing the side-to-side anastomosis of jejunum. GJA: Gastrojejunal anastomosis.

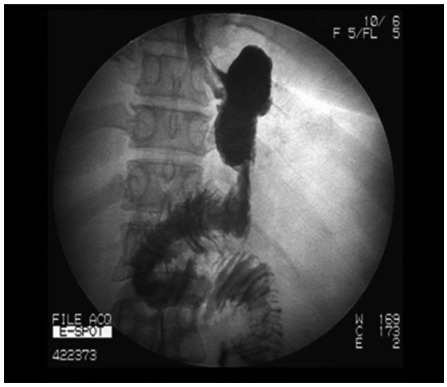

Figure 2 Gastrointestinal contrast radiograph. All patients were taken the gastrointestinal ioversol contrast radiograph on the third morning after LRYGB. The radiograph shows a typical appearance of the gastric pouch (length 5-6 cm, width 2-2.5 cm, volume 20-30 mL). LRYGB: Laparoscopic Roux-en-Y gastric bypass.

fasting glucose level < 100 mg/dL, HbA1c < 6.5% and an unwillingness to pursue medical treatment.

Patients data were collected and registered into the database of bariatric center, including the parameters of patients demographics, BMI, weight loss, length of hospital stay, glycosylated hemoglobin (HbA1c), complications, and comorbidit (hyperlipidemia, fatty liver, hypertension).

Patients body weight was measured in light clothing without shoes to the nearest 0.1 kg, and body height was measured to the nearest 0.1 cm. BMI was calculated as weight in kilograms divided by height in meters squared. Blood was drawn for laboratory examination from an antecubital vein following an overnight fast. Measurements were acquired using the Hitachi 7170 and J&J nephelometer assay (Dade Behring, United States). The serum insulin and C-peptide levels were measured using a DPC Immulite analyzer. All specimens were processed within 1 h of collection. Informed consent from the patients and ethics approval from the Institutional Research Ethics Committee was obtained.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0, with a baseline comparison by *t* tests, one-way ANOVA and chi-square tests. Continuous variables were expressed as the mean ± SD. *P* values < 0.05 were considered statistically significant.

RESULTS

All patients were successfully completed whole LRYGB procedure. Among them, 9 patients experienced concomitant cholecystectomy. Nobody was switched to the laparotomy surgery. The patients were followed up for 6-39 mo. The average operative time was 2.6 ± 0.8 (1.5-5.8) h, blood loss was 58.4 ± 31.1 (10-200) mL, and the length of postoperative hospital stay was 5.9 ± 1.4 (4-14) d.

Nobody was dead. One patient (1.3%) required

Table 2 Postoperative complications in laparoscopic Roux-en-Y gastric bypass patients

Complications	*n* (%)
Early complications (< 30 p-o d)	
JJA bleeding	1 (1.3)
Late complications (> 30 p-o d)	
GJA stenosis	1 (1.3)
Intestinal obstruction	6 (7.8)
Iron deficiency anemia	2 (2.6)
Acute pancreatitis	1 (1.3)
Upper gastrointestinal hemorrhage	1 (1.3)
Gallstones	5 (7.6)
Total	17 (23.2)

LRYGB: Laparoscopic Roux-en-Y gastric bypass; JJA: Jejunojejunal anastomosis; GJA: Gastrojejunal anastomosis.

secondary surgery because of bleeding of side-to-side anastomosis between intestinal 34 h after first surgery. No patient occurred the complications of gastrointestinal anastomotic bleeding, fistula, obstruction, deep vein thrombosis, incision infections, intra-abdominal hernia and any others during early stage after surgery (< 30 d).

One patient (1.3%) was carried out the repeated laparoscopic operation ascribe to the stricture of gastrojejunal anastomosis 6 mo later following first surgery. The patient presented the symptoms and signs of anastomosis stenosis 6 wk after surgery and failed to endoscopic dilation management. Six patients (7.8%) arose the incomplete intestinal obstructions, and were treated conservatively by bowel rest and parenteral nutrition. Iron deficiency anemia was appeared in two patients (2.6%) at 9 and 18 mo after operation, and was treated by ferrous preparation. One patient (1.3%) showed upper gastrointestinal hemorrhage associated with oral aspirin intake. One patient (1.3%) developed acute pancreatitis 22 mo after surgery and was treated by medications. In addition, five patients (7.6%) were newly diagnosed as cholelithiasis in 66 patients after surgery. Nine and two patients were individually experienced cholecystectomy during or before LRYGB (Table 2).

Fifty-six patients (72.7%) were followed up for 12 mo and 33 patients (42.9%) were followed up for 24 mo after surgery. BMI and HbA1c determined at 3, 6, 12, 24 mo during following up period after surgery were significantly reduced compared with those before operation respectively (Table 3). BMI achieved to minimal level at 1 year after surgery.

The portions of patients who maintained HbA1c (%) < 6.5% without antidiabetic agents were 61.0%, 63.6%, 75.0%, and 63.6% at 3, 6, 12, 24 mo respectively after operation. The outcome parameters of T2DM were significantly improved compaired with that measured before operation (Tables 4 and 5).

Fifty-six patients (72.7%) were followed up for 12 mo postoperatively. Concomitantdisorders such as hypertension, dyslipidemia and fatty liver were significantly alleviated after surgery than the baselines determined prior to surgery (Table 6).

DISCUSSION

The key technology of laparoscopic gastric bypass are gastric pouch formation and gastrojejunostomy, in which the madding of a lesser curvature of independent gastric pouch has reached a consensus[19]. Currently, the surgical techniques applied for anastomosis in LRGB include circular-stapled gastrojejunostomy, linear-stapled gastrojejunostomy, and full hand-sewing gastrojejunostomy. The circular-stapled gastrojejunostomy was first described by Wittgrove in 1994[20], which possesses the characteristic of easily control of anastomosis size. However, this surgical method demands prolonged length of operation time because it is difficult to insert a stapling anvil block inside the gastric pouch and find the active bleeding spot at anastomotic site. Although the technique of full hand-sewing gastrojejunostomy was first reported by Higa *et al*[4] in 2000, it was not expediently accepted by surgeons due to the requirement of longer learning curve and stronger laparoscopic suture skill. Schauer *et al*[5] first introduced the linear-stapled gastrojejunostomy in LRYGB, which triumphed over the complexity of stapling anvil block placement in circular-stapled gastrojejunostomy and improved the management of anastomosis bleeding. Several studies show that there was no difference in anastomotic related complication for 25 mm circular-stapled ,full hand-sewing and linear-stapled[6-10]. It is important to control the size of anastomotic for laparoscopic gastric bypass and which in gastrojejunostomy was certainly difficult, but there has no standrad for the methods of gastrojejunostomy nowadays.

The classical linear-stapled gastrojejunostomy anastomoses the posterior of gastric pouch with jejunum and continuous or discontinuous closes the common opening[5]. This method has the advantages of omitting to insert a stapling anvil block inside the gastric pouch as well as easily finding and handling the active bleeding spot at anastomotic site. But it needs a skilled laparoscopic technique to close the common opening, for improper suture may cause the anastomotic stenosis. Considering the shortcomings above, we improve the method of Torres[17] which using circular-stapled gastrojejunostomy in lesser curvature. We take the linear stapler instead of Torres'circular stapler to perform the gastrojejunostomy in lesser curvature of stomach and close the common opening. The width of anastomosis was 2.5 cm (equal to the diameter of 25 mm circular-stapled). It has three advantages, firstly, it not only retaines the linear-stapled advanage, but also easily to standardized, therefore easy to control the anastomosis size, avoiding the laparoscopic suture operation, such shortened the learning curve. Secondly, because we use liner-stapled for side to side anastomosis of the lesser curvature of stomach and jejunum, which insured the width of gastric pouch, so it is easy to formation narrow and long gastric pouch ,and

Table 3 Determination of body mass index and glycosylated hemoglobin before and after laparoscopic Roux-en-Y gastric bypass surgery

Follow-up		Pre-op.	3 mo Post-op.	6 mo Post-op.	12 mo Post-op.	24 mo Post-op.	*F* value	*P* value
12 mo (*n* = 56)	BMI (kg/m^2)	32.3 ± 5.0	26.6 ± 4.5	25.3 ± 4.1	24.9 ± 4.0	/	33.738	0.000
	HbA1c (%)	8.1 ± 1.6	6.2 ± 1.0	5.7 ± 0.6	6.1 ± 1.1	/	47.506	0.000
24 mo (*n* = 33)	BMI (kg/m^2)	31.2 ± 3.5	25.8 ± 3.5	24.3 ± 3.1	24.0 ± 2.9	24.4 ± 2.9	29.550	0.000
	HbA1c (%)	8.5 ± 1.7	6.2 ± 0.9	5.5 ± 0.7	5.7 ± 0.6	6.2 ± 1.0	41.135	0.000

BMI and HbA1c determined at 3, 6, 12, 24 mo postoperationwere significantly reduced compared with the preoperative baselines. BMI: Body mass index; HbA1c: Glycosylated hemoglobin; LRYGB: Laparoscopic Roux-en-Y gastric bypass.

Table 4 Diabetes mellitus parameters in patients combined with/without anti-diabetemedications after Laparoscopic Roux-en-Y gastric bypass surgery *n* (%)

HbA1c level	Preop (*n* = 77)	3 mo postop. (*n* = 77)	6 mo postop. (*n* = 77)	12 mo postop. (*n* = 56)	24 mo postop. (*n* = 33)
Without anti-diabetes medications	6 (7.8)	54 (70.1)	56 (72.7)	47 (83.9)	26 (78.8)
HbA1c < 7.0%	3 (3.9)	51 (66.2)	54 (70.1)	45 (80.4)	22 (66.7)
HbA1c ≤ 6.5%	1 (1.3)	47 (61.0)	49 (63.6)	42 (75)	21 (63.6)
HbA1c ≤ 6.0%	0 (0)	34 (44.2)	37 (48.1)	36 (64.3)	15 (45.5)
With anti-diabetes medications	71 (92.2)	23 (29.9)	21 (27.3)	9 (16.1)	7 (21.2)
HbA1c < 7.0%	15 (19.5)	13 (16.9)	10 (13.0)	5 (8.9)	5 (15.1)
HbA1c ≤ 6.5%	9 (11.7)	9 (11.7)	8 (10.4)	3 (5.4)	4 (12.1)
HbA1c ≤ 6.0%	4 (5.2)	3 (3.9)	3 (3.9)	3 (5.4)	1 (3.0)

The percentage of patients who maintained HbA1c (%) < 6.5% without antidiabeticmedicine were 61.0%, 63.6%, 75.0%, and 63.6% after 3, 6, 12, and 24 mo of surgery. T2DM parameters were significantly improved compared with preoperative baselines. T2DM: Diabetes mellitus; LRYGB: Laparoscopic Roux-en-Y gastric bypass; HbA1c: Glycosylated hemoglobin.

Table 5 Patients with improved diabetes mellitus after laparoscopic Roux-en-Y gastric bypass *n* (%)

HbA1c level No. of patients	Preop (*n* = 77)	3 mo postop. (*n* = 77)	6 mo postop. (*n* = 77)	12 mo postop. (*n* = 56)	24 mo postop. (*n* = 33)	*P* value			
						3 mo postop *vs* Preop	6 mo postop *vs* Preop	12 mo postop *vs* Preop	24 mo postop *vs* Preop
HbA1c < 7.0%	18 (23.4)	64 (83.1)	64 (83.1)	50 (89.3)	27 (81.8)	0.000	0.000	0.000	0.000
HbA1c ≤ 6.5%	10 (13.0)	56 (72.7)	57 (74.0)	45 (80.4)	25 (75.8)	0.000	0.000	0.000	0.000
HbA1c ≤ 6.0%	4 (5.2)	37 (48.1)	40 (51.9)	39 (69.6)	16 (48.5)	0.000	0.000	0.000	0.000

The resolution of T2DM after 3, 6, 12, 24 mo of surgery was significantly improved compared with preoperative baseline. T2DM: Diabetes mellitus; LRYGB: Laparoscopic Roux-en-Y gastric bypass; HbA1c: Glycosylated hemoglobin.

reduces gastric jejunum anastomotic tension. Lastly it can maximize the distance of two closed line, so the anastomosis has better blood supply.

The surgical technique of side-to-side anastomosis of gastric lesser curvature and jejunum in LRYGB was implemented in a total of 77 patients in this study. No patient was switched to laparotomy and no death case was existed. 9 patients received the laparoscopic cholecystectomy at same time during operation. The mean operation time was declined from 2.6 h to 1.5-2.0 h. The mean volume of blood loss during operation was decreased from 58.4 mL to 10-30 mL later stage. The average length of hospital stay was reduced from 5.9 d to 4.0 d at later stage. No gastrointestinal anastomotic bleeding, fistula, stenosis and other complications were observed in whole perioperative duration. All patients did not occur the surgery - related complications during 6-39 mo follow up period except one case (1.3%) showing anastomotic stenosis of gastric jejunum at the sixth week after operation. Compared with the rate of LRYGB gastrointestinal anastomotic complications reported in previous literature[3-16], the surgical technique of linear cutting closer side to side anastomosis of gastric lesser curvature and jejunum adopted in this study is a safe and feasible procedure in clinic. This study also demonstrates that the operation - related complications such as new gallbladder stone, iron deficiency anemia and so on will happen after surgery. The appropriate medical instructions and follow-up guidance to patients after surgery are of great importance.

Previous studies disclosed that bariatric surgery occupied the advantages of long term glycemic remission for obesity patient with type 2 diabetes[21-24]. Schauer *et al*[21] and Mingrone *et al*[22] reported the

Table 6 Patients with concomitant disorders before and after 12 mo of laparoscopic Roux-en-Y gastric bypass surgery (n = 56)

Concomitant disorders (n)	Pre-surgery	12 mo post-surgery	P value
Dyslipidemia	38	7	0.000
Fatty liver	31	4	0.000
Hypertension	29	8	0.000

Concomitant disorders in 56 patients were significantly relieved after 12 mo of surgery compared with those before surgery. LRYGB: Laparoscopic Roux-en-Y gastric bypass.

effects of bariatric surgery versus conventional medical therapy for diabetes patients in the prospective, randomized, controlled studies, which indicated that the combination of surgical intervention with conventional medicine would possess the superior therapeutic effectiveness of blood glucose reduction. This result was further confirmed in an additional study of Schauer[25] by 3 years follow-up. In our study, we provide the important evidences of bariatric surgery beneficial to type 2 diabetes management. Gastric bypass surgery is not only able to decline the blood glucose level in type 2 diabetes, but also can improve the management of related complications than any medications alone[25-27]. Furthermore, gastric bypass and other bariatric surgery are capable of alleviating the mortality of diabetes complications[28,29]. This study showed that at the 3, 6, 12, 24 mo postoperative, the BMI (kg/m^2) were reduced to 25.8 ± 3.5, 24.3 ± 3.1, 24.0 ± 2.9, and 24.4 ± 2.9 respectively, indicating significant difference compared to the baseline of 31.2 ± 3.5 determined preoperation, The BMI would reach to the minimum level within 1 year after surgery. Similarly, the HbA1c (%) levels at 3, 6, 12, and 24 mo after operation were declined to 6.2 ± 0.9, 5.5 ± 0.7, 5.7 ± 0.6, 6.2 ± 1.0 respectively with significant difference in comparison with the baseline of 8.5 ± 1.7 pre-operation. The portions of patients who maintained HbA1c (%) < 6.5% without antidiabetic medicine achieved 61.0%, 63.6%, 75.0%, and 63.6% at 3, 6, 12, and 24 mo respectively. In addition, the improvement of concomitant symptoms, hyperglycemia, lipid metabolism disorder, hypertension and fatty liver were comparable to those in LRYGB patients reported previously.

In conclusion, we have proved in this study that the side-to-side anastomosis of the lesser curvature of stomach and jejunum applied with linear cutting closer in LRYGB is a safe, feasible, and effective therapeutic option in the treatment of obesity patients with type 2 diabetes and complications. But its long-term effect requires more cases and long-term observation. Therefore, the multicenters, larger sample size and longer observation studies are required to further confirm the clinical efficacy of LRYGB to the fat diabetic patients, including the impact on microangiopathy and nephrosis.

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COMMENTS

Background

With the increasing incidence of obesity and type 2 diabetes mellitus (T2DM), bariatric surgery has becoming more and more popular as a therapeutic option. At present time, laparoscopic Roux-en-Y gastric bypass (LRYGB) is generally applied in the treatment of obesity patient with T2DM. Following 20 years clinical practice, the advantages of this surgery in aspects of curative effectiveness, weight loss, diabetes control and safety are gradually recognized worldwide and has become a gold standard mode for patients indicated.

Research frontiers

LRYGB procedure mainly contains 3 processes including gastric pouch formation, gastrojejunostomy and jejunojejunostomy. Wherein, gastro-jejunostomy is the most critical step because it needs proficient and skilled surgeon to control the size of anastomosis, which is usually associated with the occurrence of complications. However, the surgical mode has not been standardized. Currently, the diverse surgical techniques applied for anastomosis in LRYGB are circular-stapled gastrojejunostomy (43%), linear-stapled gastrojejunostomy (41%), and full hand-sewn (21%) gastrojejunostomy individually.

Innovations and breakthroughs

In this study, the modified side to side anastomosis of the lesser curvature of stomach and jejunum was applied with linear cutting closer in LRYGB. It has three advantages. Firstly, it not only retaines the linear-stapled advantage, but also easily to standardized, therefore easy to control the anastomosis size, avoiding the laparoscopic suture operation, such shortened the learning curve. Secondly, because we use liner-stapled for side to side anastomosis of the lesser curvature of stomach and jejunum, which insured the width of gastric pouch, so it is easy to formation narrow and long gastric pouch ,and reduces gastric jejunum anastomotic tension. Lastly it can maximize the distance of two closed line, so the anastomosis has better blood supply.

Applications

This study suggests that the modified side to side anastomosis of the lesser curvature of stomach and jejunum should been applied with linear cutting closer in LRYGB.

Peer-review

The article is thoughtfully written and has a restricted question it tries to answer. The authors have been careful about not imposing their techniques as the standard.

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ORIGINAL ARTICLE

Observational Study

Impact of hepatitis C virus core mutations on the response to interferon-based treatment in chronic hepatitis C

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Abstract

AIM

To determine whether hepatitis C virus (HCV) core substitutions play a role in the response to interferon-

based treatment in Caucasian patients.

METHODS

One hundred eight HCV chronically infected patients initiating treatment with pegylated IFN plus ribavirin for 48 wk were tested for baseline substitutions at codons 70 and 91 of the viral core protein (BigDye Terminator vers.3.1, Applied Biosystems,) and for genetic polymorphisms in host *IL28B* gene rs12979860 (Custom TaqMan 5' allelic discrimination assay; Applied Biosystems).

RESULTS

Of the patients, all were infected with HCV genotype 1b, 44.4% had low baseline HCV viral load, and 37.9% had mild/moderate fibrosis. Only 38.9% achieved therapeutic success, defined as sustained virological response (SVR). Eighty-eight percent of the patients presented at least one substitution at core position 70 (R70Q/H) or/and position 91 (L91M). The favorable IL28B CC polymorphism was detected in only 17.6% of the patients. In the univariate analysis, young age ($P < 0.001$), urban residence ($P = 0.004$), IL28B CC genotype ($P < 0.001$), absence of core mutations ($P = 0.005$), achievement of rapid virologic response ($P < 0.001$) and early virological response ($P < 0.001$) were significantly correlated with SVR. A multivariate analysis revealed three independent predictors of therapeutic success: young age ($P < 0.001$), absence of core substitutions ($P = 0.04$) and IL28B CC genotype ($P < 0.001$); the model correctly classified 75.9% of SVR cases with a positive predictive value of 80.7%.

CONCLUSION

HCV core mutations can help distinguish between patients who can still benefit from the affordable IFN-based therapy from those who must be treated with DAAs to prevent the evolution towards end-stage liver disease.

Key words: Chronic hepatitis C; Caucasian patients; Core substitutions; *IL28B* polymorphism; Treatment

Core tip: The high cost of the newly introduced direct acting antivirals precludes universal replacement of the suboptimal interferon-based therapy for chronic hepatitis C. Therefore, a series of host- and virus-related factors are used as prognostic markers of treatment response. In Asian patients, a newly described viral factor is represented by amino acid substitutions in the hepatitis C virus core protein at positions 70 and 91. The present study confirms that core substitutions are also found in Caucasian patients and, together with age and IL28B genotype, can be used as predictors of the outcome of interferon-based therapy.

Sultana C, Oprişan G, Teleman MD, Dinu S; HepGen 88/2012 Project Team; Oprea C, Voiculescu M, Ruta S. Impact of hepatitis C virus core mutations on the response to interferon-based treatment in chronic hepatitis C. *World J Gastroenterol* 2016; 22(37): 8406-8413 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i37/8406.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i37.8406

INTRODUCTION

Hepatitis C might become the first curable chronic disease due the remarkable efficacy of the newly introduced direct acting antiviral drugs (DAAs). Interferon-free regimens, based on combinations of DAAs with pan-genotypic activity, allow for shorter courses of treatment without severe side effects[1]. Nevertheless, the high cost of DAAs continues to preclude universal replacement of the classic treatment consisting of PEGylated-interferon and ribavirin (PEGIFN/RBV). This therapeutic combination is effective in approximately 50% of hepatitis C virus (HCV) chronically infected patients, with the response rate strongly dependent on the infecting genotype[2] and correlated with a series of other viral and host factors, *e.g.,* baseline/on-treatment viral load, liver fibrosis, host IL28B polymorphisms[3-6].

Across the 9.6 kb genome of HCV, several regions (specifically HVR1, IFN sensitivity-determining region and an IFN/ribavirin resistance-determining region in NS5A[7,8]) have been extensively analyzed in relation to treatment outcome, whereas the more conserved core gene has been mostly used for HCV genotyping and classification. Nevertheless, the core region has been reported to antagonize the antiviral response induced by IFN by interacting with the IFN-activating and signaling pathways[9,10]. Substitutions in certain less conserved sites of the core region can give rise to viral quasispecies resistant to interferon treatment[11]. Several reports, mainly from Japan, have indicated that amino acid substitutions in positions 70 and 91 of the core protein are associated with the outcome of interferon-based therapy[12,13].

Because most of the studies related to these core mutations have been conducted in Asian populations, the aim of the present study is to determine whether HCV core substitutions are present and play a significant role in the outcome of interferon-based treatment in Caucasian patients, as well to inform better selection and prioritization of those patients who can still benefit from this affordable therapy.

MATERIALS AND METHODS

Study population

An observational study was conducted on 108 HCV chronically infected Caucasian patients treated for the

Table 1 Demographic, clinical and virological characteristics of study patients *n* (%)

Characteristics	Total *n* = 108	IL28B CC *n* = 19	IL28B non-CC *n* = 89	*P* value
Age (yr), median	53.5 (22.0-68.3)	48.2 (22.1-66.4)	53.8 (22.1-68.3)	0.137
Female	64 (59.3)	8 (42.1)	56 (62.9)	0.094
Residence urban	78 (72.2)	17 (87.5)	61 (68.5)	0.090
Baseline ALT (mg/dL), median	76.5 (16-890)	103 (16-890)	74 (17-256)	0.031
Baseline HCV-RNA (log10IU/mL), median	6.1 (4.15-7.3)	6.2 (4.15-7.2)	6.1 (4.15-7.3)	0.333
Mild/moderate fibrosis	38 (58.5)	9 (69.23)	29 (55.8)	0.470
Absence of core mutations - DW	13 (12.0)	6 (31.6)	7 (7.9)	0.011
RVR (%)	14 (13.0)	7 (36.8)	7 (7.9)	0.003
EVR (%)	43 (39.8)	16 (84.2)	27 (30.3)	< 0.001
SVR (%)	42 (38.9)	17 (89.5)	25 (28.1)	< 0.001

HCV: Hepatitis C virus; DW: Double wild-type; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virologic response.

first time with a combination of PEGylated IFN-a2a (180 μg per week) or PEGylated IFN-a2b (1.5 μg/kg per week) plus ribavirin (1000 or 1200 mg, dependent on body weight) in two tertiary care facilities in Bucharest, Romania. All patients met the following inclusion criteria: 18-65 years of age, detectable HCV viremia, and previously untreated. The exclusion criteria were: HBV or HIV co-infections' malignancies; coexistent liver disease of other etiology; organ recipients, clinically significant pulmonary, renal, cardiovascular or hematological diseases; current pregnancy and lactation. Each patient provided informed consent and the Bioethics Committee of the Institute of Virology approved the study.

Measurement of HCV-RNA

HCV-RNA was performed at baseline, weeks 4 and 12 of treatment and 24 wk after treatment completion using COBAS AmpliPrep/COBAS TaqMan Quantitative Test, version 2.0 (Roche Diagnostics GmbH, Germany) with a linear range of HCV-RNA quantification between 15 and 100000000 IU/mL. Rapid virologic response (RVR) was defined as undetectable HCV-RNA at week 4 of treatment. Early virological response (EVR) was defined as undetectable HCV-RNA at week 12 of treatment, and sustained virological response (SVR) was defined as undetectable HCV-RNA 6 mo after treatment completion.

Viral genotyping and detection of substitutions at codons 70 and 91 in core protein

Viral RNA was extracted from 140 μL serum using a commercial kit (QIAamp Mini Viral Kit, Qiagen). Reverse transcription was performed as described previously[14] and the cDNA was used in a semi-nested PCR yielding a 422 bp amplicon spanning the HCV core region[14,15]. The amplicons were sequenced (BigDye Terminator v3.1 and 3130 Genetic Analyzer, Applied Biosystems) and the resulting sequences were edited with BioEdit version 7.0.5.3[16] and used for genotyping (NCBI BLAST) and to assess the presence of substitutions at positions 70 and 91 in the core protein.

Genetic polymorphism in the IL28B gene (rs12979860)

Genetic polymorphism in the *IL28B* gene (rs12979860) was investigated using Custom TaqMan 5' allelic discrimination assay (Assays-by-DesignSM Service for SNP Genotyping Assays, Applied Biosystems, United States) and running a real time PCR on an ABI 7300 instrument with primers and fluorescent probes predesigned by the manufacturer and interpreted using SDS software from Applied Biosystems Inc., United States.

Liver fibrosis

Liver fibrosis was assessed using a noninvasive method - transient elastography (FibroScan™) - that discriminated between mild/moderate fibrosis (F1 + F2) and advanced fibrosis (F3 + F4) by assigning a value of liver stiffness lower or higher than 9.5 kPa[17].

Statistical analysis

Statistical analysis performed with IBM SPSS Statistics version 20. Univariate analysis was performed for both categorical and continuous variables; *P* values were calculated using the independent samples Mann-Whitney *U* test for continuous variables and Pearson χ^2 or Fisher's exact test for categorical variables. Variables with statistical significance ($P < 0.05$) in the univariate analysis were introduced into a multivariate logistic regression model.

RESULTS

Characteristics of patients and response to treatment

Patients' characteristics are summarized in Table 1. The median baseline HCV-RNA was 6.1 $\log_{10}$IU/mL. Of the 108 patients, 44.4% had a baseline HCV viral load lower than 600000 IU/mL (5.8 $\log_{10}$IU/mL) and 37.9% had mild or moderate fibrosis. All patients were infected with HCV genotype 1b. Only 38.9% (42 patients) achieved SVR, and modest percentages had prompt responses during therapy: 13% had RVR and 39.8% had EVR.

The favorable *IL28B* CC genotype was detected in only 17.6% of the patients, and had no significant

Table 2 Patients characteristics related to core mutation type, univariate analysis

Characteristics	Patients with DW-type infection *n* = 13	Patients with R70Q/H and/or L91M substitutions *n* = 95	Patients with L91M substitution *n* = 40	Patients with R70Q/H substitution *n* = 8	*P* value[1]
Age (yr) median	51.5 (29.1-66.54)	53.6 (22.1-68.3)	53.3 (24.4-67.3)	50 (33.8-58.8)	a = 0.607 b = 0.694 c = 0.860
Gender Female (%)	7 (53.8)	57 (60)	22 (55)	5 (62.5)	a = 0.767 b = 1.000 c = 1.000
Residence Urban (%)	11 (84.6)	67 (70.5)	33 (82.5)	5 (62.5)	a = 0.509 b = 1.000 c = 0.325
Baseline ALT (mg/dL) median	94 (3-235)	76 (16-890)	78 (17-890)	67 (54-197)	a = 0.592 b = 0.849 c = 1.000
Baseline HCV-RNA (log_{10}IU/mL), median	6.25 (4.15-6.9)	6.05 (4.15-7.3)	6.05 (4.1-7.3)	6.3 (5.2-7.1)	a = 0.966 b = 1.000 c = 0.414
Mild/moderate fibrosis (F1 + F2, %)	4 (57.1)	34 (58.6)	14 (58.3)	4 (57.14)	a = 0.881 b = 0.925 c = 0.283
IL28B CC (%)	6 (46.2)	13 (13.7)	8 (20.0)	1 (12.5)	a = 0.011 b = 0.080 c = 0.174
RVR (%)	6 (46.2)	8 (8.4)	4 (10.0)	0 (0.0)	a = 0.002 b = 0.009 c = 0.005
EVR (%)	10 (76.9)	33 (34.7)	15 (37.5)	3 (37.5)	a = 0.005 b = 0.024 c = 0.164
SVR (%)	10 (76.9)	32 (33.7)	15 (37.5)	2 (25.0)	a = 0.005 b = 0.024 c = 0.032

[1]*P* value symbols a, b, c: cases from each category type of viral substitutions (columns 3, 4, 5) *vs* cases without viral mutation [double wild-type (DW)-type - column 2]. HCV: Hepatitis C virus; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virologic response.

correlation with patients' demographic characteristics (age, gender, urban residence; Table 1).

Patients with *IL28B* genotype CC had the highest therapeutic success rates than those with TT or CT genotypes for the following outcomes: RVR (36.8% *vs* 7.9%, OR = 6.8, *P* = 0.003); EVR (84.2% *vs* 30.3%, OR = 12.2, *P* < 0.001), and SVR (89.5% *vs* 28.1%, OR = 21.8, *P* < 0.001).

Impact of core mutations on the treatment response

According to the sequencing results, only 12% of the patients were infected with double wild-type (DW) strains - defined as presence of arginine (R) and leucine (L) at core position 70 and 91, respectively - while the rest had glutamine/histidine at position 70 (R70Q/H), or/and methionine at position 91 (L91M). Of the patients, R70Q/H substitution was present in viral isolates infecting 7.4%, and L91M was observed in 37%, while patients displaying both mutations represented 43.5% of the study population.

The presence of any substitutions at positions 70 and 91 of the core protein was associated with lower rates of RVR, EVR, and SVR (Table 2). Patients infected with DW-type strains obtained SVR more frequently than patients with R70Q/H substitution (76.9% *vs* 37.5%, OR = 5.6, *P* = 0.032), or L91M substitution (76.9% *vs* 25.0%; OR = 12, *P* = 0.032). Furthermore, looking simultaneously at both mutation sites, patients infected with HCV DW-type strains obtained SVR significantly more frequently than patients with substitution at any 70 or 91 positions (76.9% *vs* 33.7%, *P* = 0.032; OR = 6.6, *P* = 0.005).

No significant differences related to demographic characteristics or virological parameters (HCV viremia, ALT, liver fibrosis) were detected between the patients infected with DW-type strains and those with R70Q/H and L91M substitutions (Table 2).

Predictive factors for treatment success

In the univariate analysis, young age, urban residence, IL28B CC genotype, absence of core mutations and achievement of RVR and EVR were significantly correlated with the rate of therapeutic success, defined as SVR (Table 3).

Direct logistic regression was performed to assess the impact of different viral and host factors on the likelihood of achieving SVR, and the overall model contained all predictors with statistically significance in the univariate analysis. In the multivariate analysis, young age (less than 50 years old), absence of any type of core mutations, and presence of IL28B CC genotype were independently associated with

Table 3 Predictive factors for sustained virological response, univariate analysis *n* (%)

Variable	SVR *n* = 42	No SVR *n* = 66	*P* value
Age (yr), median	48.1 (22.1-68.3)	54.7 (22.1-67.3)	< 0.001
Female gender	22 (52.4)	42 (63.6)	0.246
Urban residence (%)	37 (88.1)	41 (62.1)	0.004
Baseline ALT (mg/dL), median	79 (16-890)	75 (17-231)	0.688
Baseline HCV-RNA, median	6.2 (4.15-7.3)	6.2 (4.8; 7.3)	0.167
Mild/moderate fibrosis	18 (75.0)	20 (48.8)	0.107
IL28B type CC (%)	17 (40.5)	2 (3.0)	< 0.001
Absence of core mutations - DW	10 (23.8)	3 (4.5)	0.005
Presence of any core mutation, (%)	32 (76.2)	63 (95.5)	0.005
Viral mutation in core 91 only (%)	15 (60.0)	25 (84.3)	0.024
Viral mutation in core 70 only (%)	2 (16.7)	6 (66.7)	0.032
RVR (%)	13 (31.0)	1 (1.5)	< 0.001
EVR (%)	37 (88.1)	6 (9.1)	< 0.001

HCV: Hepatitis C virus; DW: Double wild-type; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virologic response.

Table 4 Multivariate analysis on independent predictive factors for sustained virological response

Variable	Adjusted OR	95%CI	*P* value
Age (< 50 yr)	0.976	0.967-0.986	< 0.001
Absence of core mutations	4.710	1.10-21.30	0.04
IL28B polymorphism	19.200	4.05-91.40	< 0.001

achieving SVR (Table 4).

This multivariate model correctly classified 75.9% of cases with a positive predictive value of 80.7%.

DISCUSSION

We report that the absence of substitutions in core positions 70 and 91 is a good predictor for achieving SVR after PEG-IFN/RBV treatment in Caucasian patients; together with *IL28B* polymorphisms and age, this absence can be used to stratify HCV-infected patients according to the likelihood of response to a currently suboptimal, but affordable, interferon-based therapy.

The success rate of IFN-based therapy was rather low in the present cohort, despite the fact that several baseline predictors suggested a promising patient profile (relatively young age (median: 53.5 years), low baseline HCV viral load in half of the cases, and minimal fibrosis in more than one-third of the cases). Nevertheless, the on-treatment viral kinetic response was modest, with a minority of patients achieving undetectable viral replication after 4 wk of therapy. An analysis of *IL28B* polymorphism rs12979860, the most important predictor of SVR in patients without RVR[18], revealed that non-CC *IL28B* genotypes were predominant (82.4%). These variants are associated with endogenous activation of the innate immune responses, higher baseline expression levels of IFN-stimulated genes, and a constant activation of the IFN signaling pathway that renders patients unresponsive to IFN treatment[18,19]. The exclusive presence of subtype 1b and the low prevalence of the favorable CC genotype can explain, at least in part, the low rate of virological response, but a series of others factors must be taken into consideration: questionable or inconsistent patient adherence to treatment, presence of adverse events that could determine temporary treatment interruptions (none were acknowledged by the study patients), and preexisting mutations in other genomic regions that could render the virus less susceptible to the prescribed drugs. In this study, core protein substitutions at positions 70 and 91 were present in viral isolates infecting 88% of the patients and linked with a significantly decreased probability of achieving SVR.

There has been increased evidence that substitutions at position 70 in the core protein are found regularly in Asian HCV 1b infected patients, with the mutant clone R70Q detectable even in newly infected people and no distinguishing characteristics for the mutant strain in terms of viral fitness or demographic distribution[20]. Studies investigating HCV core mutations in Caucasian patients are scarce, but two studies of very limited numbers of patients have suggested an association between R70 and an increased response to therapy[21,22].

In our study, we detected high rates of core protein substitutions at positions 70 and 91 in 108 Caucasian patients infected with HCV subtype 1b; these substitutions had no significant association with viral load, but were significantly associated with a low therapeutic success rate. Our results are in accordance with previous reports that indicated an absence of substitutions at positions 70 and 91 in core protein as a significant predictor for the success of IFN-based therapy[12,21,22]. These results have also been confirmed *in vitro*; Funaoka *et al*[23] evaluated the effect of interferon-alpha on HCV core mutants (R70Q/H and L91M) in terms of viral replication and response to treatment and found a significantly higher degree of IFN resistance compared to the wild-type virus, associated with decreased expression of the IFN-stimulated genes. The proposed hypothesis for the interferon resistance of core mutants was related to the inhibition of the interferon signaling pathway,

potentially involving SOCS3 (suppressor of cytokine signaling). These proteins are stimulated by various cytokines including IL6, which was upregulated in cells transfected with a core mutant. This mechanism can be observed *in vivo* as well, as chronically HCV infected patients have increased levels of inflammatory cytokines, including IL-6 and TNF-alpha[24].

Another interesting mechanism that might explain the role of core substitutions in interferon resistance is their potential influence on the expression of minicore proteins- isotypes of the normal core protein, that lack an N-terminal segment[25]. Two important minicore proteins terminate in the vicinity of amino acids 70 and 91; consequently, any structural changes in these amino acids can alter the expression of minicore proteins and implicitly the HCV functioning and IFN sensitivity[11].

To our knowledge, this is one of the first studies conducted on Caucasian patients that extend and confirm the results obtained in Asian populations related to the impact of amino acid substitution in the HCV core region on treatment response. Although our study does not involve a very large number of patients, further sampling is unlikely, as the clinical facilities investigated in this study treat subjects from all over the country. Performing mathematical modeling of the cost-effectiveness of sequencing for HCV core mutations would be beneficial. Although the cost of this test may be rather high, it is significantly lower than the prohibitive cost of DAAs. Several recent studies have attempted to provide an estimate on the cost-effectiveness of interferon-free regimens (assuming a price of $100000 and a success rate of 90%). Their results support a delay in treatment for patients with mild degrees of fibrosis[26-28]. As such, the potentially beneficial role of core sequencing in selecting patients from this subclass who are responsive to interferon-based therapy may outweigh the potential price limitation. This is particularly true for at this time, when there is a constant need for ethical, evidence-based criteria for the prioritization of interferon-free treatment in countries that cannot yet afford the universal introduction of the new highly active antivirals. In addition, there have been reports that HCV core substitutions can also predict the primary outcomes of therapy using first generation protease inhibitors[29] and that the *IL28B* genotype is furthermore predictive of the response to triple therapy in patients infected with HCV genotype 1[30]. Consequently, future studies will be needed to extend our results to patients treated with the novel categories of antivirals recently introduced for the treatment of HCV chronic infection.

Moreover, recent studies have also indicated that HCV core substitutions are involved in the progression of chronic hepatitis C to hepatocellular carcinoma (HCC)[31]. The R70Q variant has been associated with an increased malignancy risk[32-34], while the implication of core 91 substitution was dubitable[11]. A very recent study using deep-sequencing reported that the presence of baseline HCV strains harboring more than 42% non-R70 quasispecies or more than 98.5% non-L91 mutants was associated with an increased HCC risk[35]. As long as the residual risk for HCC development in compensated cirrhotic patients with SVR after IFN treatment remains quite high (3.4% at 5 years and 23.7% at 20 years[36]), the impact of core mutations on the transforming capacity of HCV core protein is worth studying.

In conclusion, this study reports absence of core genomic mutations associated with IL28B CC polymorphism as prognostic markers for a favorable outcome in HCV chronically infected Caucasian patients treated with interferon-based regimens. Core genomic mutations can be used to tailor treatment and distinguish between those patients who can respond to the affordable bi-therapy and those who must be urgently treated with DAAs to prevent evolution towards end-stage liver disease or HCC.

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COMMENTS

Background

Interferon-free regimens, based on combinations of direct acting antivirals (DAAs) with pan-genotypic activity, have a remarkable efficacy and might transform hepatitis C into a curable chronic disease. Nevertheless, the high cost of DAAs still precludes the universal replacement of the classic PEGylated-interferon and ribavirin therapy (PEG-IFN/RBV) that is dependent on a series of host and viral factors. In Asian patients, a newly described viral factor influencing the outcome of bi-therapy treatment is represented by amino acid substitutions in hepatitis C virus (HCV) core protein positions 70 and 91.

Research frontiers

There is very scarce information on the presence and significance of HCV core substitutions in Caucasian populations. Nevertheless, a number of studies have indicated that amino acid substitutions in the core protein play an important role in the very early dynamics of viral replication during bi-therapy and triple therapy of chronic hepatitis C as well as in the evolution toward hepatocellular carcinoma.

Innovations and breakthroughs

This is one of the first studies confirming that HCV core substitutions are not specific to the Asian population, being also found in Caucasian patients. Moreover, it demonstrates that absence of core genomic mutations, together with young age and *IL28B* CC genotype, is a prognostic marker for favorable outcome in chronic HCV-infected Caucasian patients treated with PEG-IFN/RBV.

Applications

The research hotspot is the identification of a new viral factor that can help distinguish between patients who can still benefit from the affordable IFN-based therapy from those who must be urgently treated with DAAs to prevent the evolution towards end-stage liver disease. This is an important practical instrument in countries with developing economies that cannot afford universal introduction of DAA because it can facilitate the prioritization of patients who will benefit from less expensive therapeutic regimens. Further application of these results can be derived from the recently reported role of core substitution in the progression of HCV infection to hepatocellular carcinoma. As long as a residual risk for HCC development persists even in patients successfully treated with interferon or with IFN-free regimens, the impact of core mutations on the transforming capacity of the HCV core protein is worth studying.

Terminology

HCV core gene is a conserved part of the viral genome that is mostly used for HCV genotyping and classification. Nevertheless, the core region can antagonize the antiviral response induced by IFN, interacting with the IFN-activating and signaling pathways. Substitutions in less conserved sites (positions 70 and 91 in the core region) can contribute to resistance to interferon treatment.

Peer-review

The manuscript is very well written and clearly states its aims and conclusions. It looks overall good with some limitations of low number of patients and low SVR. Questions were raised concerning the cost of core mutations testing. A paragraph responding to the study limitations and the necessity of core sequencing cost effectiveness evaluation was added by the authors in the revised version.

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Defining response to radiotherapy in rectal cancer using magnetic resonance imaging and histopathological scales

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Abstract

AIM

To define good and poor regression using pathology and magnetic resonance imaging (MRI) regression scales after neo-adjuvant chemotherapy for rectal cancer.

METHODS

A systematic review was performed on all studies up to December 2015, without language restriction, that were identified from MEDLINE, Cochrane Controlled Trials Register (1960-2015), and EMBASE (1991-2015). Searches were performed of article bibliographies and conference abstracts. MeSH and text words used included "tumour regression", "mrTRG", "poor response" and "colorectal cancers". Clinical studies using either MRI or histopathological tumour regression grade (TRG) scales to define good and poor responders were included in relation to outcomes [local recurrence (LR), distant recurrence (DR), disease-free survival (DFS), and overall survival (OS)]. There was no age restriction or stage of cancer restriction for patient inclusion. Data were extracted by two authors working independently and using pre-defined outcome measures.

RESULTS

Quantitative data (prevalence) were extracted and analysed according to meta-analytical techniques using comprehensive meta-analysis. Qualitative data (LR, DR, DFS and OS) were presented as ranges. The overall proportion of poor responders after neo-adjuvant chemo-radiotherapy (CRT) was 37.7% (95%CI: 30.1-45.8). There were 19 different reported histopathological scales and one MRI regression scale (mrTRG). Clinical studies used nine and six histopathological scales for poor and good responders, respectively. All studies using MRI to define good and poor response used one scale. The most common histopathological definition for good response was the Mandard grades 1 and 2 or Dworak grades 3 and 4; Mandard 3, 4 and 5 and Dworak 0, 1 and 2 were used for poor response. For histopathological grades, the 5-year outcomes for poor responders were LR 3.4%-4.3%, DR 14.3%-20.3%, DFS 61.7%-68.1% and OS 60.7-69.1. Good pathological response 5-year outcomes were LR 0%-1.8%, DR 0%-11.6%, DFS 78.4%-86.7%, and OS 77.4%-88.2%. A poor response on MRI (mrTRG 4,5) resulted in 5-year LR 4%-29%, DR 9%, DFS 31%-59% and OS 27%-68%. The 5-year outcomes with a good response on MRI (mrTRG 1,2 and 3) were LR 1%-14%, DR 3%, DFS 64%-83% and OS 72%-90%.

CONCLUSION

For histopathology regression assessment, Mandard 1, 2/Dworak 3, 4 should be used for good response and Mandard 3, 4, 5/Dworak 0, 1, 2 for poor response. MRI indicates good and poor response by mrTRG1-3 and mrTRG4-5, respectively.

Key words: Tumour regression; mrTRG; Poor response; Neo-adjuvant therapy; Rectal cancer

Core tip: The degree of primary tumour regression following neo-adjuvant therapy identified on final histopathological specimens is a prognostic factor and response variation has allowed risk stratification, aiding in post-surgical treatment and follow-up decisions. To do this effectively, we need to have a common language for defining good and poor response. Definitions of response using histopathology scales are heterogenous with 19 different scales. There is one pre-operative magnetic resonance imaging (MRI) scale. Outcomes of recurrence and survival histopathology regression assessments should use Mandard 1, 2/Dworak 3, 4 for good response and Mandard 3, 4, 5/Dworak 0, 1, 2 for poor response. MRI indicates good and poor response by mrTRG1-3 and mrTRG4-5, respectively.

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INTRODUCTION

Rationale

The multidisciplinary treatment of rectal cancer has markedly improved and led to better patient outcomes over the last three decades[1]. The reasons for this are multifactorial, but one important factor is the use of neo-adjuvant or adjuvant therapies[2].

The degree of primary tumour regression following neo-adjuvant therapy, identified on final histopathological specimens, has been shown to be a prognostic factor[3,4]. The variation in response allows clinicians to risk-stratify patients after surgery, which may help in post-operative decisions, such as who to treat with adjuvant chemotherapy and the intensity of follow-up.

Clinical studies use a number of different tumour regression grade (pTRG) scales to classify the degree of tumour response to neo-adjuvant chemo-radiotherapy (CRT). This often results in confusion as to whether a good or poor response has been achieved, with subsequent uncertainty regarding treatment and prognostic implications. This problem was highlighted by MacGregor *et al*[1] who stressed the importance of a universally accepted standard.

There has been no review of the reported pTRG scales to date. It is necessary to highlight the heterogeneity in these scales, in order to consolidate the current definitions with the purpose of converging towards a set of consensus definitions.

A newer method of assessing tumour regression relies on MRI (mrTRG), which has been validated as a prognostic tool. This may supercede pTRG, as it has the advantage of assessing tumour response before surgery. As such, it has the potential for enabling response-orientated tailored treatment, including alteration of the surgical planes, additional use of chemotherapy, or deferral of surgery[5-7].

Objective

This article investigates all the pathology tumour regression scales used to define good and poor response after neo-adjuvant chemotherapy for rectal cancer, to establish the true prevalence of poor responders and to identify the best scales to use in relation to outcomes.

MATERIALS AND METHODS

Protocol and registration

The title, methods and outcome measures were stipulated in advance and the protocol is available in the PROSPERO database[8].

Types of studies

All clinical, histopathological and imaging studies that define or attempt to define good and poor responders after neo-adjuvant therapy for colorectal cancers were identified. Included studies were those investigating rectal cancer response to neo-adjuvant therapy incorporating chemotherapy, radiotherapy or chemo-radiotherapy with different protocols. All clinical studies were chosen that defined good and poor response in relation to TRG or degree of response according to histopathology using terms such as "poor response", "minor response", "less response", "good response", "major response" or "more response".

Types of participants

All rectal cancer patients treated with long course radiotherapy or an interval period to surgery were selected for this review. All sensitizing chemotherapy protocols were included. Any surgical resection was included. Studies were also included with any post-operative adjuvant practice.

Exclusion criteria

Excluded studies were those that did not specifically state whether a response was good or poor, or that qualify it with some form of inference in the paper. Further exclusions were for: non-conventional deliveries of neo-adjuvant therapy, such as endo-rectal brachytherapy; trans-anal endoscopic microsurgery (commonly known as TEMS) and local excisions; and, when the reporting scale was in obvious contradiction with the order given in the original studies[9].

Types of variable of interest

The original papers reporting the various pTRG scales were identified and articles that used the scales in clinical, pathological and MRI studies were used in the current study.

Hypotheses and types of outcome measures

The primary hypothesis was that there is an optimal histopathological TRG scale that appropriately distinguishes between good and poor response. The secondary hypothesis was that the mrTRG scale differentiates between good and poor response. This was investigated by first reviewing the clinical studies examining the response of rectal cancer to neo-adjuvant therapy. These studies were used to show the range of definitions of good and poor response according to histopathology and MRI. This was then utilised to identify the optimal scale for identifying good and poor response after neo-adjuvant therapy for rectal cancer based on recurrence and survival outcomes.

Information sources

The Cochrane library, CENTRAL, EMBASE, CINAHL and PubMed databases were searched between January 1935 and December 2015. Relevant articles referenced in these publications were obtained and the "related article" function was used to widen the results. This was complemented by hand searches and cross-references from papers identified during the initial search. No language restriction was applied.

Searches

The text words "preoperative", "neo-adjuvant", "tumour regression", "poor responder", "good responder", "regression grading", "regression grade" and "rectal cancer" were used in combination with the medical subject headings "adjuvant combined modality therapy" and "rectal cancer". Irrelevant articles not fulfilling the inclusion criteria were excluded.

Study selection and data collection process

Each included article according to our review criteria was reviewed by two researchers (MRSS and JB). Where more specific data or missing data was required, the authors of the manuscripts were contacted. Data was entered onto an Excel worksheet and compared between authors. Any disagreements that arose between the reviewers were resolved through discussion, and if no consensus could be reached a third author (GB) would decide.

Data items

Data were extracted that related to the definition of good and poor response according to the TRG scales reported in clinical, histopathological and imaging studies. The ranges of permutations of each TRG scale to define good or poor response were also documented and the most commonly used definitions identified. The primary hypothesis was proven by examining all of the studies on response to neo-adjuvant therapy and there is a single definition (which may include other scales) that consistently differentiates between good and poor responses as defined by local recurrence (LR), distant recurrence (DR), disease-free survival (DFS) and overall survival (OS).

Risk of bias and quality assessment

Quality assessment and risk of bias was not formally assessed due to the exploratory nature of this review. Validity of other studies was benchmarked to studies that identified a significant difference. Clinical heterogeneity can be seen in the table of characteristics presented as Table 1.

Summary measures and data synthesis for summative and comparative meta-analyses

As part of assessing overall prevalence of poor responders, cumulative meta-analytical techniques were used. Analyses were performed using Comprehensive Meta-Analysis 2006 (Version 2, Biostat, Englewood, NJ, United States) for Windows 10[10]. In a sensitivity analysis, 0.5 was added to each cell frequency for

Table 1 Characteristics of studies reporting on good or poor response based upon histopathology

Ref.	Year	Chemotherapy protocol with radiotherapy	Radiotherapy protocol (Gy)	Surgical procedures	TME	Time to surgery (wk)	Cancer stage pre neo-adjuvant therapy	Adjuvant therapy
Gambacorta *et al*[21]	2004	Ralitrexed	50.4	APR/AR/Col-Anal resection/ Stoma	Y	6-8	Stage 2 or 3	Y
Pucciarelli *et al*[28]	2004	Fluorouracil, leucovorin carboplatin, oxaliplatin	45-50.4	APR/AR/ Hartmann's	Y	2-8	T2/3/4, N0/1/2	Y
Beddy *et al*[17]	2008	Fluorouracil	45-50	APR/AR	Y		T3/4, N1/2	
Giralt *et al*[22]	2008	Tegafir uracil, leucovorin	45 + 9 boost	APR/AR	Y	4-6	T3/4, N0/1/2	Y
Horisberger *et al*[24]	2008	Capecitabine, irinotecan	50.4	APR/AR/stoma	Y	4-7	T2/3/4, N+	
Suárez *et al*[31]	2008	Fluoropyridine-based	50.4	APR/AR/ Hartmann's	Y	6	Stage 2 or 3	Y
Bujko *et al*[18]	2010	Fluorouracil, leucovorin	50.4	APR/AR/ Hartmann's	Y	4-6	Stage 2 or 3	Y
Avallone *et al*[13]	2011	Fluorouracil, levo-folinic acid, ralitrexed, oxaliplatin	45.0	APR/AR/Stoma	Y	< 8	T3/4, N0/1/2	Y
Eich *et al*[19]	2011	Fluorouracil	50.4	APR/AR/TEMS/ Intersphincteric Surgery	Y	4-6	Stage 1,2 or 3	Y
Min *et al*[27]	2011	Fluorouracil, leucovorin	50.4	APR/AR	Y	6	T3/4, N0/1/2	
Shin *et al*[30]	2011	Fluorouracil	25-50.4	APR/AR/Pan		4-6	T3/4	
Huebner *et al*[25]	2012	Fluorouracil		APR/AR			T1/2/3/4, N0/1/2	Y
Lim *et al*[26]	2012	Capecitabine, fluorouracil, leucovorin	44-46+4.6 boost	Radical Proctectomy	Y		T3/4, N+	Y
Roy *et al*[29]	2012	Capecitabine, fluorouracil	45-50		Y	4-6	T1/2/3/4, N0/1/2	Y
Vallböhmer *et al*[32]	2012	Fluorouracil	50.4	APR/AR	Y		T3/4, N0/1/2	
Winkler *et al*[33]	2012	Capecitabine, oxaliplatin	45-50.4		Y	4-6	Stage 2 or 3	Y
Elezkurtaj *et al*[20]	2013	Fluorouracil	50.4		Y	4-6		
Hermanek *et al*[23]	2013			APR/AR/ Hartmann's	Y			Y
Fokas *et al*[14]	2014	Fluorouracil	50.4	APR/AR	Y	4-6	T3/4 or any T and N+	Y
Santos *et al*[16]	2014	Fluorouracil	50.4	APR/AR	Y	< 8	T2N+ or T3/4	Y
Hav *et al*[15]	2015	Fluorouracil, cetuximab, oxaliplatin	25-45	AR/Hartmann's	Y	6-8	T3/4 or any T and N+	

APR: Abdominoperineal resection; AR: Anterior resection; Pan: Panproctocolectomy; Col-Anal: Colorectal and anal resection; TME: Total mesorectal excision; Gy: Gray.

trials in which no event occurred, according to the method recommended by Deeks *et al*[11] and was not considered to affect the overall result necessitating the Peto method[12]. Where only a single patient was present in any of the groups, this was excluded due to the excessive effect of zero cell correction. Outcomes were reported as event rates. Forest plots were used for the graphical display.

Publication bias

For the outcome of prevalence, publication bias was assessed using funnel plots. We used the plots to subjectively assess asymmetry and conducted an Egger test for quantitative assessment.

RESULTS

Study selection and characteristics

There were 328 references. Full texts of 85 papers were reviewed. Overall, 21 articles were of relevance and reported 25 definitions for poor response in accordance with the TRG[13-33]. Of these, 16 articles also defined good response. Table 1 shows the characteristics of individual studies.

Qualitative and quantitative syntheses

Histopathological methods of classifying regression: There were 19 TRG scales reported across the studies[18,25,34-51] (Table 2). Only one TRG system incorporated whether a response was poor or good[36] and used a categorical TRG scale based on the one described by Dworak *et al*[35].

Which scales are used to define poor response using histopathological methods?

From the search, nine scales[18,25,34-36,38,40,43,44,46] were used in 25 reports (21 articles) to define poor response[13-33]. From these 25 reports, the nine scales were used in different combinations to produce 16 individual definitions of poor response (Table 3).

Table 2 Summary of histopathological tumour regression grade scales available in the literature for rectal cancer after neo-adjuvant treatment

TRG scale	Mandard (Low no. - More regression)[43]
0	
1	Complete regression - absence of residual cancer and fibrosis
2	Presence of rare residual cancer
3	An increase in the number of residual cancer cells, but predominantly fibrosis
4	Residual cancer outgrowing fibrosis
5	Absence of regressive changes
TRG scale	Modified Mandard (Ryan) (Low no. - More regression)[37]
0	
1	TRG 1 and 2 of the Mandard scale
2	TRG 3 of the Mandard scale
3	TRG 4 and 5 of the Mandard scale
4	
5	
TRG scale	Werner and Hoffler (Low no. - More regression)[41]
0	
1	0% viable tumour cells
2	< 10% viable tumour cells
3	10%-50% viable tumour cells
4	> 50% viable tumour cells
5	No regression
TRG scale	Dworak (Low no. - Less regression)[35]
0	No regression
1	Dominant tumour mass with obvious fibrosis and/or vasculopathy
2	Dominant fibrotic change with few tumour cells or groups(easy to find)
3	Very few tumour cells in fibrotic tissue with or without mucous substance
4	No tumour cells, only fibrotic mass (total regression or response)
5	
TRG scale	Modified Dworak (Low no. - Less regression)[38]
0	No regression
1	Regression ⩽ 25% of tumour mass (dominant tumour mass with obvious fibrosis and/or vasculopathy)
2	Regression > 25%-50% of tumour mass (dominantly fibrotic changes with few tumour cells of groups, easy to find)
3	Regression > 50% of tumour mass (very few tumour cells in fibrotic tissue with or without mucous substance)
4	Complete (total) regression (or response): no vital tumour cells
5	
TRG scale	AJCC 7th Edition[48]
0	Complete-no viable cells present
1	Moderate-single cells/small groups of cancer cells
2	Minimal-residual cancer outgrown by fibrosis
3	Poor-minimal or no tumour kill, extensive residual cancer
4	
5	
TRG scale	Memorial Sloan-Kettering (Low no. - Less regression)[47]
0	0%-85% regression
1	86-99% regression
2	100% regression
3	
4	
5	
TRG scale	Cologne (Low no. - Less regression)[40]
0	
1	> 50 % Viable rectal tumour cells
2	10%-50% Viable rectal tumour cells
3	Near complete regression with < 10% Viable rectal tumour cells
4	Complete regression (pathologic complete remission and ypT0)
TRG scale	Bujko/Glynne Jones (Low no. - More regression)[18,44]
0	No cancer cells
1	A few cancer foci in less than 10% of tumour mass
2	Cancer seen in 10%-50% of tumour mass
3	Cancer cells seen in more than 50% of tumour mass
4	
TRG scale	College of American Pathologists[50]
0	Complete response: No residual tumour
1	Marked response: Minimal residual cancer
2	Moderate response: Residual cancer outgrown by fibrosis
3	Poor or no response: Minimal or no tumour kill; extensive residual cancer
4	
TRG scale	RCPath system (Low no. - More regression)[42]
0	
1	No residual cells and/or mucus lakes only
2	Minimal residual tumour *i.e.*, microscopic residual tumour foci only
3	No marked regression
4	
TRG scale	RCRG system (Low no. - More regression)[34]
0	
1	Sterilisation or only microscopic foci of adenocarcinoma with marked fibrosis
2	Marked fibrosis but macroscopic disease present
3	Little or no fibrosis with abundant macroscopic disease
4	
TRG scale	Mod RCRG system (Low no. - More regression)[45]
0	
1	Macroscopic features may be varied. Microscopy reveals no tumour or < 5% of area of abnormality
2	Macroscopic features may be varied. Microscopy reveals combination of viable tumour and fibrosis. Tumour comprises 5%-50% of overall area of abnormality
3	Macroscopic or microscopic features may not be significantly different. Over 50% comprises tumour. Some fibrosis may be present but no more than untreated cases
4	
TRG scale	Japanese (Low no. - Less regression)[25]
0	No regression
1a	Minimal effect (necrosis less than 1/3)
1b	Mild effect (necrosis less than 2/3 but more than 1/3)
2	Moderate effect (necrosis more than 2/3 of the lesion)
3	No tumour cells
TRG scale	Ruo (Low no. - Less regression)[39]
0	No evidence of response
1	1% to 33% response
2	34% to 66% response
3a	67% to 95% response
3b	96% to 99% response
4	100% response (no viable tumour identified)
TRG scale	Junker and Muller (Low no. - Less regression)[46]

1	No regression
2a	> 10% residual tumour cells
2b	< 10% residual tumour cells
3	Total regression (no viable tumour cells)
TRG scale	Rodel (Low no. - Less regression)[36]
Poor	TRG 1 and 0 of the Dworak scale
Intermediate	TRG 2 and 3 of the Dworak scale
Complete	TRG 4 of the Dworak scale
TRG scale	Four point scale Swellengrebel *et al*[49]
pCR	Pathological complete response without residual primary tumour
Near pCR	Isolated residual tumour cells/small groups of residual tumour cells
Response	Stromal fibrosis outgrowing tumour
No response	No regression or those with stromal fibrosis outgrown by tumour
TRG scale	Modified Mandard TRGN by Dhadda *et al*[51]
TRGN 1	Complete regression with absence of residual cancer and fibrosis extending through the wall
TRGN 2	Presence of rare residual cancer cells scattered through the fibrosis
TRGN 3	An increased number of residual cancer cells, but fibrosis is still predominant

The overall proportion of poor responders after neo-adjuvant CRT was 37.7% (95%CI: 30.1-45.8) (Table 4, Figure 1). Study characteristics can be seen in Table 1. Table 5 shows the scales that define poor response with their permutations. Most studies used the Mandard or Dworak TRG scales. The studies using the Mandard scale[13,16,21,22,28-31] defined poor response as Mandard TRG 3 to 5, 4 or 4 to 5. The Dworak scale uses a similar numerical scale in the opposite direction to the Mandard system. From the articles that use the Dworak classification for their definitions[14-16,20,25,26,29,33], a poor response was defined as Dworak 0 to 1, 1, 1 to 2 or 0 to 2.

Outcomes of poor response defined by histopathological scales

Fourteen studies that defined poor response reported on outcomes (Table 5). LR at 5 years ranged from 2% to 26%[17,18,23,26,27,31], DR was 14.3% to 47%[18,23,26,27,31]. One study reported 10-year LR and DR of 3.6% and 39.6%, respectively[14]. Two-year DFS was 60.3% to 83.6%[19,29,31], 3-year DFS was 72.6% to 73.8%[30,31], 4-year DFS was reported by a single study as 47%[18], 5-year DFS was reported as 56% to 71%[13,16,17,23,26], and 10-year DFS was documented as 63%[14]. OS at 2 years was 87.3% to 92.6%[29] and at 5 years was 60.7% to 75.8%[16,23,26].

Which scales are used to define good response?

Six scales[18,25,35,40,43,44,46] were used in 20 reports (16 articles) to define good response[13-16,18,20,21,24-26,28-33]. These six scales produced 12 different definitions of good response (Table 2). The characteristics of these studies are shown in Table 1. Table 6 shows the scales defining good response along with their permutations.

Table 3 Permutations of regression scales to define poor and good response

Poor response		Good response	
TRG grading system	**Studies that used the scale**	**TRG grading system**	**Studies that used the scale**
Mandard TRG 3,4,5	Suárez *et al*[31] Santos *et al*[16]	Mandard TRG 1,2	Suárez *et al*[31] Gambacorta *et al*[21] Santos *et al*[16]
Mandard TRG 4	Gambacorta *et al*[21] Giralt *et al*[22]	Mandard TRG 2,3	Avallone *et al*[13]
Mandard TRG 4,5	Avallone *et al*[13] Roy *et al*[29] Pucciarelli *et al*[28] Shin *et al*[30]	Mandard TRG 1,2,3	Roy *et al*[29] Pucciarelli *et al*[28] Shin *et al*[30]
Dworak 1	Winkler *et al*[33]	Dworak TRG 2,3,4	Huebner *et al*[25] Roy *et al*[29]
Dworak TRG 0,1	Huebner *et al*[25] Roy *et al*[29] Fokas *et al*[14]	Dworak TRG 2,3	Fokas *et al*[14]
Dworak TRG 1,2	Lim *et al*[26]	Dworak TRG 3,4	Lim *et al*[26] Elezkurtaj *et al*[20] Santos *et al*[16] Hav *et al*[15]
Dworak TRG 0,1,2	Elezkurtaj *et al*[20] Hav *et al*[15] Santos *et al*[16]	Dworak TRG 3	Winkler *et al*[33]
Rodel TRG 3 [Dworak 0,1]	Min *et al*[27]	Japanese TRG 2,3	Horisberger *et al*[24]
Rodel TRG 3 [Wittekind (mod Dworak 0,1)]	Hermanek *et al*[23]	Japanese TRG 3	Vallböhmer *et al*[32]
Japanese TRG 0,1a,1b	Horisberger *et al*[24]	Miller Junker TRG 2a and 2b	Vallböhmer *et al*[32]
Japanese TRG 1	Vallböhmer *et al*[32]	Cologne TRG 3 and 4	Vallböhmer *et al*[32]
Miller Junker TRG 1	Vallböhmer *et al*[32]	Glynne Jones TRG 1	Bujko *et al*[18]
Miller Junker TRG 1,2a	Eich *et al*[19]		
Cologne TRG 1,2	Vallböhmer *et al*[32]		
Glynne Jones TRG 3	Bujko *et al*[18]		
Wheeler RCRG TRG 2	Beddy *et al*[17]		

Outcomes of good response defined by pathological scales

Ten studies reported on outcomes (Table 6). Most studies defined good response as Mandard 1 to 2, 1 to 3, 2 to 3 or Dworak 2 to 4, 3 to 4 or 2 to 3. LR at 5 years after a good response ranged from 0% to 9%[16,18,26,31] and DR was reported as 0% to 34%[16,18,26,31]. One study reported 10-year LR and DR of 8.0% and 29.3%, respectively[14]. Two-year DFS was 86.1% to 91.7%[29], 3-year DFS was 74.1%[30], 4-year DFS was 67%[18], 5-year DFS was 78.4% to > 90%[13,16,26], and 10-year DFS was 73.6%[14]. OS at 2 years was 89.2% to 92.2%[29], and at 5 years OS was

Table 4 Proportion of poor responders in the literature according to regression grades

TRG grading system	No. of reports (total 25 reports from 21 studies)	Proportion of poor responders	Lower limit of confidence Interval	Upper limit of confidence Interval
Mandard	8	34.9	22.8	49.4
Dworak	8	47.4	32.5	62.7
Junker/Muller	2	50.8	28.8	72.5
Japanese	2	35.0	20.4	52.9
Wheeler	1	38.9	30.8	47.7
Bujko/Glynne-Jones	1	22.1	15.8	30.0
Rodel based on Dworak	1	52.2	44.9	59.5
Rodel based on Wittekind (modified Dworak)	1	14.7	10.6	19.9
Cologne	1	7.1	3.2	14.8

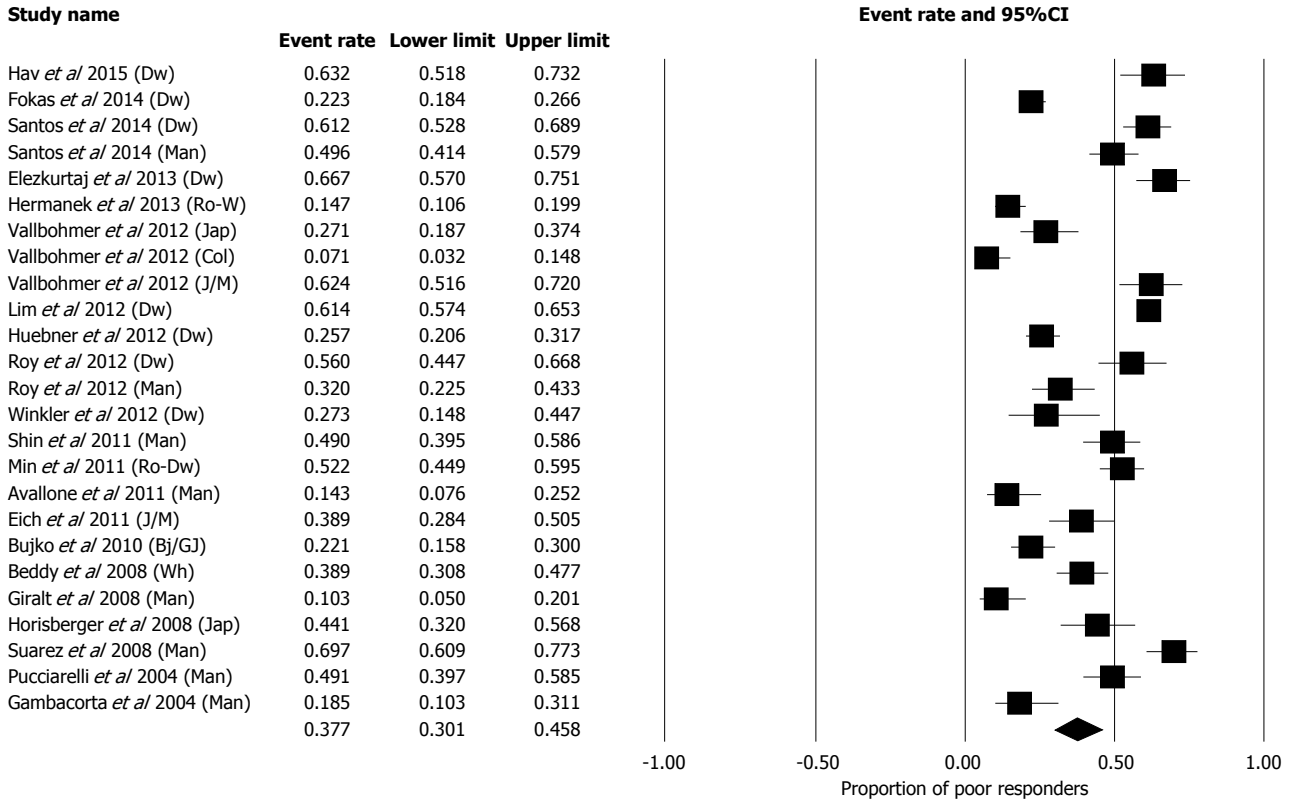


Figure 1 Proportion of patients who responded poorly to neo-adjuvant therapy.

77.4% to 88.2%[16,26].

Considerations and comparison between good and poor responders

A range of survival outcomes existed for good and poor response (Table 7). There were 15 reports (11 articles) comparing outcomes from good and poor response[13-16,18,26,28-32]. Four outcome measures were examined in detail: LR, DR, DFS and OS.

Studies differentiating between good and poor responders for LR

Six reports from five studies[14,16,18,26,31] compared good and poor response in relation to LR (Figure 2). Of these, one study reported a non-significantly higher LR in good responders compared with poor responders[14]. Five reports[16,18,26,31] showed LR was higher in poor responders, of which only one study showed a significant difference[26]. Using the definition given by Lim *et al*[26] there were three other studies with similar definitions[16,31]. The reported LR for good response ranged from 0% to 1.8%[16,26,31]. There were no studies that agreed with Lim *et al*[26] for the definition of poor response. Three studies[16,31] agreed with each other for poor response and reported LR of 3.4% to 4.3%. Lim *et al*[26] (which showed a significant difference between good and poor) gave LR rate in poor responders of 9.5%. This indicates that either Mandard 1 to 2 or Dworak 3 to 4 should be used to define good response for LR and Mandard 3 to 5 or Dworak 0 to 2 or 1 to 2 should be

Table 5 Study definitions of poor response according to histopathological tumour regression grade scales

Ref.	Year	TRG scale used (original disease application)	Are the scales reported accurately?	Poor response definition	Total (*n*)	Poor responders (*n*)	Average F/up in months	LR (%) 5 yr	DR (%) 5 yr	DFS (%)	OS (%)
Gambacorta *et al*[21]	2004	Mandard (oesophagus)	Yes	TRG 4	54	10	25				
Pucciarelli *et al*[28]	2004	Mandard (oesophagus)	Yes	TRG 4 and 5	106	52	42				
Beddy *et al*[17]	2008	Wheeler (rectal)	Yes	TRG 2	126	49	37	2[1]		Yr. 5: 71	
Giralt *et al*[22]	2008	Mandard (oesophagus)	No	TRG 4	68	7					
Horisberger *et al*[24]	2008	Japanese Society for Cancer of the Colon and Rectum (rectal)	Yes	TRG 0 and 1a and 1b	59	26					
Suárez *et al*[31]	2008	Mandard (oesophagus)	Yes	TRG 3 and 4 and 5	119	83	33	3.4[1]	14.3[1]	Yr. 2: 83.6 Yr. 3: 73.8	
Bujko *et al*[18]	2010	Glynne Jones/Bujko (rectal)	Yes	TRG 3	131	29	48	26	47	Yr. 4: 47	
Avallone *et al*[13]	2011	Mandard (oesophagus)	Yes	TRG 4 and 5	63	9	60			Yr. 5: Prob free of recurrence 56[2]	
Eich *et al*[19]	2011	Müller and Junker (lung)	Yes	TRG 1 and 2a	72	28	28			Yr. 2: 76 ± 14.8	
Min *et al*[27]	2011	Rodel (rectal based on Dworak)	Yes	Categorised as poor according to Rodel and based on TRG 0 and 1 on Dworak scale	178	93	43	21	31		
Shin *et al*[30]	2011	Mandard (oesophagus)	Yes	TRG 4 and 5	102	50	40.3			Yr. 3: 72.6	
Huebner *et al*[25]	2012	Dworak (rectal)	Yes	TRG 0+1	237	61					
Lim *et al*[26]	2012	Dworak (rectal)	Yes	TRG 1+2	581	357	61	9.5	27.2	Yr. 5: 63.6	Yr. 5: 71.3
Roy *et al*[29]	2012	Dworak (rectal)	Yes	TRG 0 and 1	75	42				Yr. 2: 68.9	Yr. 2: 92.6
Roy *et al*[29]	2012	Mandard (oesophagus)	Yes	TRG 4 and 5	75	24				Yr. 2: 60.3	Yr. 2: 87.3
Vallböhmer *et al*[32]	2012	Japanese Society for Cancer of the Colon and Rectum (rectal)	Yes	TRG 1	85	23					
Vallböhmer *et al*[32]	2012	Junker Miller (lung)	Yes	TRG 1	85	6					DNE
Vallböhmer *et al*[32]	2012	Cologne (oesophageal)	Yes	TRG 1 and 2	85	53					DNE
Winkler *et al*[33]	2012	Dworak (rectal)	No	TRG 1	33	9					DNE
Elezkurtaj *et al*[20]	2013	Dworak (rectal)	Yes	TRG 0,1 and 2	102	68					
Hermanek *et al*[23]	2013	Rodel (rectal based on Wittekind and Tannapfel (rectal based on Dworak)	Yes	Categorised as poor according to Rodel and based on TRG 0and1 on Wittekind and Tannapfel (a modified Dworak scale)	225	33	92	15.9	27.9	Yr. 5: 63.6	Yr. 5: 75.8
Fokas *et al*[14]	2014	Dworak (rectal)	Yes	TRG 0+1	386	90	132	Yr. 10: 3.6	Yr. 10: 39.6	Yr. 10: 63%	
Santos *et al*[16]	2014	Dworak (rectal)	Yes	TRG 0,1 and 2	144	85	56	3.5	16.4	Yr. 5: 68.1	Yr. 5: 69.1
Santos *et al*[16]	2014	Mandard (oesophagus)	Yes	TRG 3 and 4 and 5	144	69	56	4.3	20.3	Yr. 5: 61.7	Yr. 5: 60.7
Hav *et al*[15]	2015	Dworak (rectal)	Yes	TRG 0,1 and 2	76	48	20			No specific data but no correlation with DFS	

[1]Overall rate for total follow-up time; [2]Probability of being free from recurrence (DFS rate not given). LR: Local recurrence; DR: Distant recurrence.

Table 6 Study definitions of good response according to histopathological tumour regression grade scales

Ref.	Year	TRG scale used (original disease application)	Are the scales reported accurately?	Good response definition	Total (*n*)	Good responders (*n*)	Average F/up in months	LR (%) 5 yr	DR (%) 5 yr	DFS (%)	OS (%)
Gambacorta *et al*[21]	2004	Mandard (oesophagus)	Yes	TRG 1 and 2	54	24	25				
Pucciarelli *et al*[28]	2004	Mandard (oesophagus)	Yes	TRG 1 and 2 and 3	104	52	42			DNE	DNE
Horisberger *et al*[24]	2008	Japanese Society for Cancer of the Colon and Rectum (rectal)	Yes	TRG 2 and 3	59	33					
Suárez *et al*[31]	2008	Mandard (oesophagus)	Yes	TRG 1 and 2	119	36	33	0	0	DNE	
Bujko *et al*[18]	2010	Glynne Jones/ Bujko (rectal)	Yes	TRG 1	131	40	48	9	34	Yr. 4: 67	
Avallone *et al*[13]	2011	Mandard (oesophagus)	Yes	TRG 2 and 3	63	20	60			Yr. 5: Prob free of recurrence > 90%	
Shin *et al*[30]	2011	Mandard (oesophagus)	Yes	TRG 1 and 2 and 3	102	52	40.3			Yr. 3: 74.1	
Huebner *et al*[25]	2012	Dworak (rectal)	Yes	TRG 2 and 3 and 4	237	176					
Lim *et al*[26]	2012	Dworak (rectal)	Yes	TRG 3 and 4	581	224	61	1.3	11.6	Yr. 5: 86.7	Yr. 5: 88.2
Roy *et al*[29]	2012	Dworak (rectal)	Yes	TRG 2 and 3 and 4	75	33				Yr. 2: 91.7	Yr. 2: 89.2
Roy *et al*[29]	2012	Mandard (oesophagus)	Yes	TRG 1 and 2 and 3	75	51				Yr. 2: 86.1	Yr. 2: 92.2
Vallböhmer *et al*[32]	2012	Japanese Society for Cancer of the Colon and Rectum (rectal)	Yes	TRG 3	85	23					DNE
Vallböhmer *et al*[32]	2012	Junker Miller (lung)	Yes	TRG 2aand2b	85	65					DNE
Vallböhmer *et al*[32]	2012	Cologne (oesophageal)	Yes	TRG 3 and 4	85	26					DNE
Winkler *et al*[33]	2012	Dworak (rectal)	No	TRG 3	33	6					
Elezkurtaj *et al*[20]	2013	Dworak (rectal)	Yes	TRG 3 and 4	102	34					
Fokas *et al*[14]	2014	Dworak (rectal)	Yes	TRG 2 and 3	386	256	132	Yr. 10: 8.0	Yr. 10: 29.3	Yr. 10: 73.6%	
Santos *et al*[16]	2014	Dworak (rectal)	Yes	TRG 3 and 4	144	54	56	1.8	11.1	Yr. 5: 78.4	Yr. 5: 77.4
Santos *et al*[16]	2014	Mandard (oesophagus)	Yes	TRG 1 and 2	144	70	56	1.4	8.6	Yr. 5: 81.7	Yr. 5: 79.4
Hav *et al*[15]	2015	Dworak (rectal)	Yes	TRG 3 and 4	76	28	20			No specific data but no correlation with DFS	

Overall rate for total follow-up time. LR: Local recurrence; DR: Distant recurrence; DNE: Data given but not extractable; DFS: Disease-free survival.

used for poor response.

Studies differentiating between good and poor response for DR

Six reports from five studies[14,16,18,26,31] compared good and poor response in relation to DR (Figure 3). Of these, all showed DR was higher in poor responders, of which two studies (Lim *et al*[26] and Fokas *et al*[14]) showed a significant difference; although, they used different definitions. Using the definition given by Lim *et al*[26], there were three other studies with similar definitions[16,31]; the reported 5-year DR for good response was 0% to 11.6%. Using the definition given by Fokas *et al*[14], there was one other study with a similar definition[18]; the reported 5- and 10-year DR for good response was 34% and 29%, respectively. Poor response was defined by three studies[16,31], with similar definitions reporting DR of 14.3% to 20.3%. Poor response was 47% and 39.6% for 5- and 10-year DR, respectively, by two other studies[14,18] with similar

Table 7 Comparison of outcomes between good and poor responders

Ref.	Year	Good response defn.	Poor response defn.	LR %		*P* < 0.05	DR %		*P* < 0.05	DFS %		*P* < 0.05	OS %		*P* < 0.05	DSS		*P* < 0.05	Conclusion
				GR	PR		GR	PR		GR	PR		GR	PR		GR	PR		
Pucciarelli *et al*[28]	2004	TRG 1 and 2 and 3	TRG 4 and 5							Better in GR		No	Better in GR		No				Good responders have better, non-statistically significant outcomes for DFS and OS
Suárez *et al*[31]	2008	TRG 1 and 2	TRG 3 and 4 and 5	0	3.4	NC	0	14.3	NC	Better in GR		Yes				Better in GR		No	Good responders have better, statistically significant DFS but have better, non significant LR, DR and DSS
Bujko *et al*[18]	2010	TRG 1	TRG 3	9	26	No	34	47	No	67	47	No							Good responders have better, non-statistically significant outcomes for LR, DR and DFS
Avallone *et al*[13]	2011	TRG 2 and 3	TRG 4 and 5							Prob > 90%	Prob 56%	Yes							Good responders have better, statistically significant DFS
Shin *et al*[30]	2011	TRG 1 and 2 and 3	TRG 4 and 5							74.1	72.6	No							Good responders have better, non-statistically significant outcomes for DFS
Lim *et al*[26]	2012	TRG 3 and 4	TRG 1 and 2	1.3	9.5	Yes	11.6	27.2	Yes	86.7	63.6	Yes	88.2	71.3	Yes				Good responders have better, statistically significant outcomes for LR, DR, DFS and OS
Roy *et al*[29]	2012	TRG 1 and 2 and 3	TRG 4 and 5							86.1	60.3	Yes	92.2	87.3	No				Good responders have better, statistically significant DFS but have better, non significant OS
Roy *et al*[29]	2012	TRG 2 and 3 and 4	TRG 0 and 1							91.7	68.9	No	89.2	92.6	No				Good responders had better, non-statistically significant outcomes for DFS. Good responders had poorer, non-statistically significant outcomes for OS
Vallböhmer *et al*[32]	2012	TRG 3	TRG 1										Better in GR		No				Good responders have better, non-statistically significant outcomes for OS
Vallböhmer *et al*[32]	2012	TRG 2a and 2b	TRG 1										Better in GR		No				Good responders have better, non-statistically significant outcomes for OS
Vallböhmer *et al*[32]	2012	TRG 3 and 4	TRG 1 and 2										Better in GR		No				There was no statistically significant difference for OS between good and poor responders
Fokas *et al*[14]	2014	TRG 2 and 3	TRG 0 and 1	8	3.6	No	29.3	39.6	Yes	73.6	63	Yes							Good responders have better, statistically significant outcomes for DR and DFS. Good responders had poorer, non-statistically significant outcomes for LR
Santos *et al*[16]	2014	TRG 1 and 2	TRG 3 and 4 and 5	1.4	4.3	NC	8.6	20.3	NC	81.7	61.7	Yes	79.4	60.7	Yes				Good responders have better, statistically significant outcomes for DFS and OS
Santos *et al*[16]	2014	TRG 3 and 4	TRG 0 and 1 and 2	1.8	3.5	NC	11.1	16.4	NC	78.4	68.1	No	77.4	69.1	No				Good responders have better, non-statistically significant outcomes for DFS and OS
Hav *et al*[15]	2015	TRG 3 and 4	TRG 0 and 1 and 2							Better in GR		No							Good responders have better, non-statistically significant outcomes for DFS

Where data is not given the overall result is stated. LR: Local recurrence; DR: Distant recurrence; GR: Good responders; PR: Poor responders; NC: No statistical comparison made.

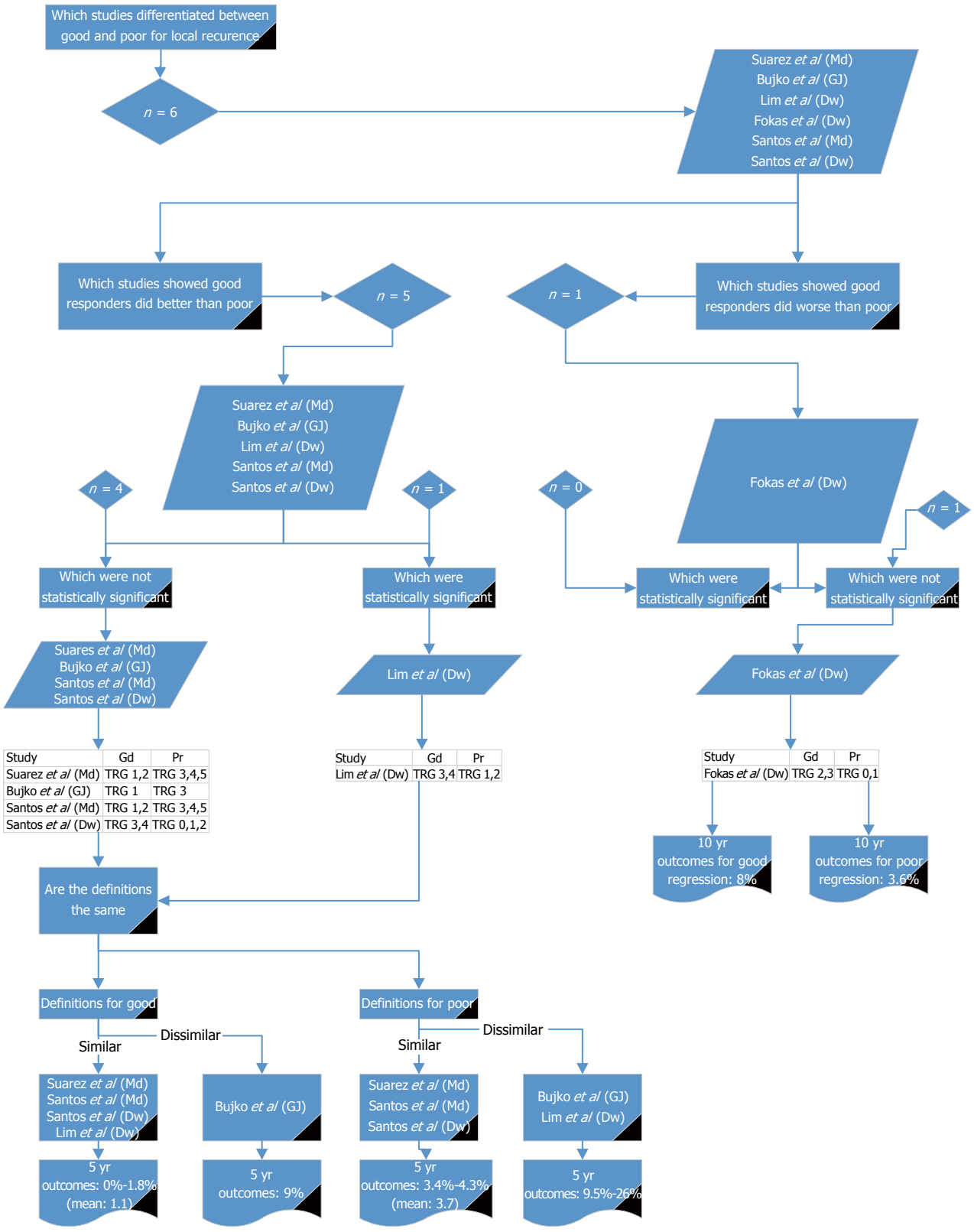


Figure 2 Studies reporting on local recurrence in good and poor responders.

definitions. Lim *et al*[26] reported 5-year DR as 27.2% for poor responders. The values reported by Fokas *et al*[14] and Bujko *et al*[18] are much higher than the other reports and do not reflect the body of literature. It would, therefore, be preferable to use either Mandard 1 to 2 or Dworak 3 to 4 for defining good response for DR and Mandard 3 to 5 or Dworak 0 to 2 or 1 to 2 for poor response.

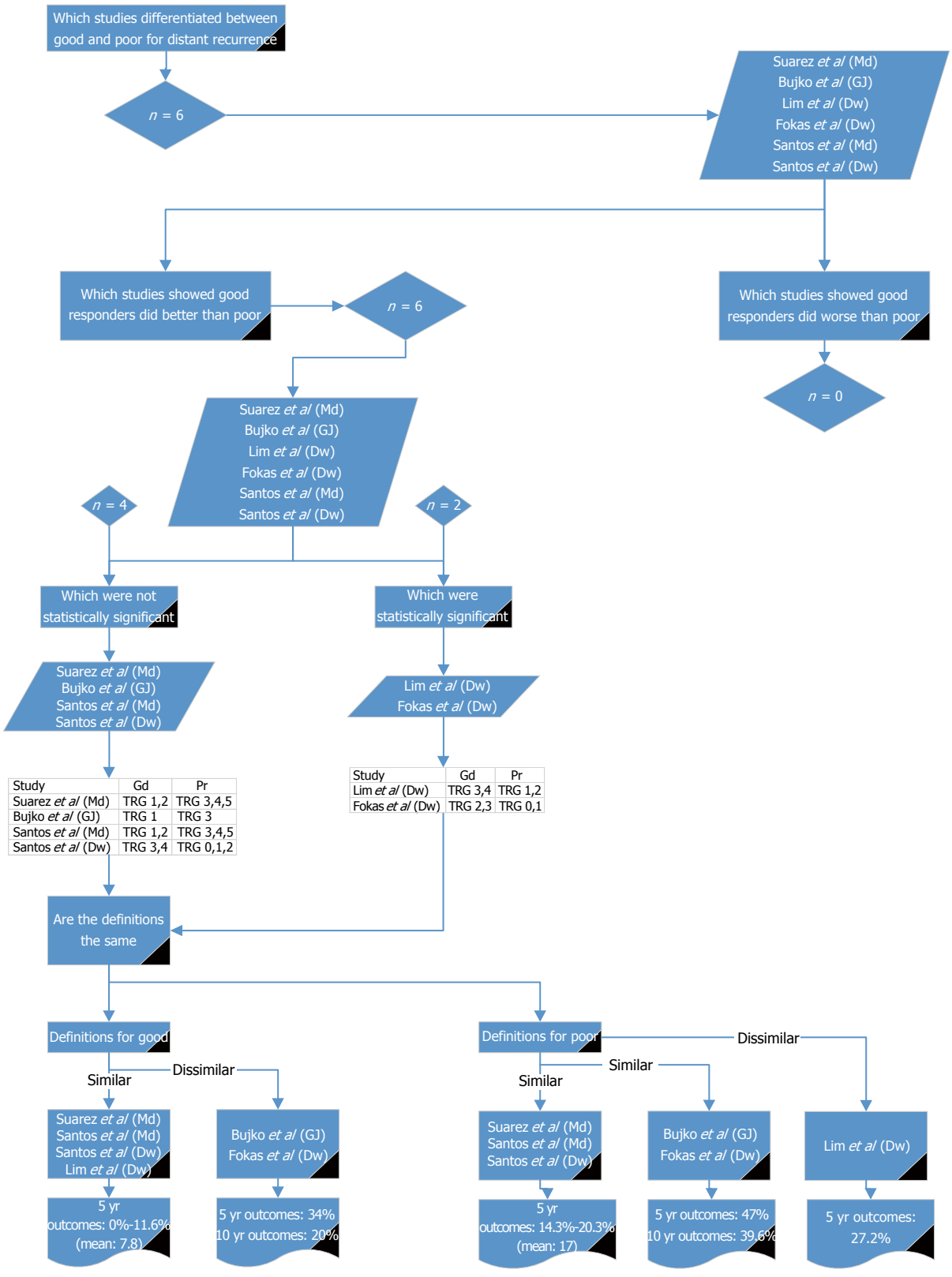


Figure 3 Studies reporting on distant recurrence in good and poor responders.

Studies differentiating between good and poor response for DFS

Twelve reports[13-16,18,26,28-31] compared good and poor response in relation to DFS (Figure 4). All of the studies showed DFS to be worse in poor responders. Six studies showed a significant difference between good and poor response[13,14,16,26,29,31]. For the definition of good response, three of the papers[16,26,31] showing a statistical significance used a similar definition to each other; two[13,14] used different definitions but were

similar to each other and one used a different definition to the other significant studies[29]. Using the definition given by Lim *et al*[26] and comparing it to studies with similar definitions[15,16,30,31], the reported DFS for good response at 5 years was 78.4% to 86.7%. Using the definition given by Fokas *et al*[14] and comparing it with the other reports with similar definitions[13], the reported 5- and 10-year DFS for good response was > 90% and 73.6%, respectively. Using the definition by Roy *et al*[29] and comparing it with the other studies with similar definitions[28-30], 2-year DFS was 86.1% to 91.7% and 3-year DFS was 74.1%.

For the definition of poor response, three of the papers[13,14,29] showing a statistical significance used a similar definition to each other, two[16,31] used different definitions but were similar to each other and one study was different in its definition of poor response[26]. Using the definition given by Avallone *et al*[13] and comparing it to the other studies with similar definitions[14,18,28-30], the reported DFS for poor response at 2 years was 60.3% to 68.9%, at 3 years was 72.6%, at 4 years was 47%, and at 5 years was 56%. Using the definition given by Suárez *et al*[31] and comparing it with the other studies with similar definitions[15,16], the reported DFS for poor response at 2 years was 83.6%, at 3 years was 73.8%, and at 5 years was 61.7% to 68.1%. Lim *et al*[26] reports a 5-year DFS of 63.6%. From these results it may be appropriate to use Mandard 1 to 2, 1 to 3 or 2 to 3 or Dworak 3 to 4, 2 to 4 or 2 to 3 for defining good response and Mandard 4 to 5, 3 to 5 or Dworak 0 to 1, 0 to 2 or Bujko 3 to define poor response.

Studies differentiating between good and poor response for OS

Nine reports[16,26,28,29,32] compared good and poor response in relation to OS (Figure 5). Of these, all but one[29] showed OS was non-significantly worse in poor responders. Six reports from four papers showed a significant difference[16,28,29,32]. For the definition of good response, two of the papers[16,32] showing a statistical significance used a similar definition to each other; two reports from one paper[32] used different definitions but were similar to each other, and a further two used similar definitions to each other but were different from the other papers[28,29]. Using the definition given by Pucciarelli *et al*[28] and comparing it with the other studies with similar definitions[29], the reported OS for good response at 2 years was 92.2%. Using the definition given by Lim *et al*[26] and comparing it with the other studies with similar definitions[16,26,32], the reported OS for good response at 5 years was 77.4% to 88.2%.

For the definition of poor response, two of the papers[28,29] showing a statistical significance used a similar definition to each other and a further two studies had similar definitions to each other[16,32]. Two reports from one study were different in their definitions of poor response[32]. Using the definition given by Pucciarelli *et al*[28] and comparing it with other reports with similar definitions[29], the reported OS for poor response was 87.3% at 2 years. Using the definition given by Vallböhmer *et al*[32] and comparing it with the studies with similar definitions[26], the reported OS for poor response was 71.3% at 5 years. Using the next definition given by Vallböhmer *et al*[32] and comparing it with studies with similar definitions[16], the reported OS for poor response was 60.7% to 69.1% at 5 years. From these results it may be appropriate to use Mandard 1 to 2, 1 to 3 or Dworak 3 to 4 or Cologne 3 to 4 for defining good response and Mandard 4 to 5, 3 to 5 or Dworak 0 to 2, 1 to 2 or Japanese 1a to 1b or Cologne 1 to 2 to define poor response.

Consensus histopathological definition of good and poor response

These results show that across the outcomes of LR, DR, DFS and OS, Mandard 1 to 2 and Dworak 3 to 4 could be used for defining good response and Mandard 3 to 5 and Dworak 0 to 2 for poor response.

MRI method of classifying regression

There was one mrTRG system using a 5-point scale[52] (Table 8). Lower mrTRG refers to greater regression and the system also divides the categories into type of response (complete, good, moderate, slight and none).

There were five papers on five studies reporting on poor response[5-7,52,53]. Characteristics of these studies can be seen in Table 9. Overall, the reported proportion of poor responders after neo-adjuvant CRT was 38.6% (95%CI: 34.5%-42.8%) and there was only moderate heterogeneity that was still significant (Q = 10.7, df = 4, I^2 = 63, P = 0.03) (Figure 6).

Definition of poor response as defined by MRI

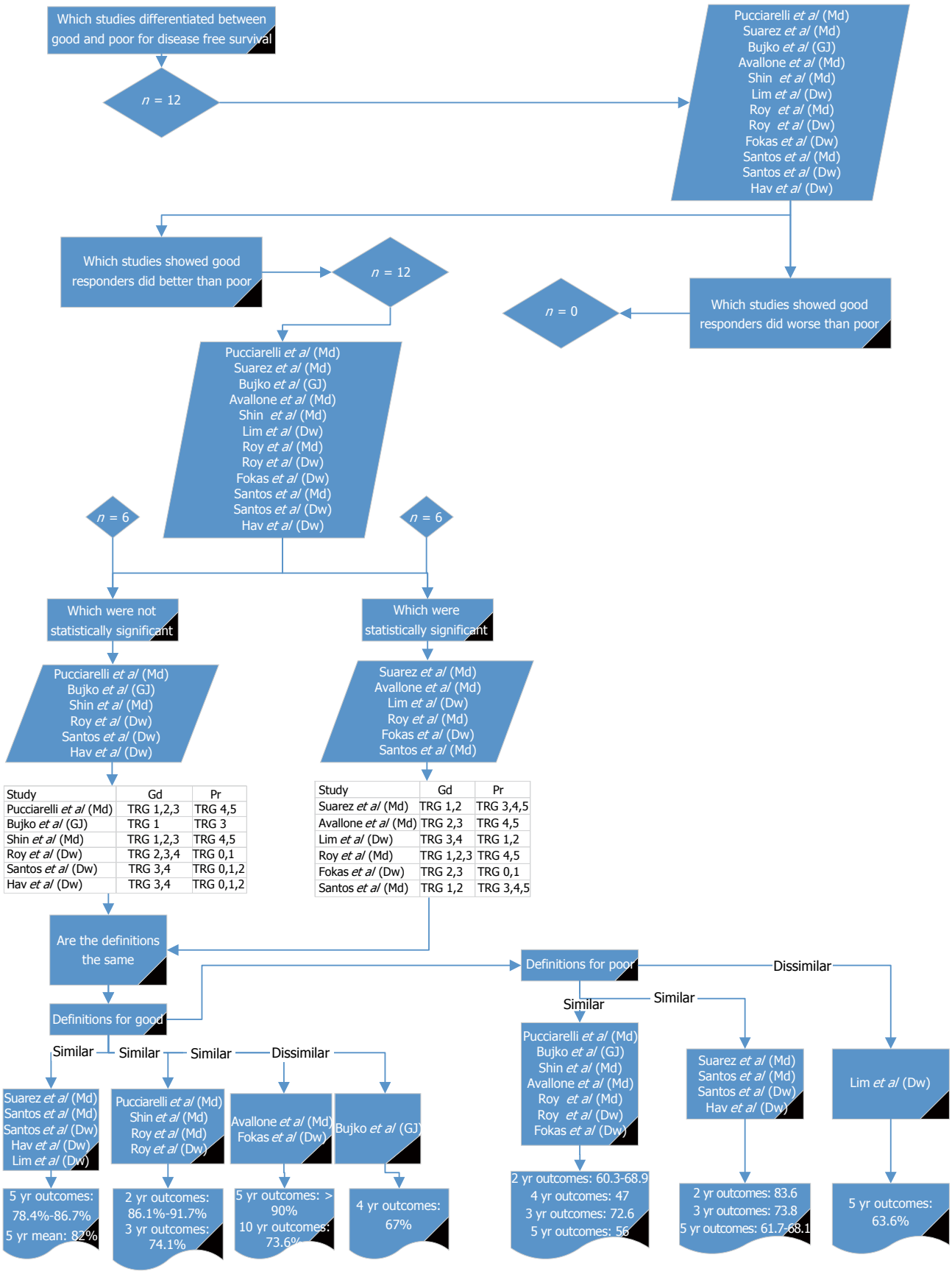
Two studies[5-7] stated that mrTRG was based on the Dworak scale, but the hierarchy actually follows that of the Mandard scale (Table 10). Three studies stated that it was based on the Mandard scale[52,53]. Poor response was defined as mrTRG 4 and mrTRG 5 by all of the papers. LR for poor responders at 5 years ranged from 4% to 29%[6,52]. Five year DR was 9%[52]. From our centres, unpublished data for 3-year DFS was 52%[53] and 5-year DFS was 31% to 68%[6,53]. OS at 3 years from this centre was 74%[53] and at 5 years was 27% to 68%[6,53].

Outcomes of good response defined by MRI TRG scales

LR rates for good responders at 5 years ranged from 1% to 14%[6,52]. Five-year DR was 3%[52] and DFS was 64% to 83%[6,53]. OS at 5 years was 72% to 90%[6,53] (Table 11).

Considerations and comparison between good and poor responders

mrTRG is a relatively new scale and the studies



Study	Gd	Pr
Pucciarelli *et al* (Md)	TRG 1,2,3	TRG 4,5
Bujko *et al* (GJ)	TRG 1	TRG 3
Shin *et al* (Md)	TRG 1,2,3	TRG 4,5
Roy *et al* (Dw)	TRG 2,3,4	TRG 0,1
Santos *et al* (Dw)	TRG 3,4	TRG 0,1,2
Hav *et al* (Dw)	TRG 3,4	TRG 0,1,2

Study	Gd	Pr
Suarez *et al* (Md)	TRG 1,2	TRG 3,4,5
Avallone *et al* (Md)	TRG 2,3	TRG 4,5
Lim *et al* (Dw)	TRG 3,4	TRG 1,2
Roy *et al* (Md)	TRG 1,2,3	TRG 4,5
Fokas *et al* (Dw)	TRG 2,3	TRG 0,1
Santos *et al* (Md)	TRG 1,2	TRG 3,4,5

Figure 4 Studies reporting on disease- free survival in good and poor responders.

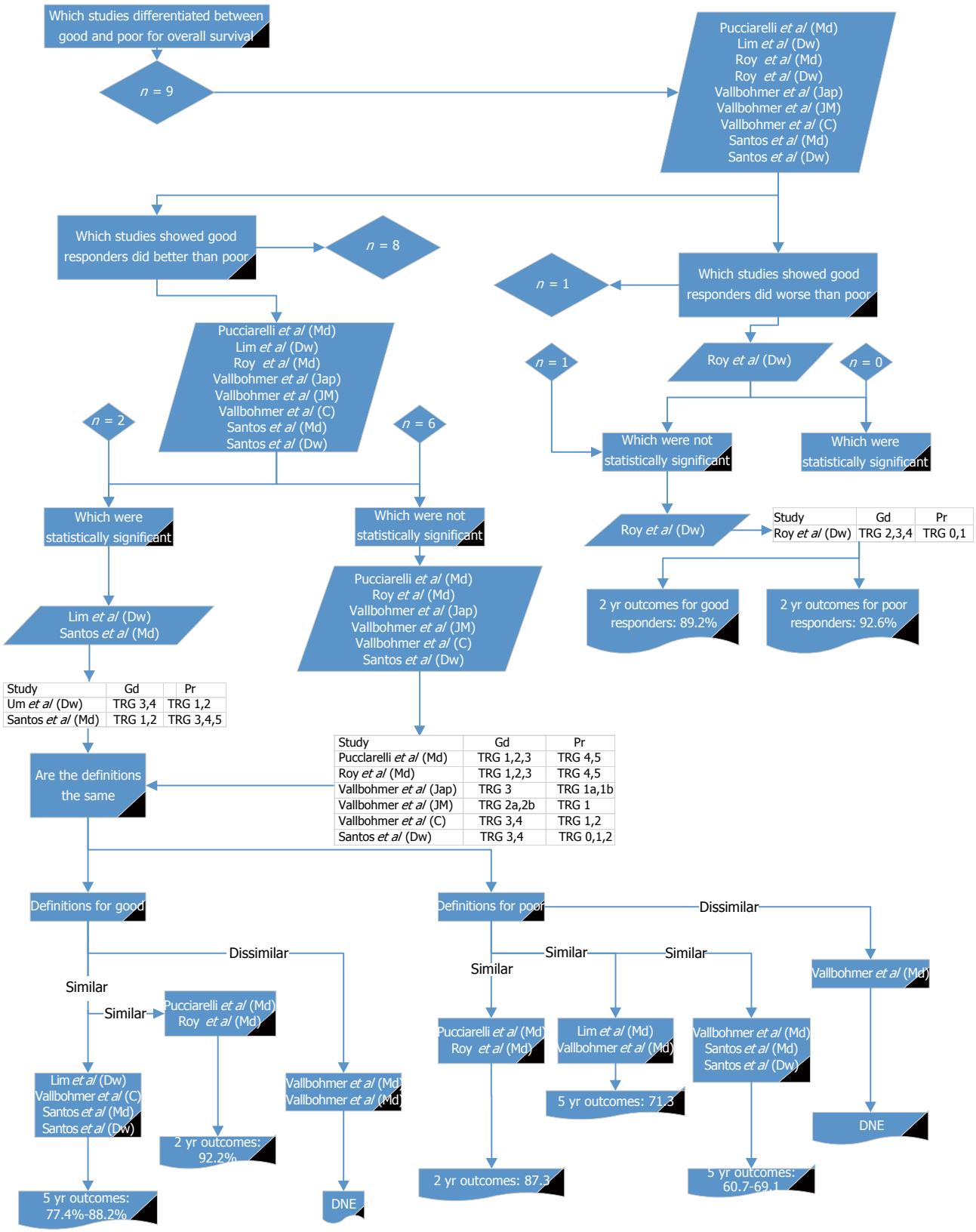


Figure 5 Studies reporting on overall survival in good and poor responders.

reporting it are from one centre; hence, consistency would be expected. Good responders were defined as mrTRG 1 to 3 or 1 to 2 and poor responders were defined as mrTRG 4 to 5 (Table 12).

Studies differentiating between good and poor responders for LR, DR, DFS and OS

There are three articles with available data comparing outcomes for good and poor responders (Table 11). In

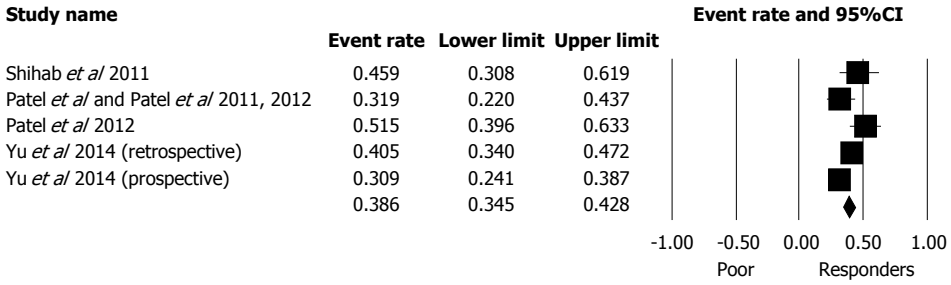


Figure 6 Proportion of patients who responded poorly to neo-adjuvant therapy according to magnetic resonance imaging.

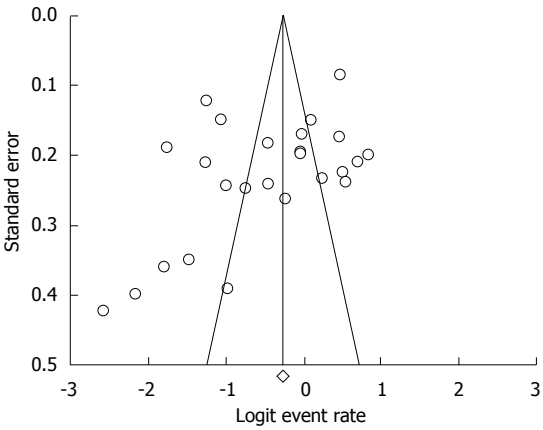


Figure 7 Funnel plot for studies reporting on the prevalence of poor response according to histology.

all three reports, good responders had better outcomes compared with poor responders in relation to LR, DR, DFS and OS. Furthermore in all but LR there was a statistically significant difference in outcomes.

Although there was a range of survival outcomes, the overall rates for survival are lower in poor responders, distinguishing them clearly from the survival figures and rates of those with good response.

Consensus mrTRG definition of good and poor response

From these results, good response may be defined as mrTRG 1 to 3 or 1 to 2 (with mrTRG3 as a separate, independent group) and poor responders as mrTRG 4 to 5. This consistency of results, therefore, indicates the secondary hypothesis is likely to be true.

Publication bias for prevalence

Publication bias for prevalence from histology was initially assessed using a funnel plot (Figure 7). There appeared to be some asymmetry on the plot and so Eggers test was used. There was statistically significant asymmetry seen (Intercept: -4.30, SE: 2.23, 95%CI:-8.90-0.31, t = 1.93, P = 0.07), indicating there is unlikely to be significant publication bias.

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Table 8 Summary of magnetic resonance imaging regression scale available in the literature

mrTRG scale	mrTRG (Low no. - More regression)[47]
1	Radiological complete response: no evidence of ever treated tumour
2	Good response (dense fibrosis; no obvious residual tumour, signifying minimal residual disease or no tumour)
3	Moderate response (50% fibrosis or mucin, and visible intermediate signal)
4	Slight response (little areas of fibrosis or mucin but mostly tumour)
5	No response (intermediate signal intensity, same appearances as original tumour)

DISCUSSION

The aim of this review was to investigate the range and method of how poor response to neo-adjuvant therapy for rectal cancer is defined in order to see which scale best distinguishes between the two groups in relation to outcomes.

Main findings

In summary, this paper has shown that across the outcomes of LR, DR, DFS and OS, Mandard 1, 2 and Dworak 3, 4 could be used for defining good response and Mandard 3, 4, 5 and Dworak 0, 1, 2 for defining poor response. There are other definitions shown above which may also differentiate good and poor response. The analysis has shown differences in the reliability of these scales in consistently identifying good and poor responders.

Summary and appraisal of evidence

Our results have shown that there are three major

Table 9 Characteristics of studies reporting on poor response based upon magnetic resonance imaging

Ref.	Year	Chemotherapy protocol	Radiotherapy protocol (Gy)	Surgical procedures	TME	Time to surgery (wk)	Cancer stage Pre neo-adjuvant therapy	Adjuvant therapy
Shihab *et al*[52]	2011			APR/AR	Y			
Patel *et al*[7] and Siddiqui *et al*[8]	2011 and 2012			APR/AR	Y			
Patel *et al*[6]	2012			APR/AR	Y		T1/2/3/4, N0/1/2	Y
Yu[53]	2014 (unpublished data from our centre)	Capecitabine, oxaliplatin ± cetuximab	50.4-54		Y		T2/3/4	Y
Yu[53]	2014 (unpublished data from our centre)	Capecitabine, oxaliplatin ± cetuximab	50.4-54		Y		T2/3/4	Y

Table 10 Study definitions of poor response according to magnetic resonance imaging tumour regression grade scales

Ref.	Year	TRG scale used (histological stage based upon)	Scales accurate?	Poor response definition	Total (*n*)	Poor responders (*n*)	Average F/up in months	LR (%) 5 yr	DR (%) 5 yr	DFS (%)	OS (%)
Shihab *et al*[52]	2011	MRI TRG (based on Mandard)	Yes	TRG 4,5	37	17		4	9		
Patel *et al*[5,7]	2012	MRI TRG (based on Dworak)	Yes	TRG 4,5	69	22					
Patel *et al*[6] and Patel *et al*[7]	2011 and 2012	MRI TRG (based on Dworak)	Yes	TRG 4,5	66	34	60	29		Yr. 5: 31	Yr. 5: 27
Yu[53]	2014 (unpublished data from our centre)	MRI TRG (based on Mandard and Dworak)	Yes	TRG 4,5	210	85				Yr. 3: 52%	Yr. 3: 74%
Yu[53]	2014 (unpublished data from our centre)	MRI TRG (based on Mandard and Dworak)	Yes	TRG 4,5	152	47				Yr. 5: 59%	Yr. 5: 68%

LR: Local recurrence; DR: Distant recurrence; DFS: Disease-free survival; OS: Overall survival; TRG: Tumour regression grade.

Table 11 Study definitions of good response according to magnetic resonance imaging tumour regression grade scales

Ref.	Year	TRG scale used (histological stage based upon)	Scales accurate?	Good response definition	Total (*n*)	Good responders (*n*)	Average F/up in months	LR (%) 5 yr	DR (%) 5 yr	DFS (%)	OS (%)
Shihab *et al*[52]	2011	MRI TRG (based on Mandard)	Yes	TRG 1,2,3	37	20		1	3		
Patel *et al*[6]	2012	MRI TRG (based on Dworak)	Yes	TRG 1,2,3	69	47					
Patel *et al*[5] and Patel *et al*[7]	2011 and 2012	MRI TRG (based on Dworak)	Yes	TRG 1,2,3	66	32	60	14		Yr. 5: 64	Yr. 5: 72
Yu[53]	2014 (unpublished data from our centre)	MRI TRG (based on Mandard and Dworak)	Yes	TRG 1,2	152	61				DFS, Yr. 5: 83%	DFS, Yr. 5: 90%

LR: Local recurrence; DR: Distant recurrence; DFS: Disease- free survival; OS: Overall survival; TRG: Tumour regression grade.

challenges when it comes to the standardization of tumour regression for rectal cancer. The first is the vast choice of regression scales available to histopathologists. The second is that studies use these varied scales to define poor response without consistency. The third is that there are marked differences between the scales. Therefore, trying to merge these systems into one, universally acceptable

Table 12 Comparison of outcomes between good and poor responders

Ref.	Year	Local recurrence (LR)	$P < 0.05$	Distant recurrence (DR)	$P < 0.05$	Progression disease-free survival (DFS)	$P < 0.05$	Disease-free survival (DFS)	$P < 0.05$	Overall survival (OS)	$P < 0.05$	Conclusion
Shihab *et al*[52]	2011	Better in GR	No	Better in GR	Yes							Good responders have better, statistically significant outcomes for DR but have better, non significant LR
Patel *et al*[5] and Patel *et al*[7]	2011 and 2012	Better in GR	No					Better in GR	Yes	Better in GR	Yes	Good responders have better, statistically significant outcomes for DFS and OS but have better, non significant outcomes for LR
Yu[53]	2014					Better in GR	Yes			Better in GR	Yes	Good responders have better, statistically significant outcomes for DFS and OS

GR: Good responders; NC: No statistical comparison made; DNI: Data not interpretable.

scale becomes unrealistic. Furthermore, studies have shown that inter-observer agreement amongst histopathologists using the existing scales is low[54]. The scales themselves do not advise on whether histopathologists should use a single worst slide for assessment or a composite assessment and adds to the challenge of defining good and poor response. This was highlighted by a study which showed poor inter-observer agreement between histopathologists assessing regression using different regression scales[54].

Some of the scales use qualitative estimates[25,39,46] for levels of fibrosis, but these overlap with regression grades in alternative scales given in other studies[35,43]. Even by trying to examine the correlation between two systems, two grades may be grouped into one grade on a different scale.

Both MRI and histopathological grading systems are open for misinterpretation if standard methods of preparation and interpretation are not employed; there has been a focused attempt to do this in relation to histopathological assessment[54,55] and mrTRG is a novel scale requiring appropriate training to ensure consistency when utilised in other centres.

Differences in the definitions of poor response are highlighted by the number of poor responders identified in each of the studies (Figures 1 and 6). This review concentrated on studies using specific terms stating what they believed to be poor response; however, there were studies that divided TRG into two groups but did not specifically state them as good and poor responders; their results are consistent with the range that is reported in this paper but differ in that they show a good correlation to outcomes for their presumed good and poor responders[56].

In relation to the original definitions, one study showed that poor responders could be either those with predominant fibrosis or patients with tumour outgrowing fibrosis[31] compared with other studies using the same Mandard scale which only defined poor responders as those with tumour outgrowing fibrosis[22]. This is then compounded by the fact that more than one grade on other scales could be combined together on an alternative system.

Importance and implications for practice

Historically, the histopathological TRG systems were developed without validation of the grading in relation to outcomes, and evolution of these scales has occurred with the presence of long-term prognostic information. Histopathological TRG is also dependent on thorough pathological sampling and comparisons are not made to the pre-treatment biopsy; therefore, high stromal content tumours are often given a better regression grade, even though the high stroma may not be due to regression. mrTRG may be one way to respond to this, as it compares and examines the whole tumour and because of the presence of one-scale heterogeneity is reduced. mrTRG also better distinguishes between good and poor response in relation to survival. LR appears to be reported with a large range using both histopathological and mrTRG and may relate to surgical factors being the most important issue in relation to this outcome.

Implications for research and further studies

Recent data from our centre would suggest that mrTRG3, whilst traditionally considered a good response, behaves more like the poor responder group[57] and could be considered as a separate group[58].

In summary, this paper has shown that across the outcomes of LR, DR, DFS and OS, Mandard 1 to 2 and Dworak 3 to 4 could be used for defining good

response and Mandard 3 to 5 and Dworak 0 to 2 for poor response. These definitions may help in achieving consensus in histopathological reporting. However, these definitions do not always produce a significant difference in the outcomes from the different studies utilizing these definitions. Furthermore, there are other definitions shown above which may also differentiate good and poor response. This casts doubt on the reliability of these scales in consistently identifying good and poor responders. A preoperative grading system, such as mrTRG, may be useful to appropriately differentiate good and poor response, thus guiding management decisions, and images attained could effectively be attained by high resolution MRI imaging.

A range of histopathological TRG scales is used in clinical studies. Good and poor response are heterogeneously described, even when using the same histopathological regression scales. Across the outcomes of LR, DR, DFS and OS, Mandard 1 to 2 and Dworak 3 to 4 could be used for defining good response and Mandard 3 to 5 and Dworak 0 to 2 for poor response. These definitions may help in achieving consensus in histopathological reporting. Preoperative mrTRG is similarly able to differentiate between good and poor response based on outcomes.

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COMMENTS

Background

Clinical studies use a number of different tumour regression grade (pTRG) scales to classify the degree of tumour response to neo-adjuvant chemo-radiotherapy (CRT). This often results in confusion as to whether a good or poor response has been achieved, with subsequent uncertainty regarding treatment and prognostic implications. This problem was highlighted by studies that stress the importance of a universally accepted standard. There has been no review of the reported pTRG scales to date. It is necessary to highlight the heterogeneity in these scales, consolidate the current definitions with the purpose of converging towards a set of consensus definitions. This article investigates all the pathology tumour regression scales used to define good and poor response after neo-adjuvant chemotherapy for rectal cancer, to establish the true prevalence of poor responders and to identify the best scales to use in relation to outcomes.

Research frontiers

A newer method of assessing tumour regression relies on MRI (mrTRG), which has been validated as a prognostic tool. This may supercede pTRG, as it has the advantage of assessing tumour response before surgery. Potential enabling response-orientated tailored treatment, including alteration of the surgical planes, additional use of chemotherapy or deferral of surgery.

Innovations and breakthroughs

The authors have found the best classification of good and poor response for rectal cancer response to neoadjuvant chemo-radiotherapy.

Applications

This systematic review has immediate application to rectal cancer care by identifying how to classify good and poor response in the context of outcomes of local recurrence, metastases, disease-free survival and overall survival

Peer-review

This is an interesting review about neoadjuvant therapy for postoperative outcome in rectal cancer.

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CASE REPORT

Ataxic gait following total gastrectomy for gastric cancer

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Abstract

A 58-year-old woman, who had undergone total gastrectomy for early gastric cancer 9 years previously, visited the outpatient clinic complaining of progressive difficulty in walking for 15 d. Laboratory examinations showed macrocytic anemia and a decreased serum vitamin B12 concentration and increased serum concentrations of folate, vitamin E and copper. Magnetic resonance imaging showed multifocal high signal intensities along the posterior column of the cervical and thoracic spinal cord. Treatment consisted of intramuscular injections of vitamin B12 for 7 d, which increased her serum level of vitamin B12 to normal. This was followed by weekly intramuscular injections of vitamin B12 for another 2 wk and oral administration of vitamin B12 three times per day. After comprehensive rehabilitation for 4 wk, she showed sufficient improvements in strength and ataxic gait, enabling her to return to her normal daily activities.

Key words: Subacute combined degeneration; Vitamin B12 deficiency; Total gastrectomy; Gastric cancer

Core tip: Subacute combined degeneration (SCD) of the spinal cord is defined as the degeneration of the posterior and lateral columns of the spinal cord due to vitamin B12 deficiency. Because vitamin B12 deficiency can occur after total gastrectomy, so can SCD. Postoperative assessments have focused on the recurrence of gastric cancer after surgery, with fewer studies focusing on vitamin B12 deficiency, especially because SCD is a long-term complication. This report describes a patient who developed SCD after total gastrectomy for gastric cancer. SCD due to vitamin B12 deficiency should be suspected in patients showing gait disturbance after gastrectomy, even as a long-term complication.

Hwang CH, Park DJ, Kim GY. Ataxic gait following total

gastrectomy for gastric cancer. *World J Gastroenterol* 2016; 22(37): 8435-8438 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i37/8435.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i37.8435

INTRODUCTION

Subacute combined degeneration (SCD) of the spinal cord is defined as the degeneration of the posterior and lateral columns of the spinal cord as a result of vitamin B12[1], vitamin E, and copper deficiencies[2]. Vitamin B12 deficiency, the most frequent cause of SCD, is associated with pernicious anemia, mal-absorption[3] of vitamin B12, intrinsic factor depletion, and exposure to nitrous oxide. Mal-absorption of vitamin B12 may also occur when intrinsic factor is not produced by the stomach, including after total or sub-total gastrectomy[4,5].

Vitamin B12 deficiency can cause a wide range of hematological, gastrointestinal, psychiatric, and neurological disorders. Neurologically, vitamin B12 deficiency can result in a multi-organ disorder involving the spinal cord, especially the dorsal and lateral columns, brain, and optic and peripheral nerves[6]. Abnormalities can include weakness in both the upper and lower extremities, changes in mentality, and decreased vision[6]. Although SCD associated vitamin B12 deficiency has been believed to be well researched disease since the first report by Knox and Delamore[7] in 1960, the connection between the SCD and gastrectomy is under-recognized, primarily because SCD usually occurs about 10 years after surgery[8,9]. This report describes a patient who developed SCD 9 years after total gastrectomy.

CASE REPORT

A 58-year-old woman, who had undergone total gastrectomy for early gastric cancer 9 years previously, visited the outpatient clinic complaining of progressive difficulty in walking for 15 d. Symptoms included allodynia (by light touch and pinprick, not by heat and cold), a tingling sensation along both lower limbs, ataxic gait disturbance, and decreased joint sense of the knees. Computerized neurocognitive tests showed reductions in memory and attention. Laboratory examinations showed macrocytic anemia and a decreased serum vitamin B12 concentration (< 30 pg/mL; normal range, 197-894 pg/mL) and increased serum concentrations of folate (32 ng/ml; normal range, 2.0-19.9 ng/mL), vitamin E (32 ng/mL; normal range, 5.5-17.0 ng/mL), and copper (157.99 mg/dL; normal range, 64-134 mg/dL). Her serum concentrations of homocysteine (5.80 nmol/mL; normal range, 3.36-20.44 nmol/mL) and methylmalonic acid (3.4 mg/d; normal range, 0.00-10.0 mg/d) 15 d after starting treatment were within normal ranges (Table 1).

Magnetic resonance imaging (MRI) showed multi-focal high signal intensities along the posterior column of the cervical and thoracic spinal cord (Figures 1 and 2)[10]. MRI of the brain showed minimal numbers of small vessel ischemic lesions in the white matter and mild ventriculomegaly. A nerve conduction study showed sensory-motor peripheral polyneuropathy, and a sensory evoked potential test showed a conduction delay from the bilateral posterior tibial sensory nerve to the somatosensory cortex. However, no definitive abnormalities were observed on the visual evoked potential test.

Treatment consisted of intramuscular injections of 5 mg/d vitamin B12 for 7 d, which increased her serum level of vitamin B12 to normal. This was followed by weekly intramuscular injections of 5 mg of vitamin B12 for another 2 wk and oral administration of 0.5 mg of vitamin B12 three times per day. After comprehensive rehabilitation for 4 wk, she showed sufficient improvements in strength and ataxic gait, enabling her to return to her normal daily activities as a housewife.

DISCUSSION

Vitamin B12 deficiency can result in a defective ability to synthesize myelin, resulting in the insidious development of demyelinating vitamin B12 deficiency neuropathy, often beginning in the peripheral nerves and progressing to involve the posterior and lateral columns of the spinal cord and the cerebellum[11]. The histo-pathological findings of SCD include patchy losses of myelin, especially in the dorsal and lateral columns of the spinal cord[1]. This can result in impaired proprioception of peripheral limbs and ataxic gait disturbance[3]. Symptoms of SCD can include abnormal sensations, including peripheral polyneuropathy, changes in mentality (*i.e.*, nutritional encephalopathy), and decreased vision (*i.e.*, optic neuritis).

Because SCD is caused by a vitamin B12 deficiency, it may occur after gastrectomy. However, its onset is usually slow and insidious. The likely sequence of events in the development of vitamin B12 deficiency due to its inadequate absorption includes early changes in bone marrow, occurring 1-2 years after inadequate absorption of vitamin B12 begins, followed by early myelin damage, occurring after 1.5-2 years[11]. Because severe myelin damage takes more than 3 years to develop, SCD first occurs many years after gastrectomy. Reports have described the development of neurological symptoms > 10 years[9] and even 37 years[8] after surgery.

Although postoperative follow-up focuses on the recurrence of gastric cancer after surgery, fewer studies have focused on vitamin B12 deficiency[12], especially because SCD is a long-term complication. Nutritional status after gastrectomy is important, even many years later. The increasing long-term survival of patients who undergo surgery for early gastric cancer, especially in the upper third of the stomach,

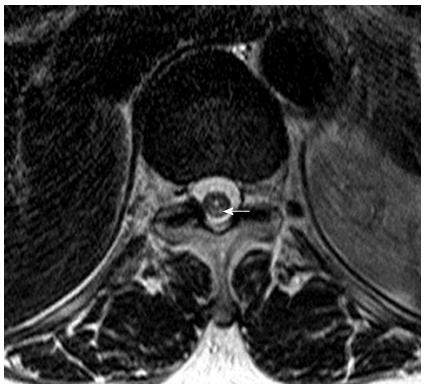

Figure 1 T2-weighted axial magnetic resonance image of the thoracic spinal cord in the patient. The white arrow indicates high signal intensity in the posterior column.

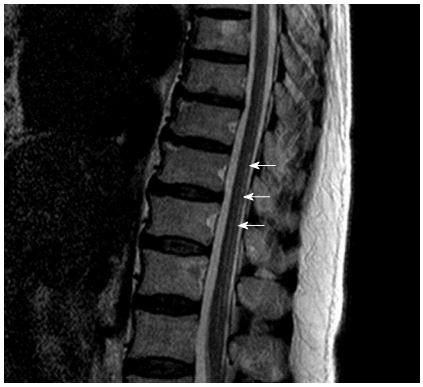

Figure 2 T2-weighted sagittal magnetic resonance image of the thoracic spinal cord in the patient. The white arrows indicate high signal intensity in the posterior column.

Table 1 Laboratory examinations of the patient

Variable	Value (normal range)
Vitamin B12	< 30 pg/mL (197-894 pg/mL)
Folate	32 ng/mL (2.0-19.9 ng/mL)
Vitamin E	32 ng/mL (5.5-17.0 ng/mL)
Copper	157.99 mg/dL (64-134 mg/dL)
Homocysteine	5.80 nmol/mL (3.36-20.44 nmol/mL)
Methylmalonic acid	3.4 mg/d (0.00-10.0 mg/d)

emphasizes the importance of long-term metabolic consequences of total gastrectomy, especially vitamin B12 deficiency.

In conclusion, this report describes a patient who developed SCD 9 years after total gastrectomy for gastric cancer. SCD due to vitamin B12 deficiency should be suspected in patients with neurological disorders, including gait disturbance, after gastrectomy, even as a long-term complication.

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We express our sincere thanks to Woo-Ram Koo, MD for his help.

COMMENTS

Case characteristics

A 58-year-old woman, who had undergone total gastrectomy for early gastric cancer 9 years previously, visited the outpatient clinic complaining of progressive difficulty in walking for 15 d.

Clinical diagnosis

Symptoms included allodynia (by light touch and pinprick, not by heat and cold), a tingling sensation along both lower limbs, ataxic gait disturbance, and decreased joint sense of the knees.

Differential diagnosis

HIV-associated vacuolar myelopathy, vitamin E deficiency, copper deficiency, Friedreich's ataxia, leukoencephalopathy

Laboratory diagnosis

Laboratory examinations showed macrocytic anemia and a decreased serum vitamin B12 concentration and increased serum concentrations of folate, vitamin E, and copper.

Imaging diagnosis

Magnetic resonance imaging showed multifocal high signal intensities along the posterior column of the cervical and thoracic spinal cord.

Treatment

Intramuscular injections of vitamin B12.

Related reports

Vitamin B12 deficiency can result in a defective ability to synthesize myelin, resulting in the insidious development of demyelinating neuropathy, often beginning in the peripheral nerves and progressing to involve the posterior and lateral columns of the spinal cord and the cerebellum.

Term explanation

Subacute combined degeneration (SCD) of the spinal cord is defined as the degeneration of the posterior and lateral columns of the spinal cord due to vitamin B12 deficiency.

Experiences and lessons

SCD due to vitamin B12 deficiency should be suspected in patients with neurological disorders after gastrectomy, even as a long-term complication.

Peer-review

This is a concise and well written paper about the possibility of SCD arising from vitamin B12 deficiency following a gastrectomy.

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CASE REPORT

Central pancreatectomy for pancreatic schwannoma: A case report and literature review

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Abstract

Schwannomas are mesenchymal tumors originating from Schwann cells in peripheral nerve sheaths. Although the tumor can be located in any part of the human body, the most common locations are the head, neck, trunk and extremities. Pancreatic

schwannomas are rare. To our knowledge, only 64 cases of pancreatic schwannoma have been reported in the English literature over the past 40 years. In this paper, we present a pancreatic schwannoma in a 59-year-old female. Ultrasound, computed tomography and magnetic resonance imaging revealed the tumor located in the pancreatic body; however, accurate diagnosis was hard to obtain preoperatively and a pancreatic cystadenoma was preliminarily considered. During laparotomy, the mass was found in the body of the pancreas. An enlarged gallbladder with multiple stones was also observed. We performed central pancreatectomy, end-to-side pancreaticojejunostomy and cholecystectomy. Notably, central pancreatectomy has been reported in only one case prior to this report. The gross specimen showed a mass with a thin capsule, 1.6 cm × 1.1 cm × 1.1 cm in size. Microscopic examination showed that the tumor was mainly composed of spindle-shaped cells with palisading arrangement and no atypia, which is consistent with a benign tumor. Both hypercellular and hypocellular areas were visible. Immunohistochemical staining revealed strongly positive results for protein S-100. Finally, the tumor was diagnosed as a schwannoma of the pancreatic body. Postoperatively, the patient recovered well and left the hospital 6 d later. During the 53-mo follow-up period, the patient remained well and free of complications.

Key words: Pancreaticojejunostomy; Schwannoma; Pancreas; Central pancreatectomy; S-100

Core tip: Over the past 40 years, only 64 cases of pancreatic schwannomas have been reported in the English literature. It is a considerable challenge to obtain a precise preoperative diagnosis, despite the application of multiple imaging modalities. We present a patient with a pancreatic schwannoma and enlarged gallbladder with multiple stones. After central pancreatectomy, end-to-side pancreaticojejunostomy and cholecystectomy, the patient recovered quickly and had a good prognosis. In this study, we focused on the diagnosis and treatment of a pancreatic schwannoma and conducted a literature review to deepen the understanding of the subject.

Xu SY, Sun K, Owusu-Ansah KG, Xie HY, Zhou L, Zheng SS, Wang WL. Central pancreatectomy for pancreatic schwannoma: A case report and literature review. *World J Gastroenterol* 2016; 22(37): 8439-8446 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i37/8439.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i37.8439

INTRODUCTION

Schwannomas are tumors originating from the Schwann cells of peripheral nerve sheaths[1]. Most schwannomas show either monosomy 22 or loss of 22q material; however, the pathogenesis of the tumor remains unclear[2]. Schwannomas are generally encapsulated tumors, and more than 90% of them are benign[2]. Schwannomas occur in patients with no obvious gender difference and at all ages. However, patients between 20 and 50 years of age were most frequently reported. Surgery is the most common treatment for schwannomas, and patients usually have a good postoperative prognosis[3]. While almost every part of the human body can be involved, the most common locations are the head, neck, trunk and extremities[3]. Pancreatic schwannomas are extremely rare. To our knowledge, only 64 cases of pancreatic schwannomas have been reported in the English literature over the past 40 years[4-61]. Although most patients with pancreatic schwannomas were symptomatic, a considerable number of patients were asymptomatic with tumors that were found incidentally. This paper presents a case of pancreatic schwannoma in a 59-year-old female and a review of the literature.

CASE REPORT

On January 7, 2012, a 59-year-old female was referred to our hospital because of a pancreatic mass found during a routine health examination. The abdomen was soft and nondistended without evidence of a palpable mass. Her family history was not significant. Abnormal laboratory results included: Unconjugated bilirubin, 2 μmol/L (normal, 3-14) and serum kalium, 3.42 mmol/L (normal, 3.5-5.2). Ultrasound revealed a 1.4 cm × 1.3 cm, well-defined cystic lesion in the pancreatic body (Figure 1A), as well as a 6.8 cm sized strong echo in the gallbladder (Figure 1B). An unenhanced computed tomography (CT) scan showed a 1.6 cm × 1.1 cm well-defined hypodense mass in the pancreatic body (Figure 2A). On the contrast-enhanced CT, the mass was not enhanced (Figure 2B). On magnetic resonance imaging (MRI), the mass in the pancreatic body and gallbladder appeared hypointense on T1 weighted images (Figure 3A). The mass in the pancreatic body appeared inhomogeneously hyperintense and the enlarged gallbladder appeared hyperintense on T2 weighted images (Figure 3B). We also performed endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). However, the tumor sample was difficult to acquire, and the procedure was unsuccessful. According to these results, a pancreatic cystadenoma and an enlarged gallbladder with multiple stones were preliminarily considered.

After sufficient preparation and obtaining consent from the patient and her family members, a laparotomy was performed. A 1.5 × 1.0 cm mass surrounded by a thin fibrous capsule was found in the pancreatic body. An enlarged gallbladder with multiple stones was also found. We performed central pancreatectomy, end-

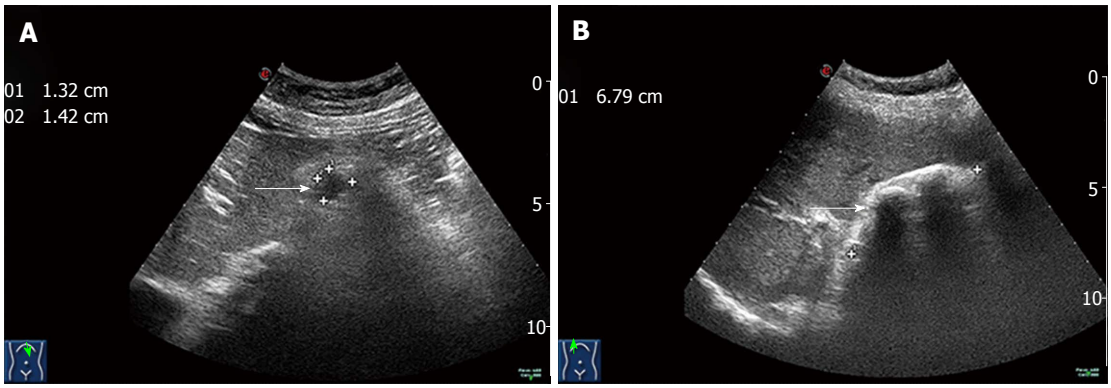

Figure 1 Ultrasound findings. A: Ultrasound revealed a 1.4 cm × 1.3 cm, well-defined cystic lesion (arrow) in the pancreatic body; B: A 6.8 cm sized strong echo (arrow) was shown in the gallbladder.

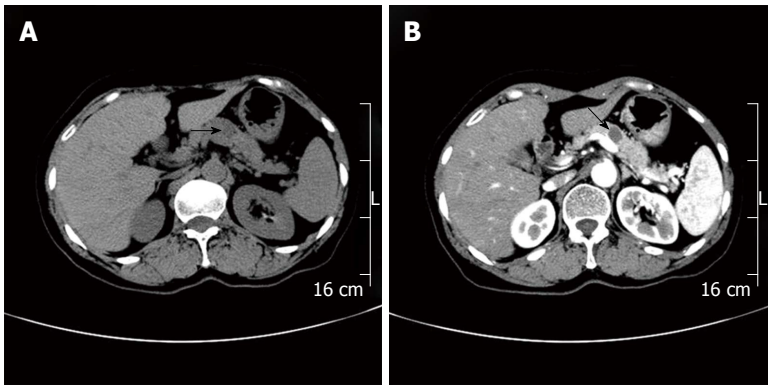

Figure 2 Computed tomography findings. A: An unenhanced CT scan showed a 1.6 cm × 1.1 cm well-defined hypodense mass (arrow) in the pancreatic body; B: On the contrast-enhanced CT, the mass (arrow) was not enhanced. CT: Computed tomography.

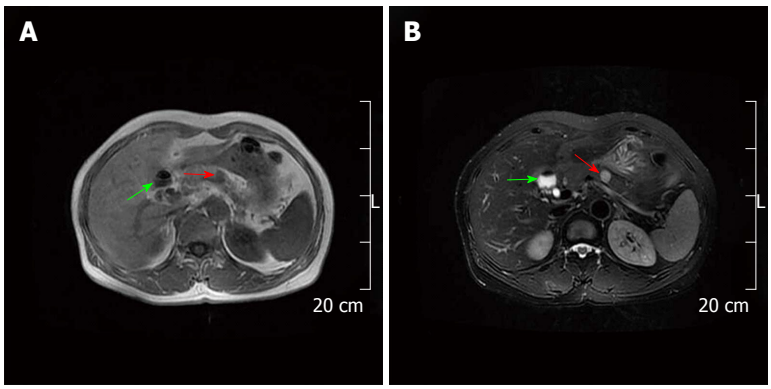

Figure 3 Magnetic resonance imaging findings. A: The mass in the pancreatic body (red arrow) and gallbladder (green arrow) appeared hypointense on T1 weighted images; B: The mass in the pancreatic body (red arrow) appeared inhomogeneously hyperintense and the enlarged gallbladder (green arrow) appeared hyperintense on T2 weighted images.

to-side pancreaticojejunostomy, cholecystectomy and inserted a pancreatic stent. Intraoperative frozen pathology revealed a schwannoma in the pancreatic body.

Macroscopically, the mass in the pancreatic body measured 1.6 cm × 1.1 cm × 1.1 cm. Microscopically, the tumor, surrounded by a thin capsule, was mainly composed of spindle-shaped cells with palisading arrangement and no atypia, which was consistent with a benign schwannoma. Both hypercellular and hypocellular areas were visible (Figure 4). Immunohistochemical staining was strongly positive for protein S-100 (Figure 5) and negative for SMA, CD34 and CD117. The final diagnosis was a schwannoma of the

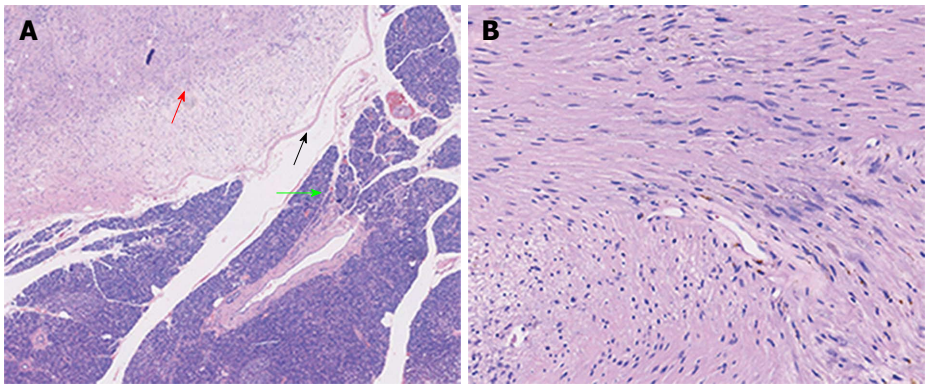

Figure 4 Microscopic examination. A: A thin capsule (black arrow) was found between the tumor (red arrow) and pancreatic (green arrow) tissues (HE, 40 ×). B: The tumor was mainly composed of spindle-shaped cells with palisading arrangement and no atypia, which is consistent with a benign schwannoma. Both hypercellular and hypocellular areas were visible (HE, 200 ×). HE: Hematoxylin and eosin.

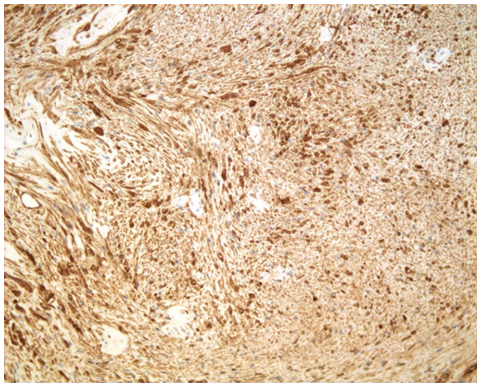

Figure 5 Immunohistochemical staining. The tumor revealed strongly positive staining for S-100 (HE, 200 ×). HE: Hematoxylin and eosin.

pancreatic body. Postoperatively, the patient recovered well and left the hospital 6 d later with no evidence of postoperative pancreatic fistula. During the follow-up period of 53 mo, the patient remained well without any complications.

DISCUSSION

Schwannomas are neoplasms that originate from the Schwann cells of nerve sheaths[62]. More than 90% of schwannomas are benign and manifest in approximately 5% of cases as benign soft-tissue neoplasms[63]. Schwannomas can occur in patients of any age with no obvious gender difference. However, they are most commonly found in patients aged between 20 years and 50 years. Although nearly any part of the human body can be involved, the head, neck and extremities are the most common sites[64]. In the abdominal cavity, the retroperitoneum (6% of primary retroperitoneal tumors)[65] and stomach[66] are the most common sites involved. Schwannomas of the pancreas are rare. Table 1 summarizes the important available clinicopathological characteristics of the 64 cases reported in the English literature over the past 40 years[4-61] and the case presented in our study. Continuous variables were summarized as the mean ± SD and range. The Student's *t* test was used for comparisons of continuous variables. Statistical analyses were conducted using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, United States). All tests for significance were two-sided, and P values < 0.05 were considered statistically significant.

Precise preoperative diagnosis of a pancreatic schwannoma is challenging because the clinical symptoms and radiological characteristics of schwannomas are nonspecific. Definitive diagnosis can be achieved only based on the combined results of histopathological and immunohistochemical examinations of surgical specimens. Microscopically, pancreatic schwannomas are encapsulated tumors that consist of hypercellular (Antoni type A area) and hypocellular areas (Antoni type B area) with varying amounts of these histological components[22]. The hypercellular area consists of closely packed spindle cells with occasional nuclear palisading as well as Verocay bodies. The hypocellular area is composed of loosely arranged tumor cells and abundant myxoid stroma[6]. Occasionally these may become cystic, hemorrhagic and calcified[18]. More than 90% of pancreatic schwannomas are benign. However, malignant pancreatic schwannomas have been reported in 5 cases (7.69%)[22,57,59-61]. Immunohistochemically, schwannomas show strongly positive staining for S-100 and negative staining for desmin, smooth muscle myosin, SMA, CD34 and CD117[16,67].

Accurate diagnosis of a pancreatic schwannoma prior to operation is nearly impossible. US, CT and MRI can be performed to establish a probable diagnosis. A pancreatic schwannoma usually appears as a well-defined hypodense lesion on US and shows no echoic enhancement with color Doppler. On unenhanced CT scan, schwannomas are usually well-defined hypodense lesions with encapsulation and/or cystic degeneration. Schwannomas with high Antoni A areas show high density and have a heterogeneous appearance due to high cellularity and increased lipid content. Antoni B areas of schwannomas appear cystic and show low

Table 1 Summary of clinicopathological data from all 65 cases of pancreatic schwannoma

	n (%) or mean ± SD (range)
Age (yr) (*n* = 64)	
Mean	55.22 ± 15.26 (20-87)
Sex (male/female), (male %) (*n* = 64)	29/35 (45.31)
Symptoms[1] (*n* = 64)	
Asymptomatic	24 (37.50)
Symptomatic	
Abdominal pain	28 (43.75)
Weight loss	8 (12.50)
Back pain	4 (6.26)
Nausea/vomiting	3 (4.69)
Anorexia	2 (3.13)
Abdominal mass	1 (1.56)
Anemia	2 (3.13)
Melena	2 (3.13)
Jaundice	2 (3.13)
Abdominal discomfort	1 (1.56)
Location (*n* = 65)	
Head	26 (40.00)
Head/body	3 (4.62)
Body	15 (23.08)
Body/tail	7 (10.78)
Tail	7 (10.78)
Uncinate process	7 (10.78)
Mean size (cm)	5.83 ± 4.59 (1-20)
Benign	56 (5.27 ± 3.95) (1-20)
Malignant	4 (13.75 ± 6.24) (7-20)
Operation (*n* = 65)	
PD[2]	20 (30.77)
PPPD	2 (3.08)
DP[3]	16 (24.62)
Enucleation	9 (13.85)
Central pancreatectomy	2 (3.08)
Unresectable	2 (3.08)
Refused	1 (1.54)
Not specified	13 (20.00)
Histology (*n* = 65)	
Malignant	5 (7.69)
Benign	59 (90.77)
Not specified	1 (1.54)
Nature of tumor (*n* = 65)	
Solid	17 (26.15)
Cystic	28 (43.08)
Solid and cystic	14 (21.54)
Not specified	6 (9.23)
Mean follow-up months (*n* = 29)	20.59 ± 17.76 (3-66)
Died of disease	0

[1]Some patients had several symptoms; [2]One patient underwent resection of portal vein; [3]One patient underwent resection of transverse colon. M: Male; F: Female; NA: Not available; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus preserving pancreaticoduodenectomy.

density due to loose stroma and low cellularity[10]. On contrast-enhanced CT, the Antoni A areas are usually enhanced, whereas the Antoni B areas are unenhanced[7]. On MRI, a typical schwannoma appears hypointense in T1-weighted images and appears inhomogeneously hyperintense in T2-weighted images[16]. EUS-FNA may greatly contribute to precise preoperative diagnosis. In a case reported by Li *et al*[28], a pancreatic schwannoma was accurately diagnosed preoperatively by EUS-FNA. In the present study, we also attempted EUS-FNA. However, a sample of the tumor was difficult to acquire because of the small tumor size and the procedure was unsuccessful.

Surgery is the optimal treatment for pancreatic schwannoma. As tumors could be located in different parts of the pancreas, surgical methods vary accordingly. In the present case, laparotomy permitted discovery of the mass in the body of the pancreas. An enlarged gallbladder with multiple stones was also found. We performed central pancreatectomy, end-to-side pancreaticojejunostomy, cholecystectomy and inserted a pancreatic stent. To date, central pancreatectomy had been reported in only one case[5] prior to this study. Compared with traditional distal pancreatectomy and splenectomy for tumors in the body or tail of the pancreas, central pancreatectomy can not only completely resect the tumor, but also preserve the distal pancreas and spleen, which is beneficial to patients. Following complete tumor excision, patients with pancreatic schwannomas generally have a good prognosis.

In conclusion, a schwannoma of the pancreas is rare. To our knowledge, only 64 cases of pancreatic schwannoma have been reported in the English literature over the past 40 years. Precise preoperative diagnosis is challenging despite the application of multiple imaging modalities. Surgery is the most effective treatment for pancreatic schwannoma. As tumors could be located in different parts of the pancreas, surgical methods vary accordingly. Following complete tumor removal, patients with pancreatic schwannomas generally have a good prognosis.

COMMENTS

Case characteristics

A 59-year-old female was referred to our hospital because of a pancreatic mass found during a routine health examination.

Clinical diagnosis

The abdomen was soft and nondistended without evidence of a palpable mass.

Differential diagnosis

Differential diagnoses included intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, solid pseudopapillary tumor, pancreatic endocrine tumor or pancreatic ductal adenocarcinoma.

Laboratory diagnosis

Abnormal laboratory results included: Unconjugated bilirubin, 2 μmol/L (normal, 3-14) and serum kalium 3.42 mmol/L (normal, 3.5-5.2).

Imaging diagnosis

Ultrasound revealed a 1.4 cm × 1.3 cm, well-defined cystic lesion in the pancreatic body, as well as a 6.8 cm sized strong echo in the gallbladder. An unenhanced computed tomography (CT) scan showed a 1.6 cm × 1.1 cm well-defined hypodense mass in the pancreatic body. On the contrast-enhanced CT, the mass was not enhanced. On magnetic resonance imaging, the mass in the pancreatic body and gallbladder appeared hypointense on T1 weighted images. The mass in the pancreatic body appeared inhomogeneously hyperintense and the enlarged gallbladder appeared hyperintense on T2 weighted images. We also performed Endoscopic ultrasound-guided fine needle aspiration. However,

the tumor sample was difficult to acquire, and the procedure was unsuccessful. According to these results, a pancreatic cystadenoma and an enlarged gallbladder with multiple stones were preliminarily considered.

Pathological diagnosis

Microscopic examination revealed a tumor composed mainly of spindle-shaped cells with palisading arrangement and no atypia, which is consistent with a benign tumor. Both hypercellular and hypocellular areas were visible. Immunohistochemical staining was strongly positive for protein S-100. Finally, the tumor was diagnosed as a schwannoma of the pancreatic body.

Treatment

The patient underwent central pancreatectomy, end-to-side pancreaticojejunostomy and cholecystectomy.

Related reports

Schwannoma of the pancreas is rare. Over the past 40 years, only 64 cases of pancreatic schwannomas have been reported in the English literature.

Experiences and lessons

Precise preoperative diagnosis is challenging despite the application of multiple imaging modalities. Surgery is the most effective treatment for pancreatic schwannoma. As tumors could be located in different parts of the pancreas, surgical approach varies accordingly. Following complete tumor removal, patients with pancreatic schwannomas generally have a good prognosis.

Peer review

This manuscript is an interesting surgical case report, good literature review, and well written. This study highlights the diagnosis and treatment of a rare pancreatic schwannoma and presents a literature review to deepen the understanding of the subject. The information included is worthwhile to the reader.

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