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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1376 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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FLYLEAF

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EDITORIAL

Thermal ablation in colorectal liver metastases: Lack of evidence or lack of capability to prove the evidence?

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Abstract

Many studies suggest that combined multimodality treatments including ablative therapies may achieve better outcomes than systemic chemotherapy alone in patients with colorectal liver metastases. Nevertheless, ablative therapies are not yet considered as effective options because their efficacy has never been proved by randomized controlled trials (RCT). However, there are in literature no trials that failed in demonstrating the effectiveness of ablative treatments: what are lacking, are the trials. All the attempts to organize phase Ⅲ studies on this topic failed as a result of non accrual. Just one prospective RCT comparing radiofrequency ablation combined with systemic chemotherapy vs chemotherapy alone has been published. It was designed as a phase Ⅲ study, but it was closed early because of slow accrual, and was downscaled to phase Ⅱ study, with the consequent limits in drawing definite conclusions on the benefit of combined treatment. However, the combination treatment met the primary end point of the study and obtained a significantly higher 3-year progression-free survival than systemic chemotherapy alone. It is very unlikely that ultimate efficacy of ablation treatments will ever be tested again, and the best available evidence points toward a benefit for the combination strategy using ablative treatments and chemotherapy.

Core tip: Phase Ⅲ randomized controlled trials (RCT) on the efficacy of thermal ablation combined with systemic chemotherapy in colorectal liver metastases are lacking in literature, and it is very unlikely that ultimate efficacy of ablation treatments will ever be tested again by RCT because of the difficult accrual. However, the best available evidence points toward a benefit for the combination strategy using ablative treatments and chemotherapy.

INTRODUCTION

Every year colorectal cancer is diagnosed in at least one million people worldwide, and liver metastases (LM) will develop at some point during the course of the disease in up to 50% of the patients. Surgical resection of LM is the procedure of choice with five-year survival rates of 50%-60%[1-3]. However, surgical resection is only feasible in approximately 10%-20% of cases. In most patients, too extensive liver disease, extra-hepatic disease, or co-morbidity preclude radical resection. In these patients, systemic combination chemotherapy with or without biologic therapy is the standard of care, and it has been shown to prolong median survival to nearly two years[4-6]. Over the past decade, several techniques for local tumor destruction emerged as alternative treatments for patients with non-resectable colorectal LM, in particular thermal ablation techniques such as radiofrequency ablation (RFA), microwave ablation (MWA), and laser ablation (LA)[7-9]. They have been reported to prolong survival and to improve quality of life of patients with LM from colorectal cancer[10], and they may be indicated in patients with resectable lesions as an adjunct to resection, or inoperable lesions which demonstrate complete or partial response after chemotherapy, or recurrent and progressive lesions[11]. There is wide variability in the reported 3-year and 5-year survival rates, mainly due to the different experience with the ablative techniques, tumor biology, and patient or tumor selection criteria[12]. Moreover, it is a major challenge to determine how to integrate thermal ablation with adjuvant and/or neoadjuvant chemotherapy, in an effort to further improve disease control and survival. However, survival indexes among the ablation techniques are not significantly different. RFA is the most used ablation technique worldwide. In patients with a maximum of 5-6 LM with a maximum diameter of 5-6 cm, RFA was reported to obtain 3-year and 5-year survival rates ranging from 28% to 46%, and from 25% to 46%, respectively, with a median survival ranging from 30 to 40 mo[13-16]. Studies on the outcomes of MWA and LA are less numerous and generally involve smaller series of patients, but both techniques seem to be as effective as RFA. In subgroups of patients with similar tumor characteristics (from two to 9 LM with a maximum diameter of 6.8 cm), MWA achieved 3-year and 5-year survival rates ranging from 46% to 51%, and from 17% to 32%, respectively, with a median survival ranging from 20 to 48 mo[17-19]; and LA achieved 3-year- and 5-year survival rates ranging from 56% to 72%, and from 33% to 37%, respectively, with a median survival ranging from 35 to 54 mo[9, 20,21].

In practice, there is in literature a vast amount of studies suggesting that combined multimodality treatments including ablative therapies may achieve better outcomes than systemic chemotherapy alone in patients with LM from colorectal cancer. Nevertheless, and despite they are currently and widely being used in both eastern and western countries, ablative therapies are not yet considered as effective options in the multimodality treatment of colorectal LM, because their efficacy is suggested by single-arm, retrospective and prospective trials, but it has never been proved by randomized controlled trials (RCTs)[12].

WHERE ARE THE TRIALS?

If it is true that the efficacy of ablative therapies has never been proved by RCTs, it should also be underlined that there are in literature no trials that failed in demonstrating the effectiveness of ablative treatments: what are lacking, are the trials (Table 1). Multiple factors contribute to such a lack of RCTs investigating the outcomes of ablative therapies for LM from colorectal cancer. One factor may be the reluctance of patients to be randomly assigned. Another factor is surely the objective difficulty in adequately stratifying both patients and tumors as concerns stage of disease, size and number of LM, presence/absence of extrahepatic disease, types of previous, concomitant, or salvage chemotherapies, primary and secondary end points, and so on. Moreover, many clinicians may be reluctant to enroll patients into trials because they are convinced that currently available data from highly selected patient series provide sufficient evidence. Finally, the limited resources available to support the costs of clinical trials may represent a further obstacle. As a result of these limiting factors, an attempt to organize a prospective randomized phase Ⅲ trial comparing resection and RFA in well stratified groups of patients with LM from colorectal cancer (French FFCD 2002-02) failed (Table 1). Likewise, the United States National Surgical Adjuvant Breast and Bowel project trial comparing oxaliplatin, capecitabine, and hepatic arterial infusion of floxuridine, with oxaliplatin and capecitabine in patients with resected or ablated colorectal LM was closed as a result of nonaccrual, and results were not published (Table 1). To date, just one prospective RCT that investigated the efficacy of ablative therapies has been published. It was planned and designed as a phase Ⅲ study by the European Organization for Research and Treatment of Cancer (EORTC) in an attempt to determine the additional value of RFA, comparing RFA (associated or not to resection) plus systemic chemotherapy *vs* systemic chemotherapy

Table 1 Thermal ablation in colorectal liver metastases: When the evidence can not be very evident

Phase Ⅲ RCT	Compared arms	Status of RCT	Results
French FFCD 2002-02	Surgical resection *vs* RFA	Closed because of non accrual	Not published
United States Nat Surg Adj Br Bow trial	CT + HAI *vs* CT + Resection or RFA	Closed because of non accrual	Not published
CLOCC (median FU 4.4 yr)[22]	CT *vs* CT + RFA	Downscaled to Phase Ⅱ trial because of slow accrual	OS: *P* = 0.22 PFS: *P* = 0.025 in favour of CT + RFA
CLOCC (median FU 9.7 yr)[26]	CT *vs* CT + RFA	FU in progress	OS: *P* = 0.01 in favour of CT + RFA

RCT: Randomized controlled trials; RFA: Radiofrequency ablation; CT: Systemic chemotherapy; HAI: Hepatic arterial infusion; OS: Overall survival; PFS: Progression free survival.

alone in patients with unresectable colorectal LM (CLOCC trial)[22] (Table 1). The enrollment started in April 2002 involving 22 centers, but in June 2007 the trial was closed early because of slow accrual (119 patients recruited from 22 centers in more than five years, just one patient per center per year!), and was amended and downscaled to phase Ⅱ study, with the consequent strong limits in drawing definitive conclusions on the benefit of combined treatment RFA plus chemotherapy. However, the trial yielded some interesting results. The study design considered the patients as eligible for RFA if they had up to ten LM, with a maximum diameter of 4 cm: this is not exactly the most favorable scenario for RFA, which is known to achieve the highest rates of complete tumor destruction in presence of up to three or four tumors with a maximum diameter of 3 cm[23-25]. Nevertheless, the combination treatment RFA plus chemotherapy met the primary end point of the study [30-mo overall survival (OS) rate > 38%; OS rate observed in the arm 61.7%], and obtained significantly higher 3-year progression-free survival (PFS) rate and median PFS than systemic chemotherapy alone (27.6% and 16.8 mo, respectively, *vs* 10.6% and 9.9 mo, respectively, *P* = 0.025)[22]. However, median OS was not statistically different between the two arms, as it was higher than expected in the systemic treatment alone arm (40.5 mo *vs* 45.3 mo for combined treatment arm, *P* = 0.22). The downsizing of the CLOCC trial to a phase Ⅱ trial does not allow any direct comparison in OS, but the prolongation of median PFS of nearly 7 mo in the combination treatment arm might be translated into a higher OS after longer follow-up. However, the possible translation of improved PFS into prolonged OS would be biased by the imbalances in salvage treatments after disease progression, because patients in the systemic treatment group received more frequently systemic treatment as salvage treatment than patients in the combined treatment group. For all these reasons, despite the excellent result of a 30-mo OS of 61.7% and a significantly higher median PFS, the study concluded that the ultimate effect of RFA combined with systemic chemotherapy on OS remained uncertain, and whether PFS could be considered an acceptable surrogate end point remained debatable[22]. Nevertheless, despite these

unsatisfactory and questionable conclusions, after a longer median follow up of the patients enrolled into the CLOCC trial (9.7 years *vs* 4.4 years) OS resulted significantly better in the combination arm RFA plus chemotherapy than in the arm treated with systemic chemotherapy alone [observed median OS 45.6 mo (95%CI: 30.3-67.8) *vs* 40.5 mo (95%CI 27.5-47.7); HR = 0.58, 95%CI: 0.38-0.88, *P* = 0.01][26] (Table 1).

It is very unlikely that ultimate efficacy of RFA or other ablation techniques on OS will ever be tested again, given the proved difficult accrual of the trials designed to this aim. Moreover, the effect that ablative therapies have on OS may be difficult to isolate because multiple treatment options for colorectal cancer can be used before and/or after ablative procedures, and local recurrence-free survival or local progression-free survival have been suggested as acceptable secondary end points[12]. However, according to what observed by some of the authors of the CLOCC trial in a paper based on either the results of the CLOCC trial itself or the data reported in literature[27], the best available evidence points toward a benefit for the combination strategy using ablative treatments and chemotherapy. As a consequence of these observations, a position paper by an international panel of ablation experts has recently recommended the ablative therapies associated with systemic chemotherapy as the treatment of choice in patients with non-resectable but limited liver disease[28].

The next fields of investigations should be addressed to identify the tumor characteristics and subgroups of patients with inoperable colorectal LM who could most benefit by the ablative treatment combined with systemic chemotherapy, rather than to persist in planning randomized trials that will never be. For instance, there is in literature accumulating evidence that RFA can result in improved long-term survival in patients with up to three lesions ≤ 3 cm in size[27], but the recent technical advances in MWA technology have been reported to achieve coagulation areas significantly larger than RFA[29-31]. Could the threshold of 3 cm in size be raised to 4 or 5 cm using the most advanced MWA systems, maintaining the same efficacy achieved by RFA in lesions up to 3 cm? Furthermore, what should be the best combination treatment strategy: debulking tumor mass by thermal

ablation to reduce tumor load needed to treat with systemic chemotherapy, or downstaging with systemic chemotherapy followed by thermal ablation of the remaining lesions?

Literature regarding these topics and many other ones dealing with the selection criteria and strategies to improve the outcome of thermal ablation combined with systemic chemotherapy in the treatment of patients with colorectal LM is quite scarce or even absent, and the next trials should aim at exploring these fields of research.

CONCLUSION

The best available evidence suggests that ablation therapies are a useful adjunct to systemic treatment, and many experts worldwide recommend them as an important component of the multimodality treatment of patients with LM from colorectal cancer[28]. The unsatisfying results of the attempts to perform randomized trials aimed at investigating the efficacy of thermal ablation combined with systemic chemotherapy highlight the limits of the evidence based medicine in some particular settings, much more than the limits of the combined treatment.

About ten years ago, in a provocative and very well done systematic review of the literature, Smith and Pell observed that parachutes are widely used to prevent death and major injury after gravitational challenge, but their effectiveness has not been proven by randomized controlled trials because of the difficult accrual[32].

Sometimes, the evidence cannot be evidently proved, and needs to be supported by the common sense.

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Serrated colorectal cancer: Molecular classification, prognosis, and response to chemotherapy

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Abstract

Molecular advances support the existence of an alternative pathway of colorectal carcinogenesis that is based on the hypermethylation of specific DNA regions that silences tumor suppressor genes. This alternative pathway has been called the serrated pathway due to the serrated appearance of tumors in histological analysis. New classifications for colorectal cancer (CRC) were proposed recently based on genetic profiles that show four types of molecular alterations: *BRAF* gene mutations, *KRAS* gene mutations, microsatellite instability, and hypermethylation of CpG islands. This review summarizes what is known about the serrated pathway of CRC, including CRC molecular and clinical features, prognosis, and response to chemotherapy.

Key words: Colorectal cancer; Methylator phenotype; Serrated pathway; Chemotherapy; CIMP

Core tip: Recently, the implication among colorectal cancers with methylator phenotype has burst into the gastroenterology literature. In this review, we analyze the correlation between serrated cancers, the methylator phenotype and other genetic features in order to assess their prognosis and response to adjuvant chemotherapy.

INTRODUCTION

Colorectal cancer (CRC) is considered a major health issue: it is the most prevalent cancer and the second

Figure 1 Types of serrated polyps. Serrated polyps can be divided into four types: hyperplastic polyps (HPs), sessile serrated adenomas (SSAs), traditional serrated adenomas, and mixed polyps (MPs). The polyps can also be genetically characterized according to their CIMP, BRAF, KRAS, and MSI status, and certain patterns are found frequently for each category of polyp. Both microvesicular hyperplastic polyps (MVHPs), which are an HP subset, and SSAs have *BRAF* mutations more often than KRAS mutations. Conversely, goblet cell hyperplastic polyps (GCHPs), another HP subset, more frequently show *KRAS* mutations. *BRAF* mutations often correlate with high-grade CIMP CRC, whereas *KRAS*-mutated serrated polyps are more commonly found in low-grade CIMP. MPHP: Mucin-poor hyperplastic polyp.

largest cause of cancer death in Western countries[1]. Traditionally, colorectal carcinogenesis research has focused on the chromosomal instability (CIN) pathway, in which the APC gene mutation is the first pathogenic event that leads to allelic losses and to somatic gene amplification and translocation. This classical carcinogenetic model is responsible for about 70%-80% of all CRC cases[2-5]. A second carcinogenic pathway was described in the last decades of the 20th century. This pathway is related to inactivation of the mismatch repair (MMR) gene system, which in turn leads to inactivation of mutated tumor suppressor genes, and is called MMR or MSI pathway. Lynch syndrome is the paradigm of this alternative carcinogenetic model; this syndrome leads to diploid tumors that have a microsatellite instability (MSI) phenotype[6].

Lastly, molecular advances have identified a third pathway of colorectal carcinogenesis. This pathway does not cause changes at the chromosomal level or in the MMR system; rather, it involves hypermethylation of specific DNA regions near the promoter genes: the CpG islands. This alternative pathway is called the serrated pathway due to the serrated appearance of tumors in histological analysis. The main molecular feature of these tumors is the variable degree of methylation in promoter gene regions[7]. Like MSI tumors, serrated cancers are diploid tumors, and some genetic variability has been described for tumors caused by the serrated pathway. New classifications were proposed recently for CRC based on genetic profiles that show four types of molecular alterations: BRAF gene mutations, KRAS gene mutations, MSI, and

hypermethylation of CpG islands[8,9].

The aim of this review is to summarize what is known about the serrated pathway and CRC, including CRC molecular and clinical features, prognosis, and response to chemotherapy (CT).

SERRATED LESIONS

Serrated polyps constitute a heterogeneous group of lesions that include four types of polyps: sessile serrated adenomas (SSAs), traditional serrated adenomas (TSAs), mixed polyps (MPs), and hyperplastic polyps (HPs)[10]. HPs are the most prevalent serrated lesion type, accounting for around 80%-90% of serrated polyps. HPs are subdivided into three subtypes: microvesicular HPs (MVHPs), goblet cell HPs (GCHPs), and mucin-poor HPs (MPHPs)[11]. Although only a minority of HPs will progress to CRC, mainly when they are right-sided, there can be progression to other serrated lesions that can evolve into CRC[11]. MVHPs are more likely to localize to the right colon and may progress more frequently to SSA. Conversely, when GCHPs evolve into other lesions, they are more likely to be left-sided TSAs (Figure 1)[12].

SSAs are less common than HPs, accounting for about 10%-25% of serrated polyps. Located mainly in the right colon, they can be original tumors or can evolve from HPs[13]. TSAs account for about 1%-2% of serrated polyps and are more frequent on the left colon[13]. Finally, MPs constitute around 1%-4% of serrated polyps[14]. Mixed tumors usually comprise a dysplastic lesion (TSA or conventional adenoma) plus a non-dysplastic one, usually an HP or SSA[15].

GENETIC AND MOLECULAR FEATURES

The serrated pathway is complex and is currently poorly understood. This pathway has two key characteristics, namely aberrant hypermethylation of certain promoter regions in the genome and alterations in MAP kinase signaling pathway genes.

Aberrant hypermethylation

Functions related to the human genome are tightly regulated, and enzymatic systems control processes such as DNA mismatch repair, DNA transcription and replication. DNA transcription is the result of the regulation of genomic expression. Specifically, the transcription machinery interacts with the start codon ATG *via* DNA methyltransferases and forms methyl cytosine[16]. In humans and other mammals, only cytosine residues that precede a guanosine in the DNA sequence are modified to form CpG dinucleotides. These can change the three-dimensional configuration of the DNA and consequently affect its interaction with transcription factors. Methylation of these CpG dinucleotides usually leads to genetic silencing. CpG dinucleotides are located throughout the DNA sequence, and approximately half of all human gene promoter sequences are embedded in these CpG clusters, which are termed CpG islands[17]. The CpG island sequence is at least 200 bases long and is usually > 500 bases; the CG content is > 50%, and the ratio of observed-to-expected CpGs is > 60%[18,19]. In the genomes of cells in healthy tissue, the CpG islands are usually unmethylated, especially those associated with gene promoters. Conversely, about 80% of the CpG dinucleotides that are not part of CpG islands (*i.e.,* that are in DNA non-coding regions) are heavily methylated to prevent the expression of viral sequences that are integrated into the genome as well as the expression of many other DNA elements[20]. On the one hand, hypomethylation of these CpG dinucleotides could result in harmful gene expression; on the other hand, methylation of the promoter regions could have undesirable adverse effects.

Notably, some methylated promoters do not play any role in tumor development-these genes with methylated promoters are called "methylated in tumor" or MINT genes[16]. However, other methylated CpG islands in promoter regions silence the expression of known tumor suppressor genes by interrupting the interactions between transcription factors and the start codon. When this happens, it is considered a new phenotype of CRC that is caused mainly by epigenetic alterations rather than by DNA mutations and is called the CpG island methylator phenotype or CIMP. This term was introduced in 1999 when it was first suggested that CRC could be initiated by genetic silencing in the absence of DNA sequence modifications[21]. There are two types of CIMPs that are closely related and that are sometimes difficult to distinguish. Whereas CIMP-A is more related to

aging, CIMP-C is more related to cancer[22,23]. In addition, some authors have suggested different ways to categorize CIMPs[24]. Shen *et al*[25] defined three subgroups of CIMP CRCs: CIMP1, CIMP2, and CIMP-negative. KRAS mutations are reported to be the strongest predictor for CIMP2 (92%). Later, Ogino *et al*[26]. Proposed the use of 8 markers rather than 5 to classify methylation. They defined CRCs as CIMP-0 when none of the markers were methylated; as CIMP-low (CIMP-L) when 1 to 5 markers were methylated; and as CIMP high (CIMP-H) when 6-8 markers were methylated. The new cluster of methylated markers proposed by Weisenberger suggests that CIMP-L tumors might have high levels of methylation at other unknown loci rather than at a just a few loci. In fact, more CIMP CRCs were identified when the test panel of methylated markers was expanded[7]. Although the Weisenberg panel of loci is currently used more often than other panels, it is not yet clear which markers are the most appropriate for establishing the CIMP status of tumors.

The prevalence of CIMP tumors varies in different types of serrated lesions. CIMP-H is present in around 41%-73.3% of MVHPs but only in about 8%-18.2% of GCHPs[27]. The proportion of CIMP-H in evolved serrated lesions is similar: the proportion is about 44%-76.8% in TSAs and about 80% in SSAs[28]. Nonetheless, low grade CIMP is more often associated with TSAs than with SSAs (Figures 2 and 3)[13,27-32].

The MAPK pathway

When the serrated pathway was first described, investigators considered it a unique and linear carcinogenesis pathway. However, the current view is that the early events in this pathway include both an alteration of the MAPK pathway plus concomitant DNA epigenetic alterations[4]. The MAP kinase pathway is a *via* of intercellular signaling transmission. Mutations in their constituent proteins mainly involve the Raf and RAS families (Figure 4). There are 3 types of Raf kinases, termed types A, B, and C. B-Raf (or BRAF) is located on 7p34, is involved in serrated CRC, and is mutated in approximately 10% of CRC cases[33]. A BRAF mutation is rare in CIN tumors, and its presence almost completely excludes a Lynch syndrome diagnosis[34,35]. Therefore BRAF can be considered to be specific to CRCs arising *via* the serrated pathway[7,33,35]. The *BRAF* V600E mutation, in which valine is substituted for glutamate at codon 600 on chromosome 7[36], is the most common and the best characterized mutation. V600E leads to constitutive gene activation, thereby inducing cell proliferation and inhibiting apoptosis.

RAS mutations can also lead to dysfunctional MAPK pathway signaling. There are at least three RAS genes, namely H-RAS, N-RAS, and K-RAS or KRAS[37]. The consequences of KRAS gene mutations are similar to those of BRAF mutations in that the mutations can induce proliferation and inhibit apoptosis. Classically,

Serrated polyp type	MVMP	GCHP
Morphology	Few larger polyps	Many smaller polyps
Preferential location	Right colon	Left colon
CIMP-H phenotype (%)	41-73	8-18
MSI (%)	No reported	8
BRAF mutation (%)	67-88	20-83
KRAS mutation (%)	6-17	8-73

Figure 2 Features of microvesicular hyperplastic polyps and goblet cell hyperplastic polyps. Microvesicular hyperplastic polyps (MVHPs) and goblet cell hyperplastic polyps (GCHPs) are premalignant lesions that differ mainly in their location and morphology. MVHPs are more likely to occur as a few large polyps in the ascendant colon, and GCHPs are more likely to occur as multiple small polyps in the left colon. Each can become senescent or evolve into another type of polyp. Although they can transform into other adenomas, MVHPs more often evolve into sessile serrated adenomas (SSAs), and GCHPs more often evolve into traditional serrated adenomas (TSAs). The prevalence of *KRAS* mutations is higher in TSAs, while *BRAF* mutations are more prevalent in SSAs.

Serrated polyp	SSA	TSA
Prevalence (%)	10-25	1-2
Preferential location	Right colon	Left colon
CIMP-H phenotype (%)	44-77	43-80
MSI (%)	Rarely observed	3
BRAF mutation (%)	32-83	60-76
KRAS mutation (%)	7-25	0-28

Figure 3 Features of sessile serrated adenoma and traditional serrated adenomas. Pathological analysis shows that sessile serrated adenoma (SSAs) are more common than traditional serrated adenomas (TSAs). While TSA is preferentially located on the left colon, SSA is preferentially located on the right side. In addition, both SSAs and TSAs are found in high-grade CIMP tumors, although several studies show a relationship between low-grade CIMP tumors and TSAs. *BRAF* mutations are often observed in SSA, whereas *KRAS* mutations are often observed in TSAs. Contrary to what is found in serrated adenocarcinomas, MSI is almost never present in serrated polyps.

RAS mutation has been linked to the CIN pathway, but RAS is also impaired in some serrated cancers. KRAS mutations are found in 30%-40% of CRCs[38,39]. Activating KRAS mutations are most common (up to 80%) in codon 12 but are also found in codon 13; these include the G12D, G12V, and G13D KRAS point mutations[40]. Notably, the combination of KRAS mutation plus low grade CIMP in many serrated lesions constitutes an alternative subset of CRC that is established *via* the serrated pathway. For example, an increase in MGMT methylation rather than MLH1 methylation is associated with CIMP-L and KRAS mutations[41,42].

Serrated pathway
In the serrated pathway, *BRAF* or *KRAS* mutation initially induces a burst of cellular proliferation in the normal colorectal epithelium, and serrated aberrant hyperplastic crypt foci seem to be the earliest histological lesions[43]. Mutations in both genes,

Figure 4 MAPK pathway. This simplified diagram of the MAPK pathway shows the RAS/Raf-1/MEK/ERK pathway. RAS mutations are found in 36% of serrated polyps, and Raf-1 mutations are found in 9%-11%. These mutations promote gene transcription and cellular growth that results in cellular adhesion, invasion, metastasis, and angiogenesis.

although more frequently mutations in *BRAF*, can lead to upregulation of p16INK4a and to an increase in insulin-like growth factor binding protein 7 (IGFBP7) secretion at aberrant crypt foci[44]. p16INK4a and IGFBP7 are tumor suppressor proteins that prevent polyp growth and that drive these proliferative cells to form small senescent lesions. The silencing of these genes, for example by methylation, allows the cells to escape from cellular senescence and permits the progression to MVHPs or GCHPs and then to serrated polyps[37]. Some mRNAs have also been linked to serrated carcinogenesis. One example is microRNA-31, located at 9p21.3, which seems to correlate with mutated *BRAF* tumors. MicroRNA-31 may be involved in the progression from HPs to SSAs, since it has been detected in a high proportion of these lesions as well as in the CRCs that evolved from them[45,46].

Some genes are inhibited in the pathway to malignancy in CRC. Among them, the methylguanine methyltransferase gene, *MGMT*, and the *MLH1* gene are the best characterized. *MGMT* is frequently involved in the progression of TSA[42]. While *MLH1* silencing is linked to SSA evolution. *MLH1* is one of the main genes in the MMR system, and its inactivation leads to an accumulation of mutations in microsatellite sequences. This results in an MSI phenotype in these tumors, which is a hallmark of CRC in Lynch syndrome. However, only 3% of all CRCs and 20% of MSI CRCs are due to Lynch syndrome[18]. The remaining 80% of MSI CRCs are considered sporadic CRCs that are caused by the epigenetic inactivation of *MLH1*, which is present in approximately 15% of all CRCs[47]. These

tumors follow the serrated pathway of carcinogenesis, especially when CIMP is present[24,48]. In fact, most of the sporadic MSI CRCs are CIMP tumors, although only half of CIMP CRCs show methylation of the MMR genes (Figure 5)[49,50]. "the natural course from serrated polyps to advanced cancers can be followed in this figure".

CLINICAL FEATURES

The serrated pathway leads to a broad spectrum of CRCs. It is important to view each CRC as the result of both genetic and epigenetic alterations. In a review of the literature, serrated adenocarcinoma was frequently located on the cecum (52% of the time) and constituted around of 16% of all proximal CRCs[51-53]. Serrated adenocarcinoma is more common in Caucasians than in Hispanics or African Americans[54]. Some studies have suggested that there is an association between specific genetic profiles and certain clinical characteristics. Based on this premise, several studies have reported the clinical profiles of serrated adenocarcinoma cases[8,9].

Some studies distinguish between serrated adeno-carcinoma with *vs* without mutant *BRAF*. Serrated adenocarcinoma with mutant BRAF seems to be more frequent in older patients who are heavy smokers and in female patients[55]. It is more prevalent on the right colon in larger tumors that are usually diagnosed at an advanced stage (either pT4 or N2)[56,57]. In fact, in the right colon, there seems to be a close relationship between these features and the loss of expression of the CDX2 gene and increased levels of annexin

Figure 5 Serrated pathway. This diagram shows the chronological changes that occur during the development of neoplasia. *MAPK* mutations do not affect just one gene; rather, they affect some genes more than others. Gene methylation is successive rather than simultaneous and contributes to the progression of the serrated lesion. Depending on which genes are most affected, serrated cancers can be classified into three subtypes. GCHP: Goblet-cell hyperplastic polyp; MVHP: Microvesicular hyperplastic polyp; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma.

A10[58,59]. In addition, serrated adenocarcinoma cases show a low frequency of liver and lung involvement at the metastatic stage, but there is a higher prevalence of peritoneal involvement at the onset of metastasis[55,60]. From a pathological viewpoint, BRAF-mutated serrated adenocarcinomas are more frequently high grade tumors than other serrated adenocarcinomas[61].

Mutated KRAS tumors, represent a subset of CRCs that arise *via* the serrated pathway. Clinically, these tumors are more variable than tumors with mutated BRAF, probably due to the difficulties of differentiating mutated KRAS tumors that arise *via* the serrated pathway from those that arise *via* the CIN pathway. KRAS mutated serrated tumors are not linked with proximal location, and they are associated with higher body weight, higher body fat percentage, and with female sex[62]. Although one study found a higher prevalence in men[63]. KRAS-mutated serrated adenocarcinomas frequently show both low grade tumor differentiation and low grade CIMP.

The clinical features of CIMP and MSI tumors frequently overlap. CIMP tumors with MSS have not been described in great detail, but one study showed that they are associated with proximal CRC location, female sex, and the presence of lymph node metastasis and that they tend to present with liver metastasis (Figure 6)[64].

PROGNOSIS

Until now, the prognosis and management of CRCs has been based on the TNM staging system. However, the genetic and molecular profiles of the different CRC types should be used to improve the classification and management of these tumors. Few studies have addressed the question of the prognoses of the different CRC subtypes.

The *BRAF* mutation was the first biomarker to be investigated. It has been described as a marker of poor survival, due to its association with remodeling of the extracellular matrix, a process that is important for tumoral invasion and metastasis. Notably, its prognostic role was investigated in a meta-analysis of 26 CRC studies[65]. In general, the presence of BRAF mutation is an independent marker of a poor prognosis and is related to a decrease in 5-year disease-free survival (DFS)[66]. Its predictive value remains after stratification by age, disease stage, and degree of differentiation. However, other studies found that mutant BRAF does not affect the intrinsically good prognosis of MSI CRC. For example, Popovici *et al*[67] found BRAF to be a marker of poor prognosis only in subpopulations with microsatellite-stable left-sided tumors. Similarly, others report worse prognosis in MSS cancers in men but not in women[68]. As noted above, there seems to be a strong relationship between the CIMP status and BRAF mutations, with the prognostic value of CIMP status being related to the presence of BRAF mutations and, less frequently, to the presence of KRAS mutations[69]. Finally, regulation of SOX2 gene expression and its role as a biomarker of poor survival has been linked to tumors with BRAF mutations. This may be related to cellular migration and invasion, since an increase in SOX2 expression also correlates with CRC liver metastasis[70].

In contrast to BRAF, the MSI status is closely related to good prognosis in CRC, including in serrated tumors. The predictive power of MSI is independent of CIMP, BRAF, and KRAS status. However, it is clear that MSS tumors are associated with better DFS at any stage when no CIMP, KRAS, or BRAF mutations

Figure 6 Polyp features according to location. Half of serrated adenocarcinomas (SACs) are located on the right colon. KRAS-mutated polyps are preferentially left-sided. However, polyps with BRAF mutations dominate those found on the right side of the colon. TSA: Traditional serrated adenoma; SSA: Sessile serrated adenoma.

are found; in such cases, DFS is similar to that in familial or sporadic MSI-CRC[9] Some clinical trials have reported poor outcomes in patients with CIMP-H MSS CRC. Comparing the two MMR states, stage Ⅱ and Ⅲ CRC with MSI has a better prognosis than MSS CRC[47]. Moreover, combined MSS and CIMP CRC with mutant BRAF or KRAS frequently correlates with liver metastasis at diagnosis and has the worst prognosis of all CRCs[8,9,64]. The exactly role of CIMP status in metastatic stage has not been elucidated. It's possible that the worst prognosis of those tumors could be due to the presence of BRAS mutations as well.

The prognostic value of KRAS mutations has also been investigated. KRAS mutation is proposed to be a predictor of lower 5-year DFS in almost all studies, without any prognostic differences between right- and left-sided CRCs. However, some point mutations have been described that impact prognosis. For example, a KRAS codon 13 mutant is associated with more aggressive CRC[68].

The prognostic value of CIMP status remains controversial. There are few doubts about the relatively good prognosis of sporadic CIMP-H MSI CRC. But for sporadic MSS CRC, some studies show no effect of CIMP-H on survival prediction, while others show either a negative or a positive impact[70]. Ogino *et al*[71] described CIMP-H as a specific marker of low CRC mortality, and it appeared to counter the adverse prognostic effect of BRAF mutation in serrated cancers. Perhaps, the hypermethylation of certain genes avoided the proliferative effect of BRAF mutations. One clinical trial found it to be an independent predictor of cancer survival[72]. Despite this finding, many researchers consider CIMP a predictor of short survival, especially in proximal stage Ⅲ CRCs[69,73]. These authors discussed some possible reasons for

these controversial findings, namely differences in cohorts, the use of different panels of methylated markers, the use of different criteria to define CIMP, and confounding factors such as BRAF mutation[71]. In a recent meta-analysis of CIMP prognostic value, both DFS and overall survival (OS) were shorter in patients with CIMP CRC after adjusting for age, sex, disease stage, and treatment; it was not possible to adjust for BRAF or KRAS mutation or MMR status since not all the studies assessed these possible confounders)[70]. It seems likely that when a CIMP serrated tumor shows MSI, the MSI acts to confer a good prognosis[71].

Recent studies have revealed a link between some methylated genes and patient prognosis. MLH1 and MGMT methylation have been suggested to be predictors of good prognosis, in part due to the frequent absence of *BRAF* mutations in these subgroups of tumors. In contrast, hypermethylation of the p14 (CDKN2A^{INK4a}), RASSF1A, and p16 (CDKN2A^{ARF}) genes have been suggested to predict poorer prognosis independent of both stage and histological differentiation grade[74-76]. Aberrant methylation of genes encoding proteins involved in extracellular matrix remodeling also confers a worse prognosis, as does hypomethylation of *LINE-1* in sporadic MSI CRC[77]. Hypomethylation of the *IGF-2* gene and RAC1b overexpression are proposed markers of poor prognosis in *KRAS/BRAF* wild-type metastatic CRCs treated with FOLFOX as first-line therapy[78]. Finally, a mutated *P1K3CA* gene is associated with a worse survival rate in CRC with wild-type BRAF. Alteration of P1K3CA is found in 10%-20% of cases with CIMP-H. It is currently unknown whether P1K3CA mutation defines a new subset of CRC[79].

Genetic variability can strongly influence patient outcome, highlighting the need for a new or more fine-tuned CRC classification system. Recently, two

Table 1 Proposed[80] colorectal cancer classification

	MMR	CIMP	BRAF	KRAS
Phipps				
Subtype 1	MSI-H	+	+	-
Subtype 2	MSS or MSI-L	+	+	-
Subtype 3	MSS or MSI-L	-	-	+
Subtype 4	MSS or MSI-L	-	-	-
Subtype 5	MSI-H	-	-	-
Sinicrope				
Subtype 1	MSI	+	+	+/-
Subtype 2	MSS	+/-	+	-
Subtype 3	MSS	+/-	-	+
Subtype 4	MSS	+/-	-	-
Subtype 5	MSI	-	-	+/-

Based on the classification system proposed by Jass[80], Phipps *et al*[8] and Sinicrope *et al*[9] proposed new colorectal cancer (CRC) divisions to better stratify disease based on prognosis. (+): Mutated; (-): Wild-type.

Table 2 Summary of the treatment implications of genetic alterations in colorectal cancer

Molecular marker	Implications for CRC treatment
KRAS mutation	Broadly studied in metastatic CRC
	The most predictive biomarker for no response to anti-EGFR, either alone or with CT
	Worse OS when oxaliplatin is the first-line treatment
	Irinotecan efficacy is controversial; it may have better effects in stage Ⅱ and Ⅲ CRC
BRAF mutation	Overall, no predictive power for CT response
	No predictive power for response to 5-FU plus irinotecan/oxaliplatin or to 5-FU alone in stage Ⅱ disease
	A trend toward better survival with 5-FU plus irinotecan in stage Ⅲ disease
	Effects of anti-EGFR therapy are controversial, although most studies show a poor response
	Some studies show no differences in OS/DFS with FOLFOX-panitumumab or with FOLFIRI-cetuximab treatment
	Resistance to BRAF inhibitors
CIMP	CT results are controversial
	5-FU improves DFS and OS in some studies; in others, survival is reduced
	One study shows the benefits of 5-FU plus irinotecan in CIMP tumors after stratification by MMR status. CIMP was more strongly associated than MMR status with a better response to irinotecan
	The use of 5-FU in CIMP tumors is not currently recommended
	To date, no clinical trials have evaluated the response of CIMP tumors to anti-EGFR therapy
MMR	Prognosis is intrinsically better for MSI CRC, but MSS tumors show a better response to CT
	5-FU improves both DFS and OS in stage Ⅱ and Ⅲ MSS CRC but not in MSI CRC
	CT should only be given for stage Ⅱ MMS tumors if a high risk factor such as T4 local extension is present
	MSI CRC shows a good response to irinotecan if BAX expression has been lost

CT: Chemotherapy; DFS: Disease-free survival; anti-EGFR: Epidermal growth factor receptor antibodies; OS: Overall survival; CRC: Colorectal cancer; MMR: Mismatch repair.

publications established 5 subtypes of CRC and described differences in survival according to the subtypes' phenotypic characteristics. First, based on the classification system of Jass[80], Phipps *et al*[8] divided CRCs into MSI-H and MSS/MSI-L CRC. Later, this group further divided cases according to CIMP status and then by BRAF and KRAS mutation status. The final system describes 5 well-defined CRC subtypes (Table 1). Among the subtypes, the highest survival was found for subtype 5, which is characterized by non-CIMP MSI CRC with wild-type *BRAF* and *KRAS*; this is also known as familial MSI CRC or Lynch syndrome. Conversely, the worst prognosis was found for subtype 2, which is CIMP-positive MSS/MSI-L CRC with BRAF mutations and wild-type KRAS. Subtype 2 has the lowest probability of being diagnosed as stage Ⅰ disease and shows the worst 5-year DFS. Interestingly, subtypes 1 and 2 are very similar, with the MSI status of subtype 1 being the only difference between them.

Sinicrope *et al*[9] proposed a similar classification system based on patients with stage Ⅲ CRC. This group also divided CRC into 5 subgroups, each of which was compared to subtype 4 (MSS CRC with wild-type *BRAF* and *KRAS*, representing the classic CIN pathway). The subtypes with MSI have the best prognosis, followed by subtype 5 (which is identical to the Phipps subtype 5) and subtype 1. Regardless of BRAF mutation status, MSI confers relatively good DFS in subtype 1 CRC. In contrast, subtypes 2 and 3 do not differ in terms of prognosis, but their prognosis differs from that of subtype 4. As in Phipps' classification, subtype 2 (CIMP-H MSS CRC with wild-type *KRAS* and mutant *BRAF*) shows the worst survival rate. KRAS mutation alone, with no other apparent genetic alterations, is the major alteration in subtype 3, representing an alternative subset of CRC that arises *via* the serrated pathway. Its prognosis is 1.5-times worse than that of subtype 4 (Table 1).

These findings illustrate the importance of classification based on genetic profiling and could improve our understanding of patient prognosis and disease management.

RESPONSE TO CHEMOTHERAPY

The current trend is towards offering treatment that is individualized according to CRC subtype. The most recent advances in CRC are leading to novel approaches and classification schemes, and it is attractive to think that a particular therapy can be used to target a particular type of CRC, including serrated tumors. Unfortunately, CT is not yet fully guided by the combination of genetic alterations in a CRC, and even the most recent studies have not assessed specific therapeutic management strategies for each subtype. Additional clinical trials are needed to address this. As of now, decisions must be based on studies that evaluated the responses according to individual markers (Table 2).

KRAS mutations have mainly been studied in

metastatic CRCs. In the QUASAR study, KRAS was not a negative predictive factor for the response to standard CT based on 5-Fluoruracil (5-FU) and folinic-acid[81]. However, the major characteristic of mutant KRAS remains its resistance to anti-EGFR monoclonal antibodies: it is the most important predictive marker in terms of the response to panitumumab and cetuximab, either alone or in combination with CT. This finding, which was confirmed in a meta-analysis[82], is valid for CRC with any inactivating KRAS mutation. It is also possible that the presence of KRAS mutations plus other alterations in NRAS, exons 3 and 4, PIK3CA, PTEN, and BRAF may influence CT resistance. In fact, Tian *et al*[83] found that a significant response to anti-EGFR was possible when KRAS, BRAF, and PIK3CA were all wild-type, but not when any one of them was mutated. It is currently standard practice to investigate KRAS status in order to predict the response to anti-EGFR in metastatic CRC.

BRAF mutation has also been widely studied, but its role as a predictive marker remains unclear. BRAF has repeatedly been described as a non-predictive biomarker in CRC treated using standard CT. When CRCs with [V600E]BRAF were treated with 5-FU plus irinotecan or oxaliplatin, BRAF did not correlate with either a positive or negative response[84]. Nonetheless, there was a slightly non-significant trend toward better survival in stage III CRC when irinotecan was added to a 5-FU/leucovorin regimen[85]. Notably, regarding the response to other drugs, both a null predictive value and a poor response to anti-EGFR have been reported for CRC with mutant BRAF[65]. BRAF did not show predictive power in the OPUS or CRYSTAL trials, which used FOLFIRI ± cetuximab[86,87]. The PRIME study reported similar results with FOLFOX4 ± panitumumab treatment[88]. Regarding the poor response, Di Nicolantonio *et al*[89] found that KRAS-/BRAF+ CRC showed a 0% response rate to anti-EGFR in second or subsequent lines of treatment; in contrast, the response rate was 32% in *KRAS⁻/BRAF* CRC. Loupakis *et al*[90] reported a similar response rate with irinotecan plus cetuximab. A meta-analysis that included these last two studies as well as other studies comparing metastatic CRC with mutated *vs* wild-type *BRAF* concluded that the response to anti-EGFR could be considered poor in tumors with mutant *BRAF*[91]. However, other studies did not find differences in the responses of CRCs with BRAF mutation *vs* wild-type BRAF after treatment with cetuximab, capecitabine, oxaliplatin, and bevacizumab[92,93]. Thus, to summarize, the use of BRAF mutation status as a predictive marker of the response to EGFR-targeted treatment remains controversial.

BRAF inhibitors such as vemurafenib and dabrafenib show good efficacy in melanoma. Silencing of [V600E]BRAF correlates with an increase in epithelial differentiation, in CDX-2 and claudin-1 expression[94,95], and in cellular adhesion. However, experiments in cell lines demonstrate that CRCs are intrinsically resistant to BRAF inhibition. This resistance may be dependent or independent of the ERK MAPK signaling pathway. Activating PIK3CA and AKT1 mutations and the loss of PTEN function have been proposed as possible mechanisms that underlie this resistance[82,96,97]. CRAF may also be involved. Recently, better growth inhibition was observed when BRAF inhibitors were used in combination with anti-EGFR monoclonal antibodies. Treatment with an anti-EGFR agent (like cetuximab) or a tyrosine-kinase inhibitor (such as erlotinib or gefitinib) plus a BRAF inhibitor results in sustained MAPK pathway suppression[98,99]. Different combinations of vemurafenib, erlotinib, capecitabine and/or bevacizumab, and cetuximab and/or irinotecan have shown better responses than vemurafenib alone[100]. Moreover, using vemurafenib plus anti-EGFR could improve clinical efficacy[101]. In fact, a pilot trial with 15 patients is ongoing, and the results look promising[102]. Simultaneous inhibition of the BRAF and PIK3 cascades shows a trend towards tumor regression in both mouse and human cell cultures. Ongoing studies are assessing triple combination therapy (*i.e.,* inhibition of BRAF, EGFR, and PIK3CA).

A different question regarding serrated CRCs concerns the response of CIMP tumors to the different CT options. To date, several studies have reported conflicting results. The hypothesis that CIMP cancers inhibit gamma-glutamyl hydrolase, thus enhancing intracellular folate levels and modulating a better response to 5-FU, suggested that these CRCs would benefit from adjuvant CT regimens[103-107]. Juo *et al*[70] published the first meta-analysis to address this matter. The meta-analysis identified 7 studies that evaluated the response of serrated CRC to CT as adjuvant therapy after surgical resection. Some of the studies described the benefits of 5-FU in both stage II and III CRC. Van Rijnsoever *et al*[108] found higher DFS in patients treated with 5-FU compared with patients that received surgical resection alone, as did Donada *et al*[109] who looked at stage II CRC. However, Jover *et al*[110] described a significant response to 5-FU in terms of DFS in stage II and III non-CIMP CRCs but not in CIMP CRCs. Two studies found that CIMP status had non-significant predictive value in terms of 5-FU treatment. Kim *et al*[111] compared a FOLFIRI regimen in non-CIMP *vs* CIMP metastatic CRC, while Han *et al*[112] investigated patients with stage II and III disease treated with a FOLFOX combination. A total of 4 studies reported that CT was beneficial in these patients, whereas 2 found non-significant results and 1 concluded the opposite. In a study that was not part of the meta-analysis, Wang *et al*[113] also found worse DFS rates when 5-FU was given to CIMP-positive tumors, in agreement with the findings of Jover's study. Shiovitz *et al*[114] also analyzed OS with irinotecan therapy. They compared 5-FU plus leucovorin with and without irinotecan in stage III CRCs and found a non-significant trend towards better survival with the

triple combination. In addition, patients with CIMP tumors seemed to benefit more after the tumors were stratified according to MSI status. It could be due to the better prognosis associated to unstable tumors, but further explanations regarding this are needed. Moreover, in this study, CIMP status was more strongly associated with the response to irinotecan than was MMR status.

These conflicting results may be due in part to differences in definitions, gene panels, CIMP marker thresholds, and laboratory techniques used to assess CIMP status. Currently, the National Comprehensive Cancer Network (NCCN) treatment guidelines for colon cancer (version 3) do not recommend the use of CT for stage Ⅱ (T2N0M0) CIMP cancers[115]. Because of these controversial results, the predictive value of CIMP status regarding treatment with 5-FU remains unclear, and more randomized clinical trials are needed.

Finally, the role of MMR status in predicting the response to CT has been studied extensively[116,117]. Several studies have established that MSI tumors have an intrinsically better prognosis than MSS tumors. Jover *et al*[118] analyzed OS and DFS in a cohort of 505 patients with stage Ⅱ or Ⅲ CRC according to their MMR status after receiving 5-FU. MSS CRCs showed improved DFS and OS, but no improvement was seen in MSI CRCs[102,119]. These differences remained after multivariate analysis controlled for the TNM stage, age, and sex. A prospective study by Sargent *et al*[119] had similar findings and suggested that cancers with MSI do not benefit from 5-FU adjuvant CT. However, the decision to treat stage Ⅱ MSS or MSI-L cancers should be based on high risk factors such as T4 tumor status, perforation, and obstruction, among others. The response to irinotecan was also assessed in MSI CRC. There was a better response when the tumor had lost BAX expression, and the author proposed that this was by far the best criterion for predicting efficacy[120]. Current management may include the addition of irinotecan or oxaliplatin, but more studies are needed to evaluate different combinations of CT for MSI CRC.

FUTURE DIRECTIONS

This review enhances the current change of mind about CRC. The concept of a unique model of carcinogenesis is obsolete and the consideration of pathogenesis of CRC as only three possible pathways is changing. In the recent years, a lot of publications about several aspects, from molecular and genetic discoveries to pathologic classifications, have seen the light. Current trends seem to guide to new approaches of CRC in many aspects. Great disparity in clinical trials in prognosis and response to treatment has taken place among last years. In fact, it seems to have more than twenty-five genetic alterations to date, and increasingly. Some authors have understood the problem and are making new classifications basing on prognostic implications. Even, it's possible that

CRCs following the serrated pathway are divided into more genotypes in the near future. The complete understanding of molecular pathways is the first necessary step.

Many efforts are necessary to get many consensuses, like about the definition and method of assessing CIMP phenotype and the possibility of treating CRCs with mutant BRAF when no lymph nodes are found. Randomized clinical trials evaluating response to different CTs, drugs as EGFR-ab and other biologic treatments should take place to clarify their paper on serrated cancers. After that, assessing response to each subtype, it shall be possible to establish a more individualized prognosis and therapy to each patient.

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REVIEW

Genetic variation of occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus infection (OBI), characterized as the persistence of hepatitis B virus (HBV) surface antigen (HBsAg) seronegativity and low viral load in blood or liver, is a special form of HBV infection. OBI may be related mainly to mutations in the HBV genome, although the underlying mechanism of it remains to be clarified. Mutations especially within the immunodominant "α" determinant of S protein are "hot spots" that could contribute to the occurrence of OBI *via* affecting antigenicity and immunogenicity of HBsAg or replication and secretion of virion. Clinical reports account for a large proportion of previous studies on OBI, while functional analyses, especially those based on full-length HBV genome, are rare.

Key words: Hepatitis B virus; Occult; Variation; Hepatitis B surface antigen

Core tip: With error-prone reverse transcription during replication, hepatitis B virus (HBV) displays a high rate of mutation, and a single mutation may affect the biological properties of HBV. Occult HBV infection (OBI) is a special form of HBV infection and a frequent phenomenon. Many previous publications have explored the association of OBI with the "hot spots" mutations that occur within the immunodominant "α" determinant of S proteins. However, there are no reviews available that elaborate on the relationship between OBI and mutations throughout the entire HBV genome. This review attempts to provide a comprehensive summary of HBV genetic variants that have been associated with OBI.

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INTRODUCTION

Hepatitis B virus (HBV) is classified as a DNA virus, and it replicates *via* reverse transcription, which is an error-prone process, to generate an RNA intermediate. Thus, the mutation rate of HBV is in between those of RNA viruses and conventional DNA viruses[1]. The nucleotide mutation rate of HBV is estimated to be 10^{-5}-10^{-6} per site per year, about 10 fold higher than that of other DNA viruses, and this rate might increase 100 fold after liver transplantation[2,3]. Mutations are commonly encountered, especially in the basal core promoter (BCP), the pre-core region (Pre-C), the polymerase (P) gene, and the "α" determinant of S protein, and single or multiple mutations may affect the biological properties of HBV[4]. Occult HBV infection (OBI), a special form of HBV infection and a frequent phenomenon, was first reported in 1978 and has been studied further since then[5]. In 2008, the Taormina expert meeting defined OBI as the presence of HBV DNA in the liver of individuals negative for HBV surface antigen (HBsAg) testing with the amount of HBV DNA in the serum usually lower than 200 IU/mL or undetectable[6]. OBI is characterized by the persistence of low level viremia and HBsAg seronegativity and can be grouped into two types: seropositive [anti-hepatitis B core antigen (HBc) and/or anti-hepatitis B surface antigen (HBs) positive] and seronegative (anti-HBc and anti-HBs negative)[7]. OBI harbors the potential risk of HBV transmission through blood transfusion, organ transplantation, and hemodialysis as well as from occult infected or HBsAg-positive mothers to newborns[8,9]. Although the clinical and biochemical symptoms of most OBIs are not severe, there is still a risk of developing serious liver diseases, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[10]. In fact, a meta-analysis and other findings have revealed that OBI retains the pro-oncogenic properties of HBV and serves as a risk factor for the development of HCC[11-13]. Suppression of viral replication is reversible in the case of OBI, and reactivation of OBI and/or development of fulminant hepatitis failure (FHF) has been reported in patients following chemotherapy or immunosuppressive therapy and after transplantation as well as in patients co-infected with human immunodeficiency virus (HIV) or hepatitis C virus (HCV)[14-16].

PREVALENCE OF OBI

OBI is a health problem worldwide, and its prevalence is affected by several factors, such as the prevalence of HBV infection, the study population, and the sensitivity of diagnostic assays[17,18]. OBI is significantly associated with the endemicity of HBV infection but not restricted to countries highly endemic for HBV[10,17,18]. The prevalence of OBI in blood donors from China, South Korea, and Japan was reported to be 0.11%-0.13%, 0.016%, and 1.01%, respectively[19-22]. The incidence of OBI in anti-HBc positive donors was reported to be from 0% to 15%[23,24], and the detection rate of OBI in HIV infection was reported to be from 0% to 89%[25,26]. In the context of chronic HCV infection, the percentage of OBI ranged from 0% to 52%[27,28]. Previous studies conducted in hemodialysis patients indicated a prevalence of OBI from 0% to 36%[29,30]. OBI was detected in 32% of patients with cryptogenic LC in Hong Kong, an area with a high prevalence of HBV infection[31]. The prevalence of OBI was reported to be 70.4% among HBsAg-negative HCC patients in China[32].

The identification of OBI largely depends on the tests for HBsAg and HBV DNA. There are several commercially available HBsAg assays, which mainly depend on the use of anti-HBs antibodies, with different sensitivities and specificities. Some diagnostic HBsAg assays are based on monoclonal antibodies and recognize limited types of HBsAg variants. In comparison, assays that utilize polyclonal antibodies show higher sensitivity and specificity for the detection of various types of HBsAg mutants[33,34]. Because the level of viremia is low in OBI, a small amount of HBsAg below the detection limit of tests may not be reliably recognized. Although highly sensitive real-time quantitative polymerase chain reaction (PCR) is strongly recommended for HBV nucleic acid testing (NAT) for its capability to uncover low level of HBV DNA in serum, this method harbors a risk of false-positive results[35,36]. Anti-HBc screening can reduce the risk of HBV transmission but may possibly provide false-positive results as well. It is worth noting that the nature of the specimen tested (*i.e.*, blood sample or liver tissue), the amount of specimen, as well as contamination risks can also affect the detection of OBI.

Many studies have been conducted on OBI prevalence. However, cautions must be taken when results from different studies are compared, since differences in groups of people studied, areas selected, and diagnostic assays can significantly affect the results.

MECHANISM OF OBI

The mechanism of OBI is complicated and remains to be clarified. Both host and viral factors contribute to the occurrence of OBI. For example, the persistence of covalently closed circular DNA (cccDNA) in hepatocytes, suppression and detection failure of virion replication, and protein secretion are involved[37,38]. In addition, methylation of the HBV genome, microRNAs (miRNAs), and histone modification have been reported to affect the regulation of HBV replication and expression through epigenetic mechanisms, increasing

the possibility that epigenetic regulation may also be associated with OBI[39].

Viral factors

Genetic mutations may contribute to OBI. Mutations have an impact on the expression, secretion, and antigenicity of HBsAg and cause failure in detecting HBsAg by commercial diagnostic tests. HBV is prone to mutations and exists as quasispecies to facilitate viral survival. Point mutations and insertion as well as deletion mutations are commonly encountered in OBI, and although mutations in all open reading frames (ORFs) of HBV could directly or indirectly affect detection of HBsAg, most studies have focused on mutations in the Pre-S/S gene. Besides, OBI mutations are associated with the suppression of viral replication typical of OBI, which in turn affects HBsAg expression and secretion[40]. Circulating immune complexes consisting of HBsAg and anti-HBs may be associated with Pre-S/S gene mutations and impair the binding efficiency of HBsAg capture antibodies to HBsAg in serum, leading to immune escape[41,42].

Low levels of HBV replication may be correlated with OBI. Compared to HBsAg-positive infection, OBI always has lower (< 200 IU/mL) or even undetectable HBV DNA in serum[18]. Although HBV DNA from the liver is more reliable than circulating HBV DNA for recognition of OBI, the levels of intra-hepatic HBV DNA and cccDNA in OBI were also found to be lower than those in HBsAg-positive infection[38,43]. Thus, the low level of HBV replication may be related to OBI.

HBV genome methylation is related to the development of OBI. Hypermethylation of HBV has been observed in isolates from OBI patients, and HBV CpG islands 1 and 2 were more frequently methylated in HBsAg positive infections and OBIs, respectively[44,45].

Host factors

The alternation of host immunologic responses is associated with OBI. In patients with spontaneous and therapy-induced HBsAg clearance, residual HBV may present in the serum, liver, and lymphatic system for a long time[46,47]. Similarly, using a woodchuck model, the closet natural model of HBV infection, Michalak[48] and Mulrooney-Cousins *et al*[49] described the low-level but life-long persistence of woodchuck hepatitis B virus (WHV) DNA and cccDNA in both the liver and lymphatic system in animals recovered from acute WHV invasion. Based on these data, they suggested that the hard-to-eliminate cccDNA made the complete clearance of WBV difficult to accomplish and might contribute to WBV reactivation and further speculated that HBV cccDNA may act similarly to WBV cccDNA. Therefore, latency of HBV in liver and in extra-hepatic tissues and cells provide a favorable condition for the occurrence of OBI[50]. Reactivation of overt HBV infection and OBI has been frequently described in HBV infected patients with suppressed immune systems, suggesting that HBV was inhibited rather than cleared in the setting of immunosuppression. Colson *et al*[51] investigated 16 anti-HBc positive patients who developed HBV reactivation following chemotherapy and detected an increased variability within HBsAg and HBV reverse transcriptase (RT) in these patients. OBI has also been reported in vaccinated children from Taiwan and Iran[52,53]. It is speculated that the patients' immune response to HBV infection may be related to OBI by greatly controlling HBV activity, putting pressure on secretion of HBsAg and other HBV-secreted proteins, as well as promoting the screening of mutants.

In addition to altered host immunologic responses, host genetic factors are also linked to OBI. Related studies have focused on human leukocyte antigen (HLA), which plays an important role in the modulation of immune responses, and HLA polymorphisms are considered as vital factors in determining HBV infections outcome (persistence or clearance)[54]. Previous studies showed that HLA-A2 expression was correlated with protection against HBV infection, while significantly decreased intensity of HLA-A2 expression on peripheral blood mononuclear cells (PBMCs) was detected in some OBI patients compared to healthy controls, perhaps contributing to HBV resistance[55]. Serum level of interleukin (IL)-10 was markedly higher in some OBI patients than that in healthy controls, and polymorphisms of the IL-10 promoter were found with significant differences between OBIs and healthy controls, implying a relationship between IL-10 and OBI[56,57].

Host epigenetic modification may be related to OBI. miRNAs of host cells could regulate expression of some host genes and exert effects on HBV replication and expression[58]. A profile of HBV-specific serum miRNAs has been reported with a high accuracy to separate OBI cases from healthy controls (sensitivity: 99.9%, specificity: 99.8%), raising the possibility that they may serve as effective biomarkers in OBI detection[59]. Epigenetic modifications of cccDNA-bound histones may regulate HBV replication and transcription, with acetylation and methylation being the most common kinds of histone modification. Previous studies showed that HBV replication could be regulated by the acetylation status of cccDNA-bound H3 and H4 histones in chronic hepatitis B (CHB) patients, and this regulation has been confirmed by transfection experiments[60].

Other factors

Other factors may be connected with OBI, such as interference by other viruses. Concomitant presence of chronic HCV infection exerts a negative effect on HBV. HBV DNA levels are increased during interferon-ribavirin therapy of HCV but decreased after a viral breakthrough of HCV[61,62]. Moreover, *in vitro* studies have demonstrated the inhibitory effect exerted by HCV core protein on HBV replication and expression[63].

PRE-S/S MUTATIONS

Pre-S/S point mutation

Pre-S/S gene encodes three envelope proteins named large (L), middle (M), and small (S) protein, and S protein corresponds to HBsAg[64]. Pre-S/S has a relatively high mutation rate among all ORFs, and mutations of the Pre-S/S gene have been studied extensively. Point mutations that occur in the Pre-S/S gene may affect HBsAg in two different aspects, (1) mutations may affect antigenicity, immunogenicity, secretion, and/or expression of HBsAg, leading to detection failure of HBsAg[33,65]; (2) mutations may reduce or even abolish the replication and/or secretion of virion, exerting a negative effect on HBsAg[66,67]. Amino acid (aa) substitutions of HBsAg are mostly frequently clustered in the "α" determinant, which is located at aa124-147 of the S protein. The "α" determinant is rich in cysteine, and two loops are formed by disulfide bonds between C124 and C137 as well as C139 and C147 to maintain the conformational structure and antigenicity of HBsAg. The "α" determinant is a relatively conserved region within the major hydrophilic region (MHR) between aa100 to aa169, which serves as the most important antigenic determinant in all HBV strains and is essential to the detection of HBsAg and development of HBV vaccines[64,68]. Amino acids within the region between 120 to 123 were shown to be crucial for the antigenicity of HBsAg[69]. Therefore, single or multiple point mutations occurring within or adjacent to the "α" determinant may change the antigenicity and conformation of HBsAg, resulting in failure to detect HBsAg. Various point mutations of HBsAg have been observed in OBIs (Figure 1).

Amino acid mutations were detected mostly in the MHR in OBIs, and many previous reports have documented that the mutation rate in the MHR, especially in the "α" determinant, was higher in OBIs compared to that of HBsAg-positive patients[66,70]. A number of Pre-S/S mutations were observed in both OBIs and HBsAg-positive patients (Figure 1), while it has been speculated that they may not be associated with OBI. Clinically observed Pre-S/S mutations in OBIs summarized in Figure 1 indicate that: (1) Some mutations occurred in both OBIs and HBsAg-positive patients, while some mutations were only detected in OBIs; (2) Some mutations were found outside the "α" determinant and were located at the N-terminus or C-terminus of MHR or outside MHR; (3) Some mutations corresponded to amino acid substitutions in the P region; (4) Some amino acid substitutions were specific for genotypes, subgenotypes, or subtypes, while the clinical significance of them in OBIs remains to be determined; (5) Point mutations in the first loop of the "α" epitope (AA124-137) occurred more frequently in OBI isolates from unvaccinated patients[71], while point mutations in the second loop of the "α" epitope (AA138-147) were more

frequently isolated from OBI patients after immune prophylaxis[72]; and (6) Apart from point substitutions, there were other types of mutations or combined mutations. However, further *in vivo* and *in vitro* studies are required to explore the effects of point mutations on the occurrence of OBI.

In order to elucidate the role of mutations in the occurrence of OBI, plasmids of Pre-S/S variants isolated from the sera and liver tissues of OBI patients were constructed by site-directed mutagenesis and transfected into cell lines or introduced into animal models. The antigenicity, immunogenicity, and secretion of HBsAg as well as the replication and secretion of virions were then analyzed. This strategy has been extensively applied to point mutations in the Pre-S/S gene (Table 1 and Figure 2). Similarly, these functional analyses also can be applied to research on other HBV-related mutations[73]. To date, most *in vivo* and *in vitro* studies have been limited to the Pre-S/S gene, and only a few functional studies have been performed with full-length, replication-competent HBV genomes. It is difficult to know whether some point mutations interfere with the replication of the virion and secretion of antigens or if they play a role in the occurrence of OBI[40,74].

Many studies have documented that substitutions of wild-type cysteines and G145R in the "α" determinant cause conformational and phenotypic changes in HBsAg (Table 1 and Figure 2)[33,66,71,78]. El Chaar *et al*[80] restored the cysteines at positions 124 and 137 in recombinant surface protein (rS protein) from OBIs and expressed them in yeast and showed that antigenic reactivity of HBsAg was improved. However, restoration of cysteine at position 147 and G145R did not improve the impaired reactivity of HBsAg. Y100C was commonly found in OBIs, but transfection with Y100C plasmid resulted in higher extracellular HBsAg levels than the wild-type plasmid, implying that the mutation alone could not explain the decrease of HBsAg secretion and/or affinity to antibodies[76]. *In vitro* studies have found that some point mutations could strongly influence the secretion of HBsAg and that these mutations may interfere with the secretion of HBsAg in some cases, leading to the failure of HBsAg detection[86]. Some point mutations were found to affect HBV replication in hepatoma cell lines and/or in mice and the vesicular transport of infectious virions of HBV (*i.e.*, Dane particles) from the cell[66].

Glycosylation, the most common kind being N-linked glycosylation, is required for crucial biological functions of many enveloped viruses, since it can impart various advantages to virus survival and virulence[88]. Point mutations can affect biogenesis, stability, and antigenicity of HBV through modification of the envelope protein glycosylation pattern[89,90]. The envelope protein of HBV possesses an N-glycosylation site at N146 of the "α" determinant, and the removal of this site decreased the production of virion without affecting the synthesis and stability of HBsAg (Table 1 and Figure 2)[78-80]. Several positions in the envelope

```
                10         20         30         40         50         60         70         80
                 *          *          *          *          *          *          *          *
A    MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSPTSNHSPTSCPPICPGYRWMCLRRF
Ba   . . . A. . L . . . . . . . . . . . . . K. . . . . . . . . . . . . . T. . . . . . . . . QI. S. . . . C. . . . . . . . . . . . . . . . . .
Bc   . . . A. . L . . . . . . . . . . . . . K. . . . . . . . . . . . . . T. . . . . . . . . QI. S. . . . C. . . . . . . . . . . . . . . . . .
Bj   . . . A. . L . . . . . . . . . . . . . K. . . . . . . . . . . . . . T. . . . . . . . . QI. S. . . . C. . . . . . . . . . . Y. . . .
C1   . . . T. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A. T. P. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
C2   . . . T. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A. T. P. . . L. . . . . . . . . . . . . . . . . . . . . . . . . .
D    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . TT. . . . . . . . . . . . . . . . . . . . . T. . . . . . . . . . . .
E    . . S. . . . . . . . . . . . . . . . . K. . . . . . . . . . . . . . A. . . . . . . . . . . S. . . . . . . . . . . . . . . . . . . . .
F    . D. . . . L . . . . . VC. . . K. . . . . . . . . . . . . . L. G. P. . . . . . . . L. . . . T. . . . . . . . . . . . . . . . . . .
G    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V. . P. L. . . . . . . I. . . T. . . . . . . . . . . . . . . . . .
H    . . . . . . L . . . . . VC. . . K. . . . . . . . . . . . . VP. G. P. . . . . I. . L. . . . T. . . . . . . . . . . . . . . . . . .
OBI  . AD. . . . . HQQ. . GR. . YSS. . SF. ATSKCILWL. . PPEY. EENHARREP. . LP. AF. . . LS. WAQA. V. H. S. . P. HA
OBI  . RH. . . . . . R. . . . . V. . . L. GVS. . . . . . S. . . . I. . . . R. . . . . . . . . . . H. . V. . Q. . S
OBI  . . . . . . . . . . . . . . . . . . . . . . . . . . . . NY. . . . . . . . . . . . . . . . . . . . T. . . . . S. . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A. . . . . . . . .
CON  . . . . . . . . . . . . . . . . . . . . . . KM. . . . . LR. . . . G. . K. . . . . . . . . . . . T. . . K. . . . . . . . . . . . . .

                90        100        110        120        130        140        150        160
                 *          *          *          *          *          *          *          *
A    IIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSTTTSTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAK
Ba   . . . C. . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . . . . . . . . T. . . . . . . . . . . . . . . . . . . . . . . . .
Bc   . . . C. . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . . . . . . . . T. . . . . . . . . . . . . . . . . . . . . . . . .
Bj   . . . C. . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . . . . . . . . T. . L. . . . . . . . . . . . . . . . . . . . . . .
C1   . . . . . . . . . . . . . . . . . . . . . . . L. . TS. . . . . . . . . I. . . T. . . . . . . . . . S. . . . . . . . . . . . . . . . .
C2   . . . . . . . . . . . . . . . . . . . . . . . L. . TS. . . . . . . . . I. . . T. . . . . . . . . . S. . . . . . . . . . . . . . . R
D    . . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . R. . . . . T. . Y. . . . . . . . S. . . . . . . . . . . . . . . . G.
E    . . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . R. . . L. . T. . . . . . . S. . S. . . . . . . . . . . . . . . . G.
F    . . . . . . . . . . . . . . . . . . . . . . . L. . . . . . . . . . L. . T. . . . . . . S. . S. . . . . . . . . . . . . . . LG.
G    . . . . . . . . . . . . . . . . . . . . . . . S. . . . . . . . . . . . . . . Y. . . . . . . S. . . . . . . . . . . . . . . . . . .
H    . . . . . . . . . . . . . . . . . . . . . . . L. . . . . . . . . . L. . T. . . . . . . S. . . . . . . . . . . . . . . . . . . . G.
OBI  . NSFYL. HASITSFRG. VGCH. IALN. . IMAAPVAAGSEAKIPFAPATADKPFAAFGYYAEILVRDFARTL. . P. RTGRG
OBI  . TYR. N. PS. . . SW. . . YFK. TFRA. . MPLENLNI NGKI RLGI I HNPEEI I HKFYR. RINLMWKYY. W. R. . . . LGYTN
OBI  . . . . . V. . . . . . . LR. . PS. . . PRQKTN. NRKVKSNIMMKI VNIAFNH. WW. . . RS. GASR. . . . . . . . . . . VS
OBI  . . . . . . . . . . . . . . . R. . . S. . . QSRN. P. . TMSLYPLRTLH. LKP. LI . PY. . . TT. AS. . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . S. . . W. . . . RTSR. A. . . PTQ. TNY. NS. HS. . IC. . . . . . . . . . E. . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . T. . . . . . RRR. SA. . A. . PP. . SL. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . A. S. QS. . S. . RQ. . TN. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . V. T. . V. . . . . . R. . . R. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N. . . S. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
CON  . . . . . . . Q. . . . . A. . . . . . . . . . . . . . . . . P. . . . . E. N. . . . . SYQ. H. . . . . H. . EP. . . . . . . . . . . . DS. .
CON  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V. L. . . . . . . . . . . . . . . . . . . . .

               170        180        190        200        210        220
                 *          *          *          *          *          *
A    YLWEWASVRFSWLSLLVPFVQWFVGLSPTVWLSAIWMMWYWGPSLYSIVSPFIPLLPIFFCLWVYI
Ba   . . . . . . . . . . . . . . . . . . . . . . . . V. . . I. F. . . . . N. L. . . M. . . . . . . . . . . . .
Bc   . . . . . . . . . . . . . . . . . . . . . . . . V. . . . F. . . . . N. L. . . M. . . . . . . . . . . . .
Bj   . . . . . . . . . . . . . . . . . . . . . . . . V. . . . . . . . . . N. L. . . . . . . . . . . . . . . .
C1   F. . . . . . . . . . . . . . . . . A. . . . . . V. . . . . . . . . . N. L. . . L. . . . . Y. . . . .
C2   F. . . . . . . . . . . . . . . . . . . . . . . . V. . . . . . . . . . N. L. . . L. . . . . . . . . . . .
D    F. . . . . A. . . . . . . . . . . . . . . . . . V. . . . . . . . . . . L. . L. . . . . . . . . . . . .
E    F. . . . . A. . . . . . . . . . . . A. . . . . . V. . . . . . . . . . N. L. . . . . . . . . . . . . . .
F    . . . . . A. . . . . . Q. . . . C. . . LV. . . I. . . N. C. . L. . . . CY. . . S.
G    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N. . N. L. . . . . . . . . . . . . .
H    . . . . . . A. . . . . . Q. . . . C. . . LV. . . I. . . N. C. . L. . . . CY. . AS.
OBI  N. RKL. LFH. . . GNGS. ALSARL. . D. CTIG. VALMLIKR. . . R. FLL. N. NLLHS. . L. . L. G. ICT
OBI  S. . DS. . . . . . . . N. . . . . . . . . . R. . . . . . . T. T. . . . . . RMH. T. R. . . . . Q. . . . . . D.
OBI  . . . AR. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . P. . . . . . . . . . . . . . . . . . F.
OBI  . . . G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
OBI  . . . V. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
CON  F. . . . . . . . . . . . S. L. . . . . . . . . . I. . A. F. T. L. . . . . . K. SR. S. . . . . . . . . . . . .
CON  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . CT. . . . . . . . . . . . . . .
```

Figure 1 Comparison of the amino acid sequences of the 226AA hepatitis B virus, hepatitis B virus surface antigen between occult hepatitis B virus infections, and controls. Amino acid substitutions are indicated with one-letter codes. Mutations identified in occult hepatitis B virus infections group and control group are in red and black, respectively. Mutations detected in both groups are in blue. Black dots denote amino acid identity with any of the reference strains of genotype A to H retrieved from GenBank (accession numbers: A, X02763; Ba, D00330; Bc, GQ205440; Bj, AB073858; C1, KM999990; C2, KM999991; D, X02496; E, X75657; F, X69798; G, AF160501; H, AY090454). Genotype-specific substitutions are listed. CON indicates control.

Table 1 Summary of hepatitis B virus surface antigen variants involved in functional studies

Variants	Source	Model	Antigenicity	Immuno-genicity	HBsAg Intra-	HBsAg Extra-	Viral replication/secretion	References
W74[1]	CHB	Huh7	L		L	L		[65]
M75T		Huh7			↑	↓	↓[2]	[75]
Y100C	OBI	Huh7	↑					[76]
Y100S	OBI	Huh7	L					[77]
I110M		Huh7					L[3]	[78]
T114R		Huh7					↓[3]	[79]
T115A		Huh7					↓[3]	[79]
T116N	OBI	Huh7	↓					[77]
G119E		Huh7				↓	↓[3]	[78]
G119R	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
P120T	OBI	Huh7/mice	↓					[66]
P120T		Huh7				↓		[79]
C121S		HepG2/Hela	↓		↓	↓		[69]
C121[1]	OBI	Pichia	↓ or L					[80]
C121A		Huh7				↓	↓ or L[3]	[79]
K122I		HepG2/Hela	↓		↓	↓		[69]
K122I		Huh7	↓	↓	↓	↓		[81]
K122I		Huh7/Hela	↓ or L	↓	↓	↓		[82]
R122P	OBI	Huh7	↓					[77]
T123N		HepG2/Hela	↓		↓	↓		[69]
T123N		Huh7/Hela	↓ or L				↓[3]	[82]
C124[1]	OBI	Pichia	↓ or L					[80]
C124A		Huh7				↓	↓ or L[3]	[79]
C124R	OBI	Huh7/mice	↓				↓[3]	[66]
C124Y	OBI	Huh7/mice	↓				↓[3]	[66]
T125A	CHB	Huh7	↓			↓		[65]
I126S	OBI	Huh7/mice			↑	↓	↓[3]	[66]
P127S		Huh7					↓[3]	[79]
Q129H		Huh7					↓[3]	[79]
Q129R	OBI	Huh7/mice			↑	↓	↓[3]	[66]
G130R		Huh7					L[3]	[79]
T131I		Yeast	↓					[83]
M133T[4]		Huh7						[78]
S136P	OBI	Huh7/mice			↑	↓	↓[3]	[66]
C137[1]	OBI	Pichia	↓ or L					[80]
C138Y		Huh7				↓	L[3]	[79]
C139R	CHB	HepG2	↓					[84]
C139R	OBI	Huh7/mice	↓				↓[3]	[66]
T140I	OBI	Huh7/mice			↑	↓	↓[3]	[66]
K141E	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
K141E		Huh7				↓	↓[3]	[79]
K141E		Yeast	↓					[83]
P142S		Yeast	↓					[83]
P142S		Huh7				↓		[79]
P142A	KT	Huh7	↓					[85]
D144V	KT	Huh7	↓					[85]
D144A	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
D144G		Huh7				↓	L[3]	[79]
D144E	LC	HepG2	↓					[84]
G145R		Yeast	↓					[83]
G145R		HepG2/Hela	↓		↓	↓		[69]
G145R	LT	Huh7					↓[3]	[67]
G145R		Huh7	↓	↓		↓		[81]
G145R		Huh7				↓		[79]
G145R	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
G145A	OBI	Huh7/mice	↓					[66]
G145A	OBI	Huh7/HepG2	↓		↑	↓		[83]
G145A		Yeast	↓					[81]
G145W		Huh7		↓		↓		[81]
G145I		Huh7	↓	↓				[81]
G145P		Huh7	↓					[81]
G145N		Huh7	↓					[81]
G145D		Huh7	↓					[81]
G145E		Huh7		↓				[79]
N146S		Huh7					L[3]	[79]
C147A		Huh7					↓ or L[3]	[79]

C147R		Huh7				↓	↓[3]	[79]
C149A		Huh7				↓	↓ or L[3]	[79]
C149R		Huh7				↓	L[3]	[79]
A159G		Huh7/Hela	↓		↓		↓[3]	[82]
K160N		Huh7/Hela	↓		↑[5]		↓[3]	[82]
R169P/G		Huh7				↓	L[3]	[78]
Y100S + S143L	OBI	Huh7	L					[77]
M103I + G145A	OBI	Huh7/HepG2	↓			↓	↓	[86]
M103I + R122K + G145A	OBI	Huh7/HepG2	↓			↓	↓	[86]
T115I + T116N	CHB	HepG2	↓ or L					[87]
T116N + S143L	OBI	Huh7	↓					[77]
R122P + Q101R	OBI	Huh7	↓					[77]
R122P + S167L	OBI	Huh7	↓					[77]
R122K + G145A	OBI	Huh7/HepG2	↓			↓	↓	[86]
P142A + D144V	KT	Huh7	↓					[85]
A159G + K160N		Huh7/Hela	↓		↑[5]		↓[3]	[82]

[1]Stop codon mutation; [2]Viral replication; [3]Viral secretion; [4]Correct that I110M, G119E, G145R, N146Q/S, R169H mutations lead to the decrease of viral secretion; [5]Cell-mediated immunity. ↑: Increase; ↓: Decrease. Intra-HBsAg: Intracellular HBsAg; Extra-HBsAg: Extracellular HBsAg; L: Dramatic decrease; CHB: Chronic hepatitis B; OBI: Occult HBV infection; LC: Liver cirrhosis; KT: Kidney transplant; LT: Liver transplant; HBsAg: Hepatitis B virus surface antigen.

Figure 2 Secondary structure of the major hydrophilic region of the hepatitis B virus, hepatitis B virus surface antigen. Mutations have been confirmed by functional studies to exert negative effects on antigenicity of hepatitis B virus surface antigen (HBsAg) are shown in green and red circles. Red circles with the Δ symbol denote multiple kinds of amino acid substitutions. These have been substantiated to negatively correlate with HBsAg antigenicity at some sites. The # symbol indicates multiple genotype-specific substitutions. The numbers denote amino acid sites.

proteins can substitute for position 146 as the potential glycosylation site[78]. Previous studies reported that T123N and K160N were each capable of creating new glycosylation sites and causing a decrease of HBsAg antigenicity. These two point substitutions were found to mainly affect virion assembly and secretion without interfering with virion replication. K122I and A159G appeared to affect biological properties of HBsAg and facilitated glycosylation of HBV. A159G was shown to impair greatly the assembly and secretion of virion,

Pre-S deletions:
nt2983-3167 (Pre-S1, AA47-107)
nt3019-3201 (Pre-S1, AA58-118)
nt3025-3208 (Pre-S1, AA58-119)
nt3086-15 (Pre-S1/S2, AA78-aa9)
nt3134-54 (Pre-S1/S2, AA95-23)

Drug resistance in RT:
rtA181T (sW172*)
rtV207I (sW199*)

Pre-C/X deletions:
nt1753-1772
nt1754-1771
nt1763-1770
nt1770-1777

Figure 3 Mutations in X, P, and C genes of hepatitis B virus have been substantiated in association with the occurrence of occult hepatitis B virus infections by *in vivo* and *in vitro* studies. Plus and minus strands are denoted by + and -, respectively.

but K160N and A159G/K160N slightly reduced the virion production. Thus K160N could partially rescue the negative effect exerted by A159G on secretion of virion[82]. M133T mutation could create an additional glycosylation site [131]NST[133] and partially rescued the impaired secretion of virion induced by some point mutations, such as I110M, G119E, G145R, N146Q/S, and R169H. Preventing glycosylation could destroy the effect of the M133T mutation[78,79,82]. M133T alone was not able to generate a glycosylation site, unless there was an N at AA131 (as in the reference sequences of HBV genotype A) The T131N/M133T double mutations were frequently observed in OBIs infected with non-A genotypes and changing T131N could also abrogate the secretion-rescue effect exerted by M133T[78,79].

Among OBI related mutations, some point mutations cause HBsAg secretion deficiency by affecting the start codons of Pre-S/S gene or causing amino acid substitutions[91]. A purine at the -3 position (*i.e.*, 3-nt upstream from the translational start site) of the AUG initiator codon is essential for protein expression, and substitution with a pyrimidine at this site makes translation more sensitive to changes at positions -1, -2, and +4 (*i.e.*, 4-nt downstream from the translational start site)[92]. Point mutations around the initiator codons of HBV S gene (*i.e.*, nt155-157), such as A152T and A152G/G158C double mutations, reduced HBsAg secretion by 70% and 30%, respectively, but maintained efficient virion secretion, while A152T combined with C154A or G158A completely abolished HBsAg secretion[91]. Some point mutations have an effect on RNA splicing. Hass *et al*[93] found that ntG458A inactivated 5' splice site of the Pre-S2/S mRNA and reduced the level of Pre-S2/S mRNA as well as HBsAg expression and caused a low-replication phenotype.

Among some genotype D OBIs, ntG173T was able to activate in *cis* a previously inactive splice acceptor site at neighboring nt202 and promoted RNA splicing from nt2986 to 202. The novel splicing could abolish the L, M, and S proteins without affecting the functions of polymerase[94].

Pre-S/S deletion

Deletion mutations in Pre-S/S have been frequently reported in OBI and deletions covering the Pre-S/S promoters may affect RNA splicing and exert an effect on expression of one or more envelope proteins (Figure 3)[50,74]. In cases of OBI, in-frame 183-bp deletions (nt3019-3201 or nt2983-3167) were commonly detected in the Pre-S1 region. Functional analyses found that variants harboring the 183-bp deletion mutation did not express HBsAg or secrete viral particles when transfected in cell lines, although they appeared to be replication-competent[50,95-97]. A CCAAT element (nt3143-3147) and binding sites for SP1 transcription factor are located in the Pre-S2/S promoter (nt2960-3180), which are completely and partially overlapped by the 183-bp deletion mutation, respectively[98,99]. The CCAAT element is where cellular transcription factor NF-Y binds to and plays a key role in activating the S promoter and regulating the ratio of Pre-S and S mRNA. Mutations in the CCAAT element downregulate the level of Pre-S1 mRNA while upregulate that of Pre-S2/S mRNA, resulting in a decreased amount of L protein but increased amounts of M and S proteins[100]. The SP1 sites also regulate S promoter activity and production of Pre-S and S mRNA but exert a weaker effect than the CCAAT element. Mutations that occur at SP1 sites affect the ratio of Pre-S/S mRNA as well as expression of

envelope proteins[98,99]. The specific ratio of L protein and S protein is essential for assembly of the envelope particles, since an excessively high or low L/S proteins ratio could alter HBsAg assembly and secretion and reduce virion secretion[91,101,102]. Similarly, Xu and Yen[103] reported a 129-bp deletion within the Pre-S2/S promoter that affected Pre-S2/S mRNA production, leading to complete abolishment of HBsAg secretion. Chaudhuri *et al*[74] described several fragment deletions containing Pre-S2/S promoter in OBIs and demonstrated that full-length HBV genomes harboring these deletion variants could drastically decrease the expression of HBsAg by transfected HepG2 cells. Besides, the Pre-S1 region possesses a hepatocyte-binding site (AA21-47) that is vital to virion assembly and transport from the hepatocyte, and deletions covering this site may affect virion binding to and being secreted out of the hepatocyte[104].

In OBIs, deletions in Pre-S2 region were frequently detected at aa8-23, which have also been associated with progression of liver diseases[74,105]. These deletions overlap with the binding site for cross-linked human serum albumin (AA17-28) in the Pre-S2 region, which is involved in the attachment of HBV to the human hepatocyte membrane, and mutations affecting this site may decrease the infectivity of HBV[106]. Deletions in Pre-S2 region may influence expression of M protein, which is dispensable for formation and secretion of virion *in vivo* and *in vitro*[91,107]. Deletions in the Pre-S2 region may be related to OBI by affecting the production of L protein. Deletions in the Pre-S2 region shorten the gap between the Pre-S2/S promoter and the start codon of S mRNA, in turn leading to an overproduction of L protein and an alternation of L/S ratio, which may decrease the secretion of HBsAg[108,109]. Deletions in the Pre-S2 region may be associated with antiviral-resistance, which is in turn linked to OBI. Mutations corresponding to lamivudine (LMV)-resistance were commonly detected in OBIs[110], and Pre-S2 deletion mutants were frequently found in patients with antiviral-resistance and shown to play a supportive role in the replication of LMV-resistant viruses by transfection analysis[111].

Pre-S/S insertion

Two to 8 amino acid insertions have been observed between codons 121 and 124 located upstream of the "α" determinant in OBI patients from the Far East, and they caused detection failure of HBsAg by conventional assays utilizing monoclonal and polyclonal antibodies[112]. Insertions occurring at these sites strongly influence the binding efficiency of HBsAg antibodies and may affect the conformation of the "α" determinant as well as the subtype determinant d/y at aa122. In addition, insertions detected in the Pre-S/S gene in OBI patients may lead to frameshift mutations or stop codon mutations and interfere with HBsAg detection.

Pre-S/S stop codon

Stop codon mutations in the Pre-S/S gene result in truncations of envelop proteins and may affect the expression and/or secretion of HBsAg. A stop codon mutation at aa216 in the C-terminus of HBsAg was observed in occult HBV/HIV co-infected patients and displayed undetectable or extremely low level of HBsAg in transfection experiments[73]. Other stop codon mutations at amino acid positions 61, 69, 181 *etc*. were also detected in OBI patients. These mutations may be correlated with failure in HBsAg detection, but *in vivo* and *in vitro* studies are required to confirm this association[113]. Furthermore, the Pre-S/S gene overlaps with the RT region of the P gene, and mutations corresponding to antiviral-resistance in the RT domain commonly result in stop codon mutations in HBsAg and changes in the antigenicity and secretion of HBsAg[114].

C GENE MUTATIONS

A1762T/G1764A double mutations in the BCP region and G1896A in the Pre-C region are the most frequently observed point substitutions in the C gene. These mutations prevent the production of the hepatitis B e-antigen (HBeAg) and are linked to HCC[115]. Genotypes C and D of HBV have been reported to have relatively high mutation rates in the BCP region[116]. However, A1762T/G1764A and G1896A were not common in genotype D-infected OBI patients from Turkey and India[117,118]. A study from Taiwan conducted on OBI related HCC and HBsAg-positive HCC patients found that mutations commonly found in HBsAg-positive HCC, such as A1762T/G1764A, G1896A, *etc*., were not frequently detected in OBI related HCC patients[119]. Therefore, point mutations in BCP/Pre-C in CHB may be not associated with OBI and are required to be explored by further functional studies. Several deletion mutations in the BCP region, such as nt1753-1772, nt1751-1770, nt1754-1771, *etc*., have been reported in OBIs, and variants containing deletions of nt1754-1771 were shown to reduce virion replication and expression levels of HBsAg and HBeAg (Figure 3)[97]. The BCP region recruits initiators for the transcription of Pre-C mRNA and pregenomic (pg) RNA and is rich in AT content[120,121]. Deletion mutations in the BCP region may prevent transcription of pgRNA, leading to a reduction in HBV DNA replication and HBsAg expression. Moreover, BCP region overlaps with the X gene, and deletions in the BCP region lead to truncated X protein, which also has an effect on virion replication and antigen expression[122].

X GENE MUTATIONS

X gene is a regulatory gene and generates the X protein, which is made up of 146 to 154 aa. X protein acts as a transcription factor and trans-activates

gene expression of HBV and several other viruses and cells[123]. The X gene harbors enhancer elements and the C promoter, and deletion mutations covering the C promoter were frequently encountered in various HBV infections, such as OBI, HBeAg-negative infection, and so on[124]. The C-terminal end of X protein is essential for the trans-activation function of X protein[122]. Eight base pair (nt1763-1770) and 20-bp deletion (nt1753-1772) mutations at the C-terminal end of X protein were frequently observed in OBIs, and variants containing these two deletions led to truncation of X protein and a decrease in Pre-C promoter activity as well as secretion of HBsAg, HBeAg, and HBcAg in transfected cell lines (Figure 3)[125,126]. Eight nucleotide deletions (nt1640-1647 or nt1770-1777) were frequently detected in occult HBV/HCV co-infected patients, and they exerted a positive effect on HCV replication but a negative effect on HBV replication[127,128]. Besides, partial and large deletion mutations in the X gene have been reported in OBIs in renal dialysis and HBV-vaccinated thalassemia patients[129].

P GENE MUTATIONS

Since the P gene overlaps with the S gene, mutations that occur in one gene may affect the other gene. The RT region of the P gene completely spans the S region, thus mutations resistant to nucleos(t)ide analogues (NAs) in the RT region frequently lead to mutations of HBsAg (including stop codon mutations) and affect the antigenicity of HBsAg (Figure 3)[114,130]. Transient selection of a V542I mutant in the HBV polymerase has been described in a CHB patient with prolonged administration of famciclovir. The mutation corresponded to a stop codon mutation at aa199 in the S gene, and a transfection experiment with the full-length HBV genome harboring the mutation demonstrated a negative effect on replication capacity and failed to produce HBsAg[131]. The rtA181T variant was frequently found in patients with adefovir (ADV)-resistance, corresponding to a stop codon mutation at aaW172 in the S gene (sW172*) and leading to truncation of 55 aa at the C-terminal end of HBsAg. The variant not only resulted in secretion deficiency of the virion but also exerted a dominant negative effect on virion secretion despite co-transfection with wild-type isolates[132]. Other common antiviral-resistant mutations that lead to truncation of S proteins include entecavir (ETV)-resistant rtT184M corresponding to sL176*, rtM204I (YIDD) both resistant to LMV and telbivudine (LdT) corresponding to sW196*, and so on[114]. The rtM204V (YVDD), accompanied by rtV173L (corresponding to sI195M and sE164D) and/or rtL180M, were detected in both HBsAg-negative patients and HBsAg-positive patients untreated with LMV[133,134]. Variants harboring YIDD or YVDD caused a drastic reduction in virion replication capacity in

transfection experiments[135]. Some studies found that LMV-selected HBsAg protein changes, including E164D, I195M, and E164D/I195M, significantly reduced the binding efficiency to anti-HBs, implying the potential to escape HBV vaccine like G145R[133]. Point mutations in the RNase H domain of P gene were observed in vaccinated children with OBIs[52]. *In vitro* studies demonstrated that the RNase H region is essential for both RNA packaging and DNA synthesis and that the P gene of HBV and duck hepatitis B virus (DHBV) have similar biological properties. Point mutations in the RNase H region of DHBV, such as L697Y, V719Y *etc*., led to deficiency of RNA packaging and significant reduction of DNA synthesis[136].

Deletion mutations in the P gene have been rarely reported. A 281-bp deletion (nt2068-2349) covering the start codon of the P gene was reported in genotype B infected OBIs, but the relationship remains to be confirmed by functional studies[137].

NO OBI-RELEVANT MUTATIONS

Mutations in the "α" determinant of the S protein are "hot spots" of OBI related mutations, but some clinical reports demonstrated that no mutation was detected in the "α" determinant or the mutation rate of the "α" determinant in OBI group was not significantly different from HBsAg-positive infections[31]. In some OBI patients, no mutation was detected in the Pre-S/S gene[117]. Moreover, no OBI-relevant mutation was found throughout the entire genome of HBV strains isolated from some OBI patients[138]. As no OBI-relevant mutation was encountered in some cases of OBI and some mutations occurred in both OBIs and HBsAg-positive infections, some reports concluded that there were no OBI-specific mutations[45]. Furthermore, *in vivo* and *in vitro* studies have demonstrated that some mutations identified in clinical reports did not interfere with HBV replication and expression or HBsAg secretion and were not associated with OBI. In a recent study, we investigated a number of OBI patients with a family history of HBV infection by cloning and sequencing the Pre-S/S region of HBV DNA isolated from serum samples. Sequence comparison between OBI patients and their family members with CHB failed to identify common genetic variations that were specific for OBI[139].

PROBLEMS

The mechanism of OBI is complex and remains to be clarified. Previous related studies have several limitations, as follows: (1) It is difficult to amplify the full-length HBV genome due to the extremely low viral load in OBI patients, and most clinical reports are limited to mutations in part of the viral genome (mainly the S gene coding for HBsAg). Studies based on full-length HBV genomes are rare; (2) Most previous

studies of OBI were clinical reports with limited case numbers and no comparable controls, and conclusions about some mutations were not consistent across reports; (3) Most mutations were not studied *in vivo* or *in vitro*, and some mutations occurred both in OBIs and HBsAg-positive infections; (4) Functional studies of mutations were mostly focused on the secretion, antigenicity, or immunogenicity of HBsAg, while only a few of them addressed virion replication and secretion. Opposite conclusions may be drawn by studies on the same mutations, depending on whether a fragment or the entire genome was used (data not available); (5) Most studies focused on individual mutations, and it remained to be determined whether multiple mutations interact with each other or whether there is secondary function; (6) HBV genotype and subgenotype were ignored in most studies; (7) There is a lack of suitable reference sequences for genotype and subgenotype of HBV, and it is difficult to determine whether some amino acid substitutions were genotype-specific or OBI-related[140]; and (8) The levels of serum HBsAg in some OBI patients were fluctuant, and it remains to be elucidated whether HBV methylation, glycosylation, acetylation, or miRNA plays a role in this phenomenon.

The ability to screen for OBI is strongly affected by the assays used for detecting HBsAg and HBV DNA. For instance, the Abbott assay is highly sensitive for G145R detection. It is our opinion that multiple assays should be performed to verify the accuracy of the screening results and to reduce false positive or false negative results. NAT of HBV is highly sensitive and able to detect extremely low levels of HBV DNA but has a potential risk of false-positive results due to quality-control problems. HBV NAT is still important for recognition of OBI in HBsAg-negative blood donations worldwide especially in high-endemic countries. However, NAT has not been implemented in most of these countries due to its high cost[141]. In these countries, blood donations are screened by serological testing of HBsAg alone or combined with anti-HBc. In low-endemic regions of HBV or high-endemic regions of anti-HBc, anti-HBc testing may be not as effective as NAT[142]. Anti-HBc screening is not capable to detect pre-seroconversion window period infections and anti-HBc assays based on radioimmunoassay or enzyme immunoassay may suffer from false-positive results[143,144]. Although liver biopsy is the best way for detecting OBI, it is hard to obtain liver tissue and standardized assays for it are not yet available[145].

CONCLUSION

OBI is a special and complex form of HBV infection with worldwide distribution. Its reported prevalence significantly varies depending on study locations, detection assays used, and study population. The underlying mechanism of OBI is complex and unclear. Genetic variants may be relevant but future research

is still need both *in vivo* and *in vitro*.

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MINIREVIEWS

Liver cancer stem cell markers: Progression and therapeutic implications

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Abstract

Cancer stem cells (CSCs) are a small subpopulation in cancer, have been proposed to be cancer-initiating cells, and have been shown to be responsible for chemotherapy resistance and cancer recurrence. The identification of CSC subpopulations inside a tumor presents a new understanding of cancer development because it implies that tumors can only be eradicated by targeting CSCs. Although advances in liver cancer detection and treatment have increased the possibility of curing the disease at early stages, unfortunately, most patients will relapse and succumb to their disease. Strategies aimed at efficiently targeting liver CSCs are becoming important for monitoring the progress of liver cancer therapy and for evaluating new therapeutic approaches. Herein, we provide a critical discussion of biological markers described in the literature regarding liver cancer stem cells and the potential of these markers to serve as therapeutic targets.

Key words: Liver cancer; Cancer recurrence; Liver cancer stem cells; Cancer stem cell markers; Targeted therapy

Core tip: Liver cancer is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide despite remarkable progress in understanding hepatocarcinogenesis and new therapeutic approaches. Recently, the presence of highly resistant cancer stem cells (CSCs) in liver cancer has been proposed to be responsible for tumor growth, invasion, metastasis and recurrence. CSC involvement in liver cancer pathogenesis also highlights them as preferential targets for therapy. This review specifically focuses on the markers used to define human liver

cancer stem cells, the therapeutic implications of the expression of these markers in patient's primary tumors, and the potential of the markers to serve as therapeutic targets.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common liver cancer, is the third leading cause of cancer-related mortality worldwide[1], mainly due to its high rate of recurrence, which can be as high as 70% following conventional treatments, such as chemotherapy, arterial embolization, surgical resection, and radiofrequency ablation[2]. Some research studies have demonstrated that liver cancers are derived from liver stem cells that are present in adult liver tissue with endogenous or exogenous liver origin, where the former are oogonia located in the smallest terminal of the intrahepatic bile duct. However, the cellular origin of HCC recurrence remains poorly understood, and no specific treatment strategy has been developed that focuses on HCC recurrence. Although the cytological pathogenesis of HCC remains unclear, it has been proposed that HCCs are not created equally and display a great deal of heterogeneity[3,4], as there are abundant distinct tumor cell populations expressing different markers. Only a rare subset of cancer cells with stem cell properties, often referred to as liver cancer stem cells (LCSCs), are considered to be responsible for tumor growth, metastasis and recurrence of HCC as well as for the failure of chemotherapy and radiotherapy[5]. These findings indicate that liver cancer therapies, although killing a majority of tumor cells, may ultimately fail because they do not eliminate LCSCs, which survive to regenerate new tumors (Figure 1). Therefore, the cancer stem cell theory offers novel insight into tumor diagnosis, treatment and prevention.

Recent rapid progress in CSC research has encountered increasing challenges in which the identification of CSC-specific marker sets and targeted therapeutic destruction are the most frequently debated topics. CSC markers must be clearly defined for each tissue, and clarifying cellular and signaling functions of CSCs is key to conducting better identification and diagnosis based on CSC biomarkers for targeting CSCs, which will undoubtedly improve prevention and treatment for many types of CSCs. To achieve better understanding and treatment of LCSCs, we must better understand the markers of stemness and cell fractions associated with prognosis, metastasis, and resistance. These markers are necessary to isolate CSCs and analyze their biological characteristics to target them efficiently for therapeutic purposes. Therefore, we summarize here current knowledge on putative markers that define LCSCs, potential functional implications, and therapeutic targets of these markers and provide insights into new therapeutic approaches for more specific targeting and eradication of liver CSCs.

CELL SURFACE MARKERS OF LIVER CSCS

Current surface markers or a particular phenotype are used to identify CSCs. Several markers proposed in the literature to identify CSCs in liver cancer using cell surface antigens are enriched in LCSCs (isolated by FACS or Ab-conjugated magnetic beads). Additionally, cytokeratin 7 and 19 may also serve as relatively specific markers of LCSCs, playing significant roles in hepatocellular carcinoma[6,7].

CD133

One of the most commonly described surface markers in LCSCs is CD133. CD133, also known as Prominin-1, is a membrane glycoprotein encoded by the CD133/Prom-1 gene[8]. It was first detected as a marker of hematopoietic stem cells and has since been shown to be a marker of CSCs in the prostate, colon, and ovaries[9-11]. CD133+ HCC cells were first identified as a potential CSC subpopulation by Suetsugu *et al*[12]. They found that the isolated CD133+ HCC cells from Huh7 HCC cell lines exhibited higher proliferative and tumorigenic potential and expressed lower levels of mature hepatocyte markers than those of the CD133- counterparts. Subsequent reports from Yin *et al*[13] demonstrated a similar result, where the CD133+ population of HCC SMMC-7721 cells exhibits higher *in vivo* clonogenicity and *in vitro* tumorigenicity than those of the CD133- counterparts. We have also researched the enrichment and characterization of LCSCs through a sphere culture system and found that CD133 was significantly enriched in liver CSCs compared with that in MHCC97H cells. Additionally, liver CSCs proliferated significantly faster and induced more tumor colonies than those of MHCC97H cells[14]. Enhanced CD133 expression is also found to be an independent prognostic indicator for survival and tumor recurrence in HCC patients[15]. Furthermore, CD133-positive cells seemed to be increased with the loss of differentiation of the tumor[16].

Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, which is engaged in early differentiation of stem cells by retinol oxidation to retinoic acid[17]. ALDH activity has been found to be upregulated in murine and neural stem and human hematopoietic and progenitor cells[18]. ALDH is also widely used as a CSC marker in

Figure 1 Targeting liver cancer stem cells is necessary to prevent tumor recurrence. LCSCs are resistant to the current standard of care: surgical resection, radiotherapy and chemotherapy. Therapeutic approaches and strategies to target LCSCs in addition to the differentiated tumor cells are necessary to effectively treat the entire cancer and prevent tumor recurrence. LCSCs: Liver cancer stem cells.

many types of cancer, including colon[19], breast[20], ovary[21], bladder[22] and prostate[23]. In liver cancer, Yin *et al*[13] suggested that ALDH is expressed in LCSCs and is positively correlated with CD133 expression. The combination of these markers can define LCSCs more accurately; dual-color FACS analysis found that the majority of ALDH+HCC cells were CD133+, yet not all CD133+HCC cells were ALDH+. A hierarchical organization of cells that differentially express CD133 and ALDH exhibit descending tumorigenic potential in the order of CD133+ALDH+> CD133+ALDH-> CD133-ALDH-[13], which implies that ALDH express along CD133 can be used to characterize the tumorigenic liver CSC population more specifically.

CD90

CD90 is a 25-37 kDa heavily N-glycosylated, glyco-phosphatidylinositol (GPI)-anchored protein expressed in many cells, such as thymocytes, T-cells, neurons, endothelial cells and fibroblasts[24]. CD90 operates as an important regulator of cell-cell and cell-matrix interactions, apoptosis, adhesion, migration, cancer and fibrosis[25]. CD90 is also expressed in bone marrow-derived stem cells[26] and hepatic stem/progenitor cells from adult or fetal livers but not in adult hepatocytes[27-29]. It has been identified to be one potential marker in CSCs, including in HCC. Yamashita *et al*[30] investigated the expression patterns of three CSC makers (CD 133, EpCAM, CD90) in 15 primary HCCs with high viability, where EpCAM, CD90 and CD133 are positive in 3, 7 and 15 cell strains, respectively. Although strong correlation of CD90+ proportions in tumor cells and liver cancer distant metastasis was suggested, the intrinsic mechanics still need to be determined. Additionally, the feasibility of

eradicating cancer cells committed to mesenchymal endothelial lineages by imatinib mesylate, in which CD90+ cells are believed to be chemosensitive, is proposed. Yang *et al*[31] found that the number of CD90+ cells increased with the tumorigenicity and metastatic potential in a panel of HCC cell lines. Moreover, CD45-CD90+ cells were detected in all of blood samples from HCC patients, but none in normal subjects or patients with cirrhosis. The CD45-CD90+ subpopulation has the capacity to initiate and maintain tumor formation in SCID/Beige mice, whereas the CD90- and CD45-CD90- cells do not. In conclusion, these results provide evidence of the tumorigenicity and stem cell-like properties of CD45-CD90+ and CD90+ populations from HCC patients.

CD44

CD44 is a ubiquitous multi-structural and multi-functional cell surface glycoprotein involved in adhesive cell-cell and cell-matrix interactions, cell migration, cell homing, cell proliferation and angiogenesis[32]. All of these biological properties are essential to normal cell physiology, but under certain conditions they are associated with pathological activities, in particular, those of cancer cells[33]. Moreover, CD44 is the receptor for hyaluronic acid and has been identified as a CSC marker for several human cancers, including breast[34], gastric, colon, prostate[35], colorectal[36],pancreatic[37], and head and neck squamous cell carcinomas[38]. In human liver cancer, CD44 is also an important marker. CD44 and other markers were reported to more accurately define the surface phenotype of liver CSCs. The CD90+CD44+ cells showed a more aggressive phenotype than the CD90+CD44- counterpart and formed metastatic lesions in immunodeficient

mice. CD44 blockade prevented the formation of local and metastatic tumor nodules, which showed that concomitantly expressed CD44 modulates the biological activity of the CD90^{+} CSCs[39]. Another study demonstrated that CD44 was preferentially expressed in aCD133^{+} population in four HCC cell lines, including Huh7, SMMC7721, MHCCLM3 and MHCC97L. Compared with CD133^{+}CD44^{-} cells, CD133^{+}CD44^{+} HCC cells showed more stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. Furthermore, cells double positive for CD133 and CD44 exhibited preferential expression of some stem cell-related genes and were more resistant to chemotherapeutic agents[40].

CD13

CD13 antigen, a membrane-bound zinc-dependent type II exopeptidase, is widely distributed in many tissues of mammals, such as the intestine, kidney, and liver as well as the central nervous system[41-43]. CD13 participates in the final hydrolysis of nutrients and the degradation of bioactive molecules, such as enkephalin and endorphin. In addition, CD13 is highly expressed in many tumor cells and has been considered as a tumor marker that plays a crucial role in tumor cell growth, invasion, metastasis and angiogenesis. Importantly, CD13 is involved in angiogenesis-generating and -modulating signals and in the process of capillary tube formation and is a marker of angiogenic vessels[43-51].

Recently, Haraguchi *et al*[52] identified CD13 as a candidate liver cancer stem cell marker by a surface marker screen based on microarray analysis. CD13^{+} liver CSCs were enriched in side population (SP) cells isolated from Huh-7, PLC/PRF/5 and Hep3B cells, which is known as the multi-drug resistant cell fraction with ATP-binding cassette (ABC) transporter expression, and also localized predominantly in G1/G0 phase.These results suggested that CD13^{+}cells represent the dormant or slow-growing population that is believed to account for the chemoresistant capacity in HCC. *In vivo* chemosensitivity assays indicated the high multi-drug resistant property of CD13^{+} cells. Treatment of liver cancer cells with a CD13 inhibitor or CD13 neutralizing antibody efficiently induced cellular apoptosis *in vitro*, suggesting that CD13is a liver CSC target.

In mouse xenograft models, the combination of a CD13 inhibitor and the genotoxic chemotherapeutic fluorouracil (5-FU) more efficiently reduced tumor volume compared with either agent alone.CD13 inhibition suppressed the self-renewing and tumor-initiating abilities of LCSCs. In other respects, reactive oxygen species (ROS) have been found to be negatively correlated with surface marker CD13 in cells, where CD13^{+} cells have relatively higher ROS than in their CD13^{-} counterparts. Furthermore, CD13 inhibition is also able to increase ROS expression. These results suggest that chemo-resistant properties

of CD13^{+} cells are regulated by ROS, indicating a positive prospect of treating liver cancer with a CD13 inhibitor and ROS-inducing chemo/radiation therapy[52].

EpCAM

Epithelial cell adhesion/activating molecule (EpCAM) is encoded by the TACSTD1 gene, one of the first tumor-associated antigens identified[64]. EpCAM is highly expressed in a large variety of human adenocarcinomas and squamous cell carcinomas[53]. Yamashita *et al*[54] demonstrated that EpCAM[+] HCC showed a distinct molecular signature with features of hepatic progenitor cells, including the presence of known stem/progenitor markers, such as cytokeratin 19, EpCAM, c-Kit, and activated Wnt/β-catenin signaling, whereas EpCAM[-] HCC expressed genes related to mature hepatocytes. Because its expression is highly elevated in premalignant hepatic tissues and in a subset of HCC, EpCAM may serve as an early biomarker of HCC[55]. Similar results were observed by other researchers[56-58], reiterating the significance of EpCAM in HCC development. The EpCAM[+]/AFP[+] subtype of HCC was significantly correlated with a poor prognosis for HCC patients. Functional analysis showed that EpCAM^{+} HCC cells possessed CSC phenotypes, including the ability to self-renew, differentiate and initiate tumors[54]. Moreover, these cells demonstrated EpCAM enrichment by activation of Wnt/β-catenin signaling using the GSK-3βinhibitor BIO, suggesting that EpCAM is a downstream effector of the Wnt/β-catenin signaling pathway. These data support therapeutic strategies targeting EpCAM[+] liver CSCs through the suppression of Wnt/β-catenin signaling. EpCAM knockdown by RNA interference (RNAi) was shown to cause significant inhibition of cell invasion, sphere formation, and tumorigenicity of HCC cells[55]. In addition, knockdown of EpCAM suppressed colony-forming ability in sorted EpCAM^{+} HCC cells by EpCAM shRNA (shEpCAM) *in vitro*. EpCAM[+] liver cancer cells highly express the chromatin-remodeling enzyme CHD4, whose knockdown and overexpression separately increased chemosensitivity and chemoresistance to epirubicin *in vitro*[59]. Histone deacetylase and poly (ADP-ribose) polymerase are regulators of CHD4. The inhibitory effects of their inhibitors suberohydroxamic acid and AG-014699 were assessed by Nio *et al*[60], who proposed that either inhibitor alone reduced the number of EpCAM[+] liver cancer stem cells *in vitro* and that the combination of the two inhibitors successfully inhibited tumor growth in a mouse xenograft model.

OV-6

Hepatic oval cells are an important origin of liver stem cells. OV-6 has been found to be a useful marker for rat oval cells and is thought to be a hepatic stem cell marker[61-64]. Oval cells arise in the intraportal area of the liver after treatment with hepatocarcinogens

Table 1 Cell surface markers of liver cancer stem cells

Marker	Cell line/primary tumor	Characteristics of marker-positive CSCs	Inhibitors	Ref.
CD133	PLC8024, Huh7, Hep3B, primary HCC	Self-renewal, tumorigenicity, chemoresistance, invasiveness	Lupeol, Anti-CD133 antibody, antisense oligonucleotides	[10-14]
ALDH	Huh7, HPLC8024, Hep3B	Chemoresistance tumorigenicity	Diethylaminobenzaldehyde	[10,11]
CD90	HepG2, Hep3B, PLC, Huh7, MHCC97L, MHCC97H, Primary HCC	Tumorigenicity, metastasis, circulation	Anti-CD44 antibody	[28,29]
CD44	PLC/PRL/5	Tunorigenicity, invasiveness, chemoresistance, metastasis	RNAi interference, Anti-CD44 antibody, antisense oligonucleotides	[38]
CD13	PLC/PRL/5, Huh7, Hep3B	Tumor formation, cell cycle arrest, chemoresistance, self-renewal	Anti-CD13 antibody, CD13 inhibitor ubenimex	[51]
EpCAM	Huh1, Huh7, primary HCC	Invasiveness, Self-renewal, tumor formation, chemoresistance	RNAi interference, GSK-3β inhibitor BIO, Bispecific antibody EpCAMxCD3	[53,54,60]
OV6	Huh7, SMMC7721, primary HCC	Tumorigenicity, chemoresistance, invasiveness, metastasis	RNAi interference, targeting β-catenin	[70]
1B50-1	Huh7, HepG2Hep-12, SMMC7721	Tumorigenic, invasiveness self-renewal	RNAi interference ERK1/2 inhibitor U0126	[71]
SALL4	PLC/PRF/5, Huh7	Proliferation, chemoresistance, tumorigenic	ERK1/2 inhibitor U0126 RNAi interference	[76]
ICAM-1	Hep3B, Huh7	Tumorigenic, metastasis	RNAi interference	[78,79]

CSCs: Cancer stem cells.

or hepatotoxins in rats, these cells and their progeny have the ability to proliferate and differentiate into either biliary cells or hepatocytes[62,65-69]. Yang *et al*[70] showed that OV6$^+$ cells possessed a greater ability to form tumors *in vivo* and that these cells showed a substantial resistance to standard chemotherapy when compared with OV6$^-$ tumor cells. The OV6$^+$ population was enriched after Wnt pathway activation, whereas inhibition of β-catenin signaling led to a decrease in the OV6$^+$ population. OV6$^+$ HCC cells were more chemoresistant than the OV6$^-$ counterparts, but this characteristic was reversed upon lentivirus-delivered stable expression of a microRNA targeting β-catenin. This result suggested the importance of the Wnt/β-catenin pathway in the activation and expansion of OV6$^+$ populations within tumors. Therefore, therapies targeting Wnt/β-catenin signaling may be a promising approach to reverse the chemoresistant nature of OV6$^+$ liver CSCs[70].

1B50-1

In human HCC cells, mAb 1B50-1, which binds to α2δ1$^+$ isoform 5, selectively targeted LCSCs. Recent studies show that 1B50-1$^+$ cells could initiate tumors. 1B50-1 binds to a subpopulation of HCC cells, hereafter termed α2δ1$^+$ cells, that display stem cell-like properties, such as the expression of stem cell-associated genes (OCT4, SOX2, NANOG, and BMI1), increased self-renewal, increased invasiveness and the ability to give rise to both α2δ1$^+$ and α2δ1$^-$ cells[71]. Interestingly, 1B50-1$^+$ cells overlapped with CD133$^+$, EpCAM$^+$, CD13$^+$, and ALDH$^+$ populations of Hep-12 cells. Although the majority of 1B50-1$^+$ cells were also positive for CD133, EpCAM, CD13, and ALDH in Huh7 cells, only a small fraction of CD133$^+$, EpCAM$^+$, CD13$^+$, or ALDH$^+$ cells were 1B50-1$^+$. A similar correlation

between 1B50-1 and these reported liver CSC markers was also found in other HCC cell lines and patient-derived cells (Table 1). Thus, 1B50-1$^+$ cells represent fractions of CD133$^+$, EpCAM$^+$, and CD13$^+$ populations but not vice versa[71].

SALL4

Sal-like protein 4 (SALL4) is a member of a family of zinc finger transcription factors that regulates embryogenesis, organogenesis, and pluripotency. SaLL4 is able to elicit reprogramming of somatic cells and is a marker of stem cells. Some research studies have shown that SALL4 is constitutively expressed in hematopoietic stem cells and is a potent regulator of their expansion[72,73]. SALL4 has also been identified as a novel molecule in reprogramming of somatic cells to become iPSCs[74,75]. Recent research shows that SALL4 is a novel therapeutic target for liver cancers. Bioinformatics analyses showed that elevated expression of SALL4 in tumors is closely related to poor survival of HCC patients. *In vitro*, overexpression of SALL4 promotes cell proliferation and elevates the expression of EpCAM, cytokeratin 19 (CK19), and adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2)[76]. In summary, SALL4 may be a prognostic marker of liver cancer and an indicator of stem cells, playing roles in 5-FU resistance and growth of cells, and tumors with suppressed SALL4 results in differentiation and delayed tumor growth.

ICAM-1

Intercellular adhesion molecule 1 (ICAM-1), a 90-kD cell surface glycoprotein of the immunoglobulin superfamily, is believed to be responsible for HCC metastasis[77]. Previous studies have shown that hepatocytes are negative for ICAM-1 in cancerous

Figure 2 Proposed hierarchy of liver cancer stem cells according to the current literature. Liver cancer stem cell markers have been defined that can give rise to ALDH^{+}CD133^{+}, EpCAM^{+}CD90^{+}, CD133^{+}CD44^{+} and other early progenitor cells, each of which can subsequently divide into more differentiated progenitor cells. Rounded arrows show cells with self-renewal capacity that have the potential to serve as CSCs. Small arrows show the unproven potential for "de-differentiation". CSCs: Cancer stem cells.

areas but not on hepatocytes in noncancerous areas. The expression of ICAM-1 has been reported to mediate adhesion-dependent cell-cell interactions and facilitate the movement of cells through the extracellular matrix, and it has been shown to be positively correlated with tumor size and poor prognosis in HCC[78]. Liu *et al*[79] indicated that ICAM-1 could be used as a potential CSC marker and may therefore be helpful in developing an effective treatment against cancer. ICAM-1^{+} HCC have also been shown to be highly sphere-forming, have high tumorigenic capability and have increased expression of stemness-related genes in comparison with their ICAM-1^{-} counterparts.

ESTABLISHMENT OF A HIERARCHY IN LIVER CANCER THROUGH LIVER CANCER STEM CELL MARKERS

Some research studies have indicated that different biomolecules are used as markers to identify and isolate cell populations with liver cancer stem cell properties, including the ability to generate tumors through the stem cell processes of self-renewal, ability to differentiate into multiple cell types, ability to undergo asymmetric division, and increased resistance to radio-/chemotherapy. One important task will be to confirm the relationship among these markers, as different markers of liver CSCs may be organized as a hierarchy of liver cancer cells (Figure 2). The establishment of such a hierarchy allows for the identification of which markers regulate CSC self-renewal, proliferation and differentiation. These abilities of LCSCs represent important therapeutic targets.

In the basic research field, Ma *et al*[80] found that ALDH and CD133 as CSC markers could be used either alone or in combination to identify different chemoresistant liver CSC populations and define a simple hierarchy. ALDH+ cells with CD133^{+} or CD133^{-} phenotype could initiate tumors in mice. The study also showed that the majority of ALDH^{+} cells were

CD133^{+}, yet not all CD133^{+} HCC cells were ALDH^{+}. A hierarchical organization of cells that differentially express CD133 and ALDH exhibit an ascending tumorigenic potential in the order of CD133^{+}ALDH^{+}, CD133^{+}ALDH^{-}, and CD133^{-}ALDH^{-}. Similarly, Zhu *et al*[40] observed that CD44 was consistently preferentially expressed in CD133^{+} cells at the mRNA level compared to the corresponding CD133^{-} cells from HCC cell lines. Multimarker analyses by flow cytometry revealed similar preferential expression of CD44 in the CD133^{+} cell population. Specifically, the majority of CD133^{+} cells from the SMMC-7721, MHCC-LM3 and MHCC-97L cell lines also expressed CD44. For Huh7, although the percentage of CD133^{+} cells was more than 60%, only 1.88% of cells co-expressed CD133 and CD44, more likely representing a minority of the CSC subset. CD133^{+}CD44^{+} HCC cells showed stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. *In vivo* xenograft experiments revealed that, actually, the highly tumorigenic capacity of CD133^{+} cells as previously described was primarily attributed to the CD133^{+}CD44^{+} cell subpopulation instead of their CD133^{+}CD44^{-} counterparts. Moreover, cells double-positive for CD133 and CD44 exhibited preferential expression of some stem cell-associated genes and were more resistant to chemotherapeutic agents due to the upregulation of ATP-binding cassette (ABC) superfamily transporters, including ABCB1, ABCC1, and ABCG2, further supporting that these cells are of HCC cell origin. These findings suggest that CD133^{+}CD44^{+} cells might represent true cancer stem/progenitor cells in HCC, which could allow for a better understanding of HCC initiation and progression as well as establish a precise target for the development of more effective therapies[40]. Captivatingly, in the clinical research field, Yamashita *et al*[54] found that EpCAM[+] and EpCAM^{-} HuH1 cells equally expressed CD133, but only EpCAM^{+} cells developed large hypervascular tumors. In addition, these results suggested that EpCAM may be a better marker than CD133 for enriching HCC tumor-initiating cells from AFP[+] tumors. They also found that CD90 expression was limited to HCC cell lines that are EpCAM^{-}AFP^{-}, and Wnt/β-catenin signaling had little effect on CD90^{+} cell enrichment. These results identified that the expression patterns of various stem cell markers in tumor-initiating cells with stem/progenitor cell features may be different in each HCC subtype, possibly due to the heterogeneity of activated signaling pathways in normal stem/progenitor cells where these tumor-initiating cells may originate. Consequently, it would be useful to comprehensively research the expression patterns of stem cell markers to characterize the population of CSCs that may correlate with the activation of their distinct molecular pathways.

From all of the observations described above, asymmetric division of liver CSCs gives rise to CD133^{+}CD44^{+} or CD133^{+}ALDH^{+} early progenitor CSCs, and additional tumor-forming progenitors with more differentiated histology could be produced by further asymmetric division of these early progenitor cells.

LIVER CSC MARKERS AS THERAPEUTIC TARGETS

CSCs are defined by several markers that could represent potentially important therapeutic targets. In addition, these markers may be functionally important for CSCs, making them even more attractive as therapeutic targets. Despite reports that some markers are useful in the isolation and study of liver CSCs, other tissues may share these markers with hepatocellular carcinoma because of histological variation. The CSC phenotype might not necessarily be universal in all cancer subtypes. It thus appears relevant to identify specific CSC biomarkers, including cell surface markers, to improve prognosis and ultimately patient survival. New treatment strategies involve the development of antibodies that can target these markers. Antibody therapies against tumor cell surface antigens have improved clinical prognosis through inhibition of specific signaling pathways or enhanced activation of direct immune effectors. In some cases, these antibodies are conjugated to a bioactive drug that enables selective targeting of chemotherapeutic agents. Additionally, they block a signaling pathway in which the marker may be involved. Antibodies may also act by an antibody-dependent cytotoxicity (ADCC)/complement-dependent cytotoxicity (CDC) mechanism, thereby enhancing the immune response against CSCs[81]. CD133-expressing cells have been suggested to be critical tumorigenic progenitors in HCC, conferring chemoresistance by preferential activation of AKT/PKB and Bcl-2 cell survival response[82]. The treatment of CD133^{+} HCC cells with an AKT1 inhibitor, which is specific to the Akt/PKB pathway, significantly reduced the expression of survival proteins. In addition, suppression of CD133 by a murine antibody to human CD133 conjugated to a potent cytotoxic drug reduced the proliferation rate of Hep3B cells *in vitro* and delayed tumor growth in a SCID mouse model[83]. Potential drug-resistant cell subpopulations can hopefully be eliminated in many cancers, such as liver cancer, retinoblastoma, ovarian cancer, prostatic adenocarcinoma, pancreatic cancer, or colorectal cancer, through the development of CD133-targeting antibodies. Multimarker methods have been applied in the characterization of CSCs in breast[34] and pancreatic cancers[37]. In liver cancer, CD133^{+}/CD44^{+} HCC cells were more tumorigenic than those of CD133^{+}/CD44^{-} cells *in vivo*. A recent study suggested that the CSC phenotype could be precisely defined by co-expression of CD133 and CD44 cell surface markers. CD133^{+}/CD44^{+} HCC cells showed stem cell properties, including extensive proliferation, self-renewal and differentiation into the bulk of cancer cells. Additionally, recent studies

also revealed that blocking CD44 signaling using an anti-CD44 antibody might be a potential strategy to eradicate liver CSCs and consequently cure those patients[40]. A previous study demonstrated that CD90^{+}CD44^{+} HCC cells possess a high capacity for tumorigenicity. Researchers who have characterized this subpopulation of cells have also examined the potential benefits of targeting CD44 *via* a neutralizing antibody approach. The systemic administration of anti-human CD44 antibodies in immunodeficient mice, formed by the intrahepatic inoculation of CD90+ liver CSCs, suppressed tumor nodule formation of liver tissue and metastatic lesions in lung tissue. Furthermore, the administration of CD44 antibodies was also shown to induce apoptosis in both CD90+ and CD90- cells *in vitro*[39]. In research on CD13^{+} liver CSCs, Haraguchi *et al*[52] have also indicated that the combination of a CD13 inhibitor and 5-FU dramatically reduced tumor volume compared with that of either agent alone. 5-FU inhibited proliferating CD13^{+} semi-quiescent CSCs, and the self-renewing and tumor-initiating abilities of liver CSCs were suppressed by CD13 inhibition. These studies demonstrated a novel treatment strategy of liver cancer by combining a CD13 inhibitor with reactive oxygen species (ROS) -inducing chemo/radiation therapy. Currently, several EpCAM-targeting antibodies are in clinical development, which include Catumaxomab and Adecatumumab. Clinical trials have been conducted in various cancers, including breast, prostate and colon cancers[84,85]. In liver cells, RNAi targeting of EpCAM significantly decreased the CSC pool and reduced both tumorigenicity and invasive capacity of CSCs[52,57]. Because EpCAM expression is a downstream target of Wnt/β-catenin, these results may have implications for the development of novel target therapies.

In addition to antibody-targeted therapy, a recent discovery by Lee *et al*[86] showed that lupeol, a phytochemical present in fruits and vegetables, could target CD133^{+} liver CSCs by inhibiting their self-renewal and tumorigenic capacity. In addition, lupeol was able to sensitize HCC cells to chemotherapeutic agents (doxorubicin and cisplatin) *via* the PTEN-AKT-ABCG2 signaling pathway. The combination of lupeol, doxorubicin and cisplatin was found to exert a synergistic effect on tumor suppression, allowing the use of a lower dosage of conventional chemotherapeutic drugs, which may substantially reduce the cytotoxic side effects.

Other approaches have also been applied to target liver CSCs utilizing mechanisms that are not dependent on CSC-specific markers. Research studies targeting stem cell-related signaling pathways have shown some efficiency, and these therapeutic studies have been reviewed elsewhere[87,88].

CONCLUSION

During the past few years there has been a great quantity of work researching markers that identify liver CSCs, and these discoveries have contributed to one of the most important developments in cancer treatment. Nevertheless, some important issues still need to be resolved. For example, some of the pivotal markers that are significant to CSCs are also shared by normal stem cells; thus, drugs targeting these markers could have a negative effect on normal stem cells. To specifically target CSCs without unnecessarily affecting normal stem cells, molecular differences between them need to be delineated. In addition, in the coming years, one of the major challenges will be to determine how these different liver CSC markers relate to one another. There is growing concern that a single marker cannot isolate a LCSC population. Increasing evidence has demonstrated that combinations of multiple markers can specifically label CSC populations.

In summary, in the future, more effective liver CSC markers are required to identify and design more specific anti-CSC marker therapies. The apparent advantages of specifically targeting CSCs in improving the potency of existing therapies are revealed in current knowledge, leading to long-term clinical benefits by providing an important framework for developing a novel therapeutic regimen.

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ORIGINAL ARTICLE

Basic Study

Novel and safer endoscopic cholecystectomy using only a flexible endoscope *via* single port

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Abstract

AIM: To apply the laparoscopic and endoscopic co-operative surgery concept, we investigated whether endoscopic cholecystectomy could be performed more safely and rapidly *via* only 1 port or not.

METHODS: Two dogs (11 and 13-mo-old female Beagle) were used in this study. Only 1 blunt port was created, and a flexible endoscope with a tip attachment was inserted between the fundus of gallbladder and liver. After local injection of saline to the gallbladder bed, resection of the gallbladder bed from the liver was performed. After complete resection of the gallbladder bed, the gallbladder was pulled up to resect its neck using the Ring-shaped thread technique. The neck of the gallbladder was cut using scissor forceps. Resected gallbladder was retrieved using endoscopic net forceps *via* a port.

RESULTS: The operation times from general anesthetizing with sevoflurane to finishing the closure of the blunt port site were about 50 min and 60 min respectively. The resection times of gallbladder bed were about 15 min and 13 min respectively without liver injury and bleeding at all. Feed were given just after next day of operation, and they had a good appetite. Two dogs are in good health now and no complications for 1 mo after endoscopic cholecystectomy using only a flexible endoscope *via* one port.

CONCLUSION: We are sure of great feasibility of endoscopic cholecystectomy *via* single port for human.

Key words: Laparoscopic and endoscopic cooperative surgery; Endoscopic cholecystectomy; Single port; Safer and complete resection; Feasibility

Core tip: Endoscopic cholecystectomy *via* single port is a safety and reliable new flexible endoscopic surgery. Local injection of saline to the gallbladder bed makes it easier to resect the gallbladder bed from the liver. After complete dissection of the gallbladder bed, the neck of the gallbladder was clipped and cut safer and easier using ring-shaped thread method.

INTRODUCTION

In 2004, since Kalloo *et al* reported a study on the observation of abdominal cavity through stomach using oral flexible endoscope following incision of the stomach, natural orifice translumenal endoscopic surgery (NOTES) using flexible endoscope has attracted attention as a minimally invasive surgical method[1-4]. It was impossible to suddenly succeed in developing a surgical method equivalent to laparoscopic surgery from the viewpoint of safety. On the other hand, laparoscopy and endoscopy cooperative surgery (LECS) was rapidly developed in Japan for the partial gastrectomy of gastric and duodenal tumors[5-9]. LECS is based on excellent concept of the combination and collaboration with endoscopist and surgeon, in other words, taking advantages of both flexible and rigid endoscopes to achieve super minimal invasive surgery.

By using the LECS concept and the technique of endoscopic submucosal dissection (ESD), we investigated whether endoscopic cholecystectomy could be performed more safely and rapidly *via* only single port or not.

MATERIALS AND METHODS

Conceptual images of endoscopic cholecystectomy via single port

Only 1 blunt port was created, and a flexible endoscope with a tip attachment was inserted between the fundus of gallbladder and liver easily. Local injection of saline to the gallbladder bed enabled us to easily obtain sufficient space (Figure 1A). Resection of the gallbladder from the liver was performed without any damage or bleeding (Figure 1B). After complete resection of the gallbladder bed, the gallbladder was pulled up to resect its neck. A ring-shaped thread was placed *via* an endoscopic channel using grasping forceps. One side of the ring was clipped to the fundus of the gallbladder and the other to the abdominal wall (Figure 1C). The third clip was hooked to 1 side of the ring-shaped thread. The thread was hooked crosswise to the 2 supporting points of the gallbladder and abdominal wall and clipped (Figure 1D). The neck of the gallbladder was cut using scissor forceps (Figure 1E). Resected gallbladder was retrieved using endoscopic net forceps *via* a port (Figure 1F).

In vivo dogs' experiments

Two dogs (11 and 13-mo-old female Beagle) were used in this study (Hokuzan Rabesu Co., Nagano, Japan). Animal experiments were performed in the Preclinical Animal Laboratory of Kagawa University, Japan. A dog was maintained in a pathogen-free facility under controlled temperature (24 ± 2 ℃) and humidity (55% ± 5%), with a 12-h light/dark cycle. All experimental procedures were performed according to the guidelines for the care and use of animals as established by Kagawa University.

The items examined were as follows: (1) The operation success rate with long survival; (2) The operation times from general anesthetizing to finishing the closure of the blunt port site; and (3) The resection times of gallbladder bed.

Experimental devices

Endoscopes: OLYMPUS GIF TYPE Q260J (Olympus Co. Tokyo, Japan). CO_2 insufflation device: OLYMPUS UCR (Olympus Co. Tokyo, Japan); Endoscopic knives: Dual knife (KD-650L, Olympus Co. Tokyo, Japan); Generator device: ERBE VIO300D (Elektromedizin, Tübingen, Germany).

RESULTS

Under general anesthesia, only 1 port (12 mm in diameter) was inserted using an open technique (Figure 2A). A flexible endoscope with a tip attachment was inserted between the fundus of gallbladder and liver, and a layer of loose connective tissue was opened. Local injection of saline to the loose layer enabled us to easily obtain sufficient space and dissect the gallbladder from the liver without any damage or bleeding (Figure 2B). After complete dissection of the gallbladder bed (Figure 2C), the gallbladder was pulled up to dissect its neck. A 3-0 absorbable ring-shaped thread was placed *via* an endoscopic channel using grasping forceps. One side of the ring was clipped to the fundus of the gallbladder and the other to the abdominal wall (Figure 2D). The third clip was hooked

Figure 1 Conceptual images of endoscopic cholecystectomy *via* single port. A: Local injection of saline to the gallbladder bed; B: Resection of the gallbladder from the liver was performed without any damage or bleeding due to sufficient space of local injection; C: A ring-shaped thread was placed, and one side of the ring was clipped to the fundus of the gallbladder and the other to the abdominal wall; D: The third clip was hooked to 1 side of the ring-shaped thread. The thread was hooked crosswise to the 2 supporting points of the gallbladder and abdominal wall; E: The neck of the gallbladder was cut using scissor forceps; F: Resected gallbladder was retrieved using endoscopic net forceps.

Figure 2 *In vivo* two dogs' experiments. A: Under general anesthesia, only 1 port (12 mm in diameter) was inserted using an open technique; B: A flexible endoscope with a tip attachment was inserted between the fundus of gallbladder (blue arrow) and liver (red arrow), and a layer of loose connective tissue was opened (yellow arrow); C: Complete dissection (yellow arrow) of the gallbladder bed (blue arrow) from liver (red arrow); D: One side of the ring-shaped thread was clipped to the fundus of the gallbladder and the other to the abdominal wall (yellow arrow).

Figure 3 The ring-shaped thread technique. A: The thread was hooked crosswise to the 2 supporting points (yellow arrow) of the gallbladder (blue arrow) and abdominal wall. Red arrow revealed the liver; B: The neck of the gallbladder was cut using scissor forceps (yellow arrow: neck of the gallbladder; blue arrows: gallbladder vein; red arrow: gallbladder artery; green arrow: cystic duct); C: The gallbladder was completely resected and put upon (blue curved arrow) the liver (red arrow); D: Resected gallbladder bed was very smooth without blood and damage.

to 1 side of the ring-shaped thread. The thread was hooked crosswise to the 2 supporting points of the gallbladder and abdominal wall and clipped (Figure 3A). After the neck of the gallbladder was cut using scissor forceps extending it and placing 2 clips on the central side and 1 clip on the peripheral side (Figure 3B), successful resection of the gallbladder was completed (Figure 3C). Resected gallbladder bed was very smooth without blood (Figure 3D).

The operation times from general anesthetizing with sevoflurane to finishing the closure of the blunt port site were about 50 min and 60 min respectively. The resection times of gallbladder bed were about 15 min and 13 min respectively without liver injury and bleeding at all. Feed were given just after next day of operation, and they had a good appetite.

Two dogs are in good health now and no complications for 1 mo after endoscopic cholecystectomy using only a flexible endoscope *via* one port.

DISCUSSION

Laparoscopic cholecystectomy (LC) has been established and performed safely all over the world[10,11]. As LC is safe and well-established surgical procedure, it needs to create 4 ports (5 mm-3 ports in right hypochondriac region, 12 mm-1 blunt (camera) port in the umbilical portion). Although high volume centers are able to perform single port laparoscopic

cholecystectomy (SP-LC) safely, it is still challenging, limited and risky for surgeon to perform SP-LC in the city hospital[12]. In this experiment, to create 12 mm-1 ports in right hypochondriac region made it possible to complete flexible endoscopic cholecystectomy very easy and safety. When a flexible endoscope with a tip attachment was inserted between the gallbladder and liver, a layer of loose connective tissue was opened very easily. Moreover, local injection of saline to the loose layer to obtain sufficient space and dissect the gallbladder bed more safely. After complete dissection of the gallbladder bed, the neck of the gallbladder was clipped and cut using scissor forceps easily. The Ring-shaped thread technique was very useful to pull up the gallbladder and resect its neck. Endoscopic cholecystectomy using only one flexible endoscope *via* single port was as safe and feasible as laparoscopic cholecystectomy.

There are 3 advantageous points in this procedure (EC). We described these advantages as follows: (1) Reduction of port: In general, we perform laparoscopic cholecystectomy creating 4 ports by open technique (1 blunt port 12 mm in diameter in umbilical portion which is used to insert a rigid endoscopic camera and make pneumoperitoneum to keep stable pressure, 1 port 12 mm in diameter in epigastric region, 2 port 5 mm in diameter in Rt. hypochondriac regions to assist pulling up the fundus of the GB). In this technique (EC), the most innovative points are not

only to approach to the GB *via* single blunt port in Rt. hypochondriac region and make pneumoperitoneum keeping stable pressure, but also to use functional advantages of flexible endoscope such as cleaning its lens function, water jet function and manual insufflation/deflation function under stable pressure by pneumoperitoneum system of the blunt port. The concept of this technique (EC) isn't similar to the NOTES at all, but LECS concept which approached *via* port created at the abdominal wall. This procedure (EC) requires only 1 port *via* abdominal wall without perforation of the gut wall to approach the abdominal cavity and GB bed; (2) Direction of approach to the GB: Single incision laparoscopic surgery (SILS) needs higher surgical technique than this technique using a flexible endoscope. This is because the approach direction by SILS is from the perpendicular direction to the GB bed, and this makes it difficult to resect GB without minor injury and bleeding of the liver. On the other hand, in the EC procedure, we can approach the GB bed from the parallel direction. It is very easy to inject the natural saline to the GB bed and dissect it using attachment from parallel direction by a flexible endoscope using endoscopic submucosal dissection (ESD) method; and (3) The Ring-shaped thread technique: After dissected GB bed, it is difficult to obtain clear surgical view at the Calot triangle. However, the ring thread is inserted into the GB through the endoscopic channel using grasping forceps. After clipped at 2 points, the third clip hooks 1 side of the ring thread and slides the thread crosswise to pull up the GB and is then clipped.

In this study, there are several limitations as follows and needed more animal experiments to confirm safety of EC: (1) Small number experiments; and (2) EC requires endoscopist to learn the anatomy of only the Calot triangle and its anomaly.

We are sure of the feasibility of this surgical procedure "endoscopic cholecystectomy *via* single port" for human.

ACKNOWLEDGMENTS

COMMENTS

Background
As natural orifice translumenal endoscopic surgery (NOTES) using flexible endoscope has been expected as a minimally invasive surgery, it was difficult to perform NOTES equivalent to laparoscopic surgery from the viewpoint of safety. Recently, laparoscopic and endoscopic cooperative surgery (LECS) was rapidly developed in Japan for the partial gastrectomy, which is based on excellent concept of the combination and collaboration with endoscopist and surgeon.

Research frontiers
As the LECS was an excellent surgical concept, the authors applied the LECS concept for endoscopic cholecystectomy using only one flexible endoscope *via* single port.

Innovations and breakthroughs
Local injection of saline to the gallbladder bed made it possible to resect the gallbladder bed more rapidly and safely. The Ring-shaped thread technique was very useful to pull up the gallbladder and resect its neck.

Applications
Endoscopic cholecystectomy using only one flexible endoscope *via* single port was as safe and feasible as laparoscopic cholecystectomy.

Peer-review
Quite sure that the feasibility of this surgical procedure "endoscopic cholecystectomy *via* single port" for human.

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ORIGINAL ARTICLE

Basic Study

Apoptosis of human gastric carcinoma cells induced by *Euphorbia esula* latex

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Abstract

AIM: To investigate the effect of *Euphorbia esula* (*E. esula*) extract in inhibiting proliferation and inducing apoptosis in SGC-7901 cells.

METHODS: *E. esula* extract at different concentrations was used to inhibit proliferation and induce apoptosis of human gastric carcinoma SGC-7901 cells. Inhibition of proliferation was detected with thiazolyl blue assay, and apoptosis was detected with fluorescence microscopy, transmission electron microscopy, and flow cytometry. The mechanisms were studied by measurement of caspase-3 and caspase-8 activities and *Bax* and *Bcl2* mRNA expression.

RESULTS: The thiazolyl blue assay showed that SGC-7901 cell viability and proliferation were inhibited significantly by *E. esula* extract in a time- and concentration-dependent manner. Fluorescence microscopy revealed that the cell nuclei showed the characteristic changes of apoptosis, such as uneven staining and chromatin marginalization. Some key features of apoptosis were also observed under

transmission electron microscopy, which included cellular shrinkage and the foaming or bubbling phenomenon. When the cells were analyzed by flow cytometry, a sub-G1 peak could be seen clearly. Spectrophotometric assay of caspase-3 and caspase-8 activities in the treated cells showed an approximately two-fold increase. Reverse transcriptase polymerase chain reaction showed that *Bax* mRNA expression was upregulated, while *Bcl2* mRNA expression was downregulated.

CONCLUSION: *E. esula* extract inhibited proliferation and induced apoptosis in SGC-7901 cells, in a caspase-dependent manner, involving upregulation of *Bax* and downregulation of *Bcl2*.

Key words: *Euphorbia esula* Linn; Apoptosis; Gastric carcinoma; Caspase; Bax; Bcl2

Core tip: Latex of *Euphorbia esula* (*E. esula*) is used to treat benign growths in traditional Chinese medicine. This led us to design an experiment to establish whether latex of *E. esula* can cause apoptosis. We found that *E. esula* extract inhibited proliferation and induced apoptosis in SGC-7901 cells, and that the action was caspase dependent and involved upregulation of *Bax* gene and downregulation of *Bcl2* gene.

INTRODUCTION

Euphorbia esula (*E. esula*) Linn is a herbaceous perennial plant belonging to the family Euphorbiaceae, which is native to many countries in the world, including China[1-4]. One of the characteristic features of the plant is its abundance of latex; a kind of white milk-like fluid that readily flows out when the stem or leaf of the plant is broken. For that reason, the plant is also known as milky juice herb in some areas of China. One major use of this plant in traditional Chinese medicine is to treat skin warts (a kind of non-cancerous growth, or papilloma). The fresh latex of the plant is applied several times to the surface of the growth, which results in gradual shrinkage and final disappearance of the lump.

This prompted us to speculate whether certain ingredients in the latex cause apoptosis in the wart cells. In the present study, we collected the fresh wild plant from the mountainous area of Yanan,

Figure 1 *Euphorbia esula* Linn collected in Yan'an, China in June.

extracted the latex from the herb with water at room temperature or 4 ℃, and used the extract to induce apoptosis in the SGC-7901 cell line, a continuous cell line originally isolated from human gastric carcinoma. Gastric carcinoma is the leading cause of cancer mortality in rural China and the second most common cause worldwide, including Chinese cities[5].

Our study demonstrated that *E. esula* extract inhibited proliferation and induced apoptosis in SGC-7901 cells. The action was caspase dependent and involved upregulation of *Bax* gene transcription and downregulation of *Bcl2* gene transcription.

MATERIALS AND METHODS

Preparation of *E. esula* extract

The aboveground part of wild *E. esula* was collected from the area around Yanan City in early June (Figure 1). The fresh herb was washed clean, first with running water and then with ultrapure water, and cut into small pieces. Latex of the plant was gathered by squeezing the plant pieces in ultrapure water with a machine, and the fluid was filtered with analytical filter paper (slow type) at 4 ℃. The resulting liquid, which was clear and brown in color, was air-dried at 50 ℃. The dry substance was weighed and then dissolved and diluted with ultrapure water to obtain a concentration of 20 g/L. The final solution was made aseptic by passing through a 220-nm filter membrane (Minisart; Sartorius, Goettigen, Germany) and stored at -20 ℃.

Cell line maintenance

Human gastric carcinoma cell line SGC-7901 was obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in modified RPMI 1640 medium (Hyclone; Thermo Fisher Scientific, Beijing, China) supplemented with 2.05 mmol/L L-glutamine, 100 mL/L fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, China), 100 U/mL penicillin, and 100 mg/L streptomycin, and incubated at 37 ℃ with 50 mL/L CO_2, and 95% humidity.

Cell viability assay with thiazolyl blue reagent

SGC-7901 cells cultivated to logarithmic phase were digested with 2.5 g/L trypsin and a cell suspension of 6×10^4 cells/mL was made with RPMI 1640 containing 100 mL/L FBS. The cells were seeded in two 96-well culture plates (NUNC microwell plates, Thermo Scientific, Roskilde, Denmark), 100 μL per well and incubated at 37 ℃ with 50 mL/L CO_2 and 95% humidity. Twenty-four hours later, when the cells had grown to approximately 70% confluence, the culture medium was replaced with the same volume of fresh 100 mL/L FBS-RPMI 1640 medium containing different concentrations of *E. esula* extract at 5, 10, 20, 40, 80 and 160 mg/L; there were eight wells for each dilution. The culture medium in an additional eight wells was replaced with the same culture medium without *E. esula* extract, 100 μL per well also, to be used as a control. The cells were further incubated for 24 h for one plate and 48 h for the other. At the end of the incubation, thiazolyl blue (Sigma-Aldrich, St Louis, MO, United States) solution at 5 mg/mL was added to all of the wells at 20 μL per well. After further incubating at 37 ℃, 50 mL/L CO_2, for 4 h, the supernatant containing thiazolyl blue was discarded and dimethyl sulfoxide was added to all wells at 150 μL per well. The plate was oscillated slowly with a horizontal oscillator for 10 min to dissolve the Formazan crystals fully. After that, the optical density (OD) of the testing wells and the control wells was obtained at 490 nm wavelength with a Microplate reader (Model 680; Bio-Rad, United States). The arithmetic means of the eight wells for each dilution and the control wells were taken to draw a dose-response relationship polygon curve. Rates of cell proliferation inhibition were calculated using the following formula: cell viability inhibition rate = (average OD value of control wells - average OD value of testing wells)/average OD value of control wells.

Fluorescence microscopic detection of SGC-7901 cell apoptosis after E. esula extract treatment

SGC-7901 cells were cultured until the monolayer reached approximately 80% confluence. The cells were digested with 2.5 g/L trypsin and made into a suspension of 6×10^4 cells/mL with RPMI 1640 containing 100 mL/L FBS. A sterile coverslip was placed on the bottom of each well of a six-well culture plate, and 500 μL of the cell suspension was applied to the center of each coverslip. After 2 h incubation at 37 ℃ with 50 mL/L CO_2 and 95% humidity, 1.5 mL of cell culture medium (RPMI 1640 containing 100 mL/L FBS) was added to each well of the six-well culture plate, which was then put into the incubator for further cultivation. Twenty hours later, after removing the used culture medium, *E. esula* extract at concentrations of 40 and 80 mg/L, diluted with RPMI 1640 containing 100 mL/L FBS, was added to the testing wells; 2.0 mL per well. For control wells,

2.0 mL RPMI 1640 containing 100 mL/L FBS only was added. After incubating for 24 h under the same conditions, the used culture medium was discarded and 500 μL 5 mg/L Hoechst 33258 (Sigma-Aldrich) was added to the coverslip of each well and allowed to stain in the dark at room temperature for 5 min. The coverslips were removed from the wells and each was mounted on a glass slide with the cell side downward. Nuclear morphological changes were observed using fluorescence microscopy excited by a UV light source (excitation 352 nm, emission 461 nm).

Examination of SGC-7901 cell apoptosis using transmission electron microscopy

SGC-7901 cells in logarithmic phase, prepared at a density of 3×10^4 cells, were seeded in a six-well culture plate (2 mL per well) and cultured at 37 ℃ and 50 mL/L CO_2 for 24 h. The cell culture medium was replaced with 100 mL/L FBS-RPMI 1640 containing 40 mg/L of *E. esula* extract. For control wells, only 100 mL/L FBS-RPMI 1640 culture medium was added. After cultivation at 37 ℃ and 50 mL/L CO_2 for 24 h, cells were harvested with 2.5 g/L trypsin digestion, rinsed with PBS, and pelleted at 290 × g for 6 min. The cell pellets were fixed with 1.5 g/L glutaraldehyde for 90 min, rinsed three times with 0.18 mol/L sucrose, and, after lying overnight, post-fixed in 1 g/L osmium tetroxide for 1 h, and rinsed. The cells were dehydrated in a graded series of ethanol, soaked in a mixture of an equal volume of ethanol and embedding medium at 37 ℃ for 3 h, and embedded in epoxy resin. The blocks were cut using a Leica ultramicrotome. The slices were stained with uranyl acetate and lead citrate and examined using a JEM-1011 transmission electron microscope (JEOL, Japan); photographs of representative fields were taken.

Flow cytometry analysis of SGC-7901 cell apoptosis

SGC-7901 cells were seeded in two six-well plates at 6×10^4/mL in RPMI 1640 containing 100 mL/L FBS (2 mL per well) and cultivated at 37 ℃ with 50 mL/L CO_2 and 95% humidity. Twenty-four hours later, when the cell monolayer reached approximately 80% confluence, the old culture medium of one of the two plates was discarded, and the cells were treated with *E. esula* extract at concentrations of 40 and 80 mg/L, and diluted with RPMI 1640 containing 100 mL/L FBS. Both plates were cultivated continually under the same conditions. Twenty-four hours later, cells of the other plate were treated with *E. esula* extract in the same way. The culture medium of the control wells was replaced with the same volume of RPMI 1640 containing 100 mL/L FBS. After culturing for a further 24 h, the cells were collected with 2.5 g/L trypsin digestion, passed through a 400-mesh nylon screen (38-μm sieve opening) to eliminate any cell clumps, and washed twice with ice-cold PBS. After

Table 1 Primer sequences of *Bax*, *Bcl-2* and *β-actin* genes

Gene	Forward primer	Backward primer
Bax	TTTGCTTCAGGGTTTCATCC	GGAGGAAGTCCAATGTCCAG
Bcl-2	GGATGCCTTTGTGGAACTGT	ATTCGACGTTTTGCCTGAAG
β-actin	GAAAATCTGGCCACCCACCT	GGGGTGTTGAAGGTCTCAAA

the second wash, the cell pellets were resuspended with ice-cold 70% ethanol and placed at 4 ℃ for 30 min (ethanol fixation). The cells were washed again with ice-cold PBS and resuspended with ice-cold RNase solution (BioDev-Tech, Beijing, China, kept at -20 ℃, diluted with ice-cold PBS at 50 mg/L), and kept at room temperature for 20 min. Finally, the cells were centrifuged at 129 × *g* for 5 min, resuspended with 1 mL ice-cold PBS, and kept at -20 ℃ until use. When we were ready for flow cytometry analysis, 100 μL propidium iodide (Sigma-Aldrich) solution (100 mg/L) was added to 1 mL cell suspension and mixed thoroughly. After keeping at room temperature in the dark for 30 min, the apoptotic peak (sub-G1 peak) was detected using a flow cytometer (Beckman Coulter, United States).

Spectrophotometry of caspase-3 and caspase-8 activity in cells treated with E. esula extract

SGC-7901 cells incubated in 25-cm^2 culture flasks at 37 ℃ with 50 mL/L CO₂ and 95% humidity were allowed to grow to approximately 80% confluence, and then *E. esula* extract was added at a final concentration of 40 mg/L and the cells were incubated again under the same conditions. Twenty-four hours later, the cells were harvested with 2.5 g/L trypsin digestion and washed with ice-cold PBS. After the supernatant was removed, cell lysis buffer was added (50 μL lysis solution for 2 × 10^6 cells) and the cells were resuspended and kept in an ice bath for 30 min. During that time, vortex oscillation of the centrifugal tube was carried out four times for 10 s each. The cell lysate was centrifuged at 4 ℃, 12879 × *g* for 15 min. The supernatant was transferred carefully to a new centrifuge tube and placed on ice. Caspase-3 and caspase-8 activities were measured immediately according to the instructions of Caspase Activity Colorimetric Assay Kit (Nanjing Biobox Biotech. Co. Ltd, Nanjing, China). OD values were read at 405 nm using a Microplate reader (Model 680; Bio-Rad).

Reverse transcriptase polymerase chain reaction detection of dose-effect dependence of Bax and Bcl2 mRNA expression after E. esula extract treatment

SGC-7901 cells were seeded in four 25-cm^2 culture flasks and allowed to grow to approximately 80% confluence. The cells were treated with *E. esula* extract at concentrations of 20, 40 and 80 mg/L. One of the flasks was used as a control. After 24 h incubation, the

cells were harvested with 2.5 g/L trypsin, washed with ice-cold PBS and pelleted at 290 × *g*, for 6 min. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Diethylpyrocarbonate water was used to dissolve the RNA. A small amount was taken from all extracted total RNA samples for quality and quantity analysis using UV spectrophotometry and agarose gel electrophoresis. The extracted RNA samples were stored at -80 ℃ until further use. Reverse transcription for cDNA was performed using 1 μg total RNA in a reaction volume of 20 μL. Two microliters of cDNA product was taken to perform PCR amplification in a 25-μL reaction system. *β-Actin* gene was used as an internal reference to normalize the expression of the target genes. Primer sequences of the *Bax*, *Bcl2* and *β-actin* genes are shown in Table 1. The PCR mixture was subjected to 30 cycles with the following conditions: activation at 95 ℃ for 5 min; denaturation at 95 ℃ for 5 s; annealing at 62 ℃ for 10 s; and extension at 60 ℃ for 10 s. Finally, the PCR products were subjected to 1.5% agarose gel electrophoresis separation.

Statistical analysis

Experimental data were presented as the mean ± SD. Differences between treatments and controls, and among different treatment groups, were assessed by analysis of variance or a χ^2 test. *P* < 0.05 was considered statistically significant.

RESULTS

Viability of SGC-7901 cells after treatment with E. esula extract detected with thiazolyl blue assay

The thiazolyl blue assay showed that the viability or proliferative activity of human gastric carcinoma SGC-7901 cells was significantly inhibited by *E. esula* extract, in an obvious time- and concentration-dependent manner. SGC-7901 cells treated with different concentrations of *E. esula* extract all showed a significant inhibitory effect compared with the controls (*P* < 0.01). With the increasing drug concentration, there was a significant trend of intensification of cell growth inhibition (*P* < 0.01). The inhibitory effect was more notable with treatment for 48 h than 24 h (*P* < 0.05) (Table 2).

Apoptotic nuclear morphology observed using fluorescence microscopy after treating SGC-7901 cells with E. esula extract

SGC-7901 cells were treated with *E. esula* extract, stained by fluorescent dye Hoechst 33258, and examined under a UV-excited fluorescence microscopy. The nuclei of all cells were blue in color. The cells that were treated with *E. esula* extract showed the characteristic nuclear morphological changes of apoptosis, such as nuclear condensation and uneven

E. esula (mg/L)	OD$_{490}$ (mean ± SD)		Inhibition rate (%)	
	24 h	48 h	24 h	48 h
0 (control)	0.827 ± 0.020	0.818 ± 0.021	0	0
5	0.728 ± 0.018[b]	0.712 ± 0.020[b]	11.97[b]	12.96[b]
10	0.645 ± 0.020[b,d]	0.620 ± 0.019[b,d]	22.00[b,d]	24.21[b,d]
20	0.574 ± 0.019[b,d]	0.501 ± 0.018[b,c,d]	30.59[b,d]	38.75[b,c,d]
40	0.447 ± 0.017[b,d]	0.397 ± 0.015[b,c,d]	45.95[b,d]	51.47[b,c,d]
80	0.338 ± 0.021[b,d]	0.274 ± 0.017[b,c,d]	59.13[b,d]	66.50[b,c,d]
160	0.269 ± 0.019[b,d]	0.214 ± 0.016[b,c,d]	67.47[b,d]	73.84[b,c,d]

Table 2 Inhibition of SGC-7901 cells by *Euphorbia esula* extract

[b]$P < 0.01$ *vs* control; [d]$P < 0.01$ *vs* previous concentration; [c]$P < 0.05$ *vs* 24 h treatment. $n = 8$. E. esula: *Euphorbia esula*.

staining, chromatin marginalization, and nuclear debris. In addition, enhancement of blue fluorescence was observed in the nuclei of these cells. The apoptotic nuclear morphological changes were more prominent in the cells treated with 80 mg/L *E. esula* extract than those treated with 40 mg/L extract. In contrast, the nuclei of the control cells showed a shallower and evenly distributed blue color (Figure 2).

Morphological changes of SGC-7901 cells after treatment with E. esula extract observed with transmission electron microscope

After treatment with *E. esula* extract at 40 mg/L for 24 h, SGC-7901 cells showed some characteristic features of apoptosis under the transmission electron microscope. These included cellular shrinkage, foaming or bubbling of cytoplasm, as well as chromatin condensation and marginalization. The untreated control cells did not show these phenomena (Figure 3).

Flow cytometry showed an apoptotic peak in SGC-7901 cells treated with E. esula extract

SGC-7901 cells were treated with 40 and 80 mg/L *E. esula* extract for 24 and 48 h and were detected by flow cytometry. Sub-G1 or the apoptotic peak, which is a characteristic feature of cells undergoing apoptosis, resulting from cellular DNA loss, could be seen clearly, and was more obvious in cells treated with the higher concentration and for a longer period. The untreated SGC-7901 cells did not show this feature (Figure 4).

Caspase-3 and caspase-8 activation in SGC-7901 cells treated with E. esula extract

After treating SGC-7901 cells with 40 mg/L *E. esula* extract for 24 h, caspase-3 and caspase-8 activities were measured by a colorimetric assay, and mean OD$_{405}$ values were obtained from blank wells, control wells and *E. esula* extract-treated wells, which were 0.038, 0.195 and 0.349 for caspase-3 (Figure 5A), and 0.038, 0.162 and 0.252 for caspase-8 (Figure 5B), respectively. The fold-increases of caspase-3 and caspase-8 activity in *E. esula* extract-treated

Figure 2 Nuclear morphological changes observed using a fluorescence microscopy in SGC-7901 cells treated with *Euphorbia esula* extract at different concentrations and for 24 h, stained by Hoechst 33258. A1: Untreated, magnification × 200; A2: Untreated, magnification × 1000; B1: 40 mg/L, magnification × 200; B2: 40 mg/L, magnification × 1000; C1: 80 mg/L, magnification × 200; C2: 80 mg/L, magnification × 1000.

SGC-7901 cells were 1.98 and 1.73, respectively, as determined by the following formula: Fold increase = mean OD of testing wells - mean OD of blank wells)/ mean OD of control wells - mean OD of blank wells.

Dose-dependent alteration of Bax and Bcl2 mRNA expression in SGC-7901 cells treated with E. esula extract

RT-PCR and agarose gel electrophoresis showed a dose-dependent increase in the *Bax* mRNA expression level and a dose-dependent decrease in the *Bcl2* mRNA expression level in SGC-7901 cells treated with *E. esula* extract. *Bax* mRNA expression increased with increasing concentration of *E. esula* extract, whereas *Bcl2* mRNA expression decreased with increasing concentration of *E. esula* extract (Figure 6).

DISCUSSION

Malignant tumors are a serious threat to human health, and their morbidity and mortality are among the highest of all disease categories worldwide[5]. Looking for effective anti-tumor drugs with fewer side

Figure 3 Morphology of SGC-7901 cells observed using a transmission electron microscope. A: After treatment with 40 mg/L *Euphorbia esula* extract for 24 h; B: Untreated cells. Bar = 3 μm.

Figure 4 Flow cytometry detection of the apoptotic peak. SGC-7901 cells were treated with different concentrations of *Euphorbia esula* (*E. esula*) extract and flow cytometry was performed at three time points. A: Untreated SGC-7901 cells used as a control; B: Treated with 40 mg/L *E. esula* extract; C: Treated with 80 mg/L *E. esula* extract.

A

Caspase-3 activity

Blank	Control	Testing
Mean ODλ405 0.038	0.195	0.349

Blank: PBS; Control: SGC-7901 cells without treatment; Testing: SGC-7901 cells treated with 40 μg/mL *Euphorbia esula* extract.

B

Caspase-8 activity

Blank	Control	Testing
Mean ODλ405 0.037	0.162	0.252

Blank: PBS; Control: SGC-7901 cells without treatment; Testing: SGC-7901 cells treated with 40 μg/mL *Euphorbia esula* extract.

Figure 5 Activation of caspase-3 and caspase-8 in SGC-7901 cells after treatment with *Euphorbia esula* extract, measured using spectrophotometry. Concentration of *Euphorbia esula* extract: 40 mg/L; duration of treatment: 24 h; *n* = 3.

A

Euphorbia esula extract concentration: (1) 10 μg/mL, (2) 20 μg/mL, (3) 40 μg/mL, and (4) 80 μg/mL.

B

Euphorbia esula extract concentration: (1) 80 μg/mL, (2) 40 μg/mL, (3) 20 μg/mL, and (4) 10 μg/mL.

Figure 6 Agarose gel electrophoresis of RT-PCR products showing dose-dependent alterations of *Bax* and *Bcl2* gene expression in SGC-7901 cells treated with *Euphorbia esula* extract. A: *Bax* mRNA level increased with the concentration of *Euphorbia esula* (*E. esula*) extract; B: *Bcl2* mRNA level increased with decreased concentration of *E. esula* extract.

effects is a great challenge and an urgent task for the entire medical and pharmacological community. Currently, extracting ingredients from plants or other natural resources to be used as anti-tumor drugs is a noteworthy direction in research[6-8].

Chemically synthesized drugs reached their peak phase in the western world during the 1950s and 1960s, but since the 1970s, people have become more conscious of the undesired aspects of chemical synthetic drugs, that is, their side effects and toxicity. In the meantime, natural drugs, because of their fewer side effects and lower toxicity, have gradually been taken seriously. In addition, natural medicines have lower development costs than chemical synthetic drugs[9-12]. In entering the 21st century, under the influence of the transformation of medicine model and the new concept of "returning to nature", the exploration and research

of natural resources for medications is becoming even more prevalent. At the present time, the phrase natural medicine has become an attractive and frequently used term in the medical profession, as well as among lay people, and searching for active ingredients from natural resources for health care and for the prevention and treatment of diseases has become a major trend worldwide. Many researchers believe that in the near future, natural medicines will become the mainstream medical market[13].

In the present study, we used a fresh aqueous extract from *E. esula* Linn to induce apoptosis in human gastric carcinoma SGC-7901 cells and found that it reduced cellular viability and induced apoptosis. We also investigated the underlying mechanisms and found that the activity of caspase-8 was 1.73-fold increased, that of caspase-3 was 1.98-fold increased, the expression of *Bax* mRNA was upregulated and the expression of *Bcl2* mRNA was downregulated. These results indicated that *E. esula* extract/latex must contain some ingredients that inhibit SGC-7901 cell proliferation and induce apoptosis; the latter being dependent on activation of caspases and regulated by *Bax* and *Bcl2* gene products. The results suggested that *E. esula* extract/latex induces SGC-7901 cell apoptosis through extrinsic signaling or a membrane-receptor-dependent pathway. In this pathway, the extracellular inducer acts on membrane receptors that transduce the triggering signal intracellularly through several steps and leads to caspase-8 activation[14,15]. The process is usually regulated by a balance between the proapoptotic proteins, such as Bax, Bid, Bak and Bad, and the antiapoptotic proteins, such as Bcl2 and Bcl-XL, and might involve alteration of mitochondrial membrane permeability and release of cytochrome C[16-18]. Finally, the apoptotic effector molecule caspase-3 is activated and the cell death program is executed[19]. It is not clear whether caspase-9 is also involved in the process of *E. esula* extract-induced SGC-7901 cell apoptosis.

E. esula latex is used to treat skin warts in traditional Chinese medicine. Both skin warts and cervical cancers are caused by human papillomaviruses (HPVs); therefore, when we initially designed the experiments to test whether *E. esula* induces apoptosis, we naturally thought of using a cell line that originated from cervical cancer and the HeLa cell line was the choice. A preliminary comparative study using *E. esula* extract to induce apoptosis in the HeLa cell line and several other cancerous cell lines including human gastric carcinoma SGC-7901 cells, human lung cancer A549 cells, human liver cancer HepG2 Cells, and human breast cancer MCF-7 cells showed that *E. esula* induced apoptosis in all these cell lines, but that there were no significant differences between HeLa cells and the other cell lines in their response to *E. esula* treatment (unpublished work). The results suggested that *E. esula* might not exert its action interfering with HPV carcinogenesis or HPV oncogene/oncoprotein actions. Thus, we used human gastric carcinoma SGC-7901 cells in our research because gastric cancer is the second leading cause of cancer-related death in the world (and is the number one in most Chinese rural areas, including the countryside around the city in which the authors work). The fact that *E. esula* did not show significant differences in inducing apoptosis among different cancerous cell lines could be an advantage, because if we could isolate the effective ingredient(s), it might be used to treat cancers of other tissue origins. It is not clear yet whether *E. esula* extract induces apoptosis more effectively in cancer cells than non-cancerous cells. If the answer is yes then the drug made from *E. esula* effective ingredient(s) would be very promising; if not, a technique should be established to deliver the drug to/into cancer cells.

To study further the mechanisms of *E. esula* extract-induced SGC-7901 cell apoptosis, we could detect the subcellular distribution of cytochrome C and measure the enzymatic activity of caspase-9 in *E. esula* extract-treated SGC-7901 cells. Future experiments will also include a comparison between primary cultures of normal tissues and continuous cell lines to see if there are any differences in their responses to *E. esula*-induced apoptosis. In particular, future work should include the isolation and identification of the effective ingredient(s) in *E. esula* extract that inhibit cancer cell proliferation and trigger cancer cells to undergo apoptosis.

COMMENTS

Background

Euphorbia esula (*E. esula*) is used to treat skin warts in Chinese folk medicine. The latex of the plant is applied several times to the surface of the warts and the lump shrinks gradually and finally disappears. This prompted the authors to design the present study to determine whether the latex can cause apoptosis in tumor cells.

Research frontiers

The side effects and toxicity of chemically synthetic drugs cannot be ignored; therefore, natural medicines have gradually been taken seriously. In addition to

their advantages of fewer side effects and lower toxicity, natural medicines have lower development costs than chemical drugs. As a result, the exploration and research of natural resources for medications is becoming more prevalent.

Innovations and breakthroughs

This study showed clearly that *E. esula* extract inhibited proliferation and induced apoptosis effectively in SGC-7901 cells, a cancerous cell line originally isolated from human gastric carcinoma, and that the action was caspase dependent.

Applications

After the effective ingredient in *E. esula* that inhibits cancer cell proliferation and triggers cancer cells to undergo apoptosis is isolated or extracted, it can be used to develop medicines for clinical use.

Terminology

E. esula Linn is an herbaceous perennial plant belonging to the family Euphorbiaceae. The plant is rich in a type of milky juice or latex.

Peer-review

This is a well written and set up study. The authors give a sufficient overview of the study background and clearly stated the hypothesis of the study. The aim of the study is fulfilled. The results are presented sufficiently well and have been discussed well; the 6 figures and 2 tables give a good overview of the results and are presented correctly.

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ORIGINAL ARTICLE

Basic Study

Analysis of tumor-infiltrating gamma delta T cells in rectal cancer

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Abstract

AIM: To investigate the regulatory effect of Vδ1 T cells and the antitumor activity of Vδ2 T cells in rectal cancer.

METHODS: Peripheral blood, tumor tissues and para-carcinoma tissues from 20 rectal cancer patients were collected. Naïve CD4 T cells from the peripheral blood of rectal cancer patients were purified by negative selection using a Naive CD4$^+$ T Cell Isolation Kit Ⅱ (Miltenyi Biotec). Tumor tissues and para-carcinoma tissues were minced into small pieces and digested in a triple enzyme mixture containing collagenase type Ⅳ, hyaluronidase, and deoxyribonuclease for 2 h at room temperature. After digestion, the cells were washed twice in RPMI1640 and cultured in RPMI1640 containing 10% human serum supplemented with L-glutamine and 2-mercaptoethanol and 1000 U/mL of IL-2 for the generation of T cells. Vδ1 T cells and Vδ2 T cells from tumor tissues and para-carcinoma tissues were expanded by anti-TCR $\gamma\delta$ antibodies. The inhibitory effects of Vδ1 T cells on naïve CD4 T cells were analyzed using the CFSE method. The cytotoxicity of Vδ2 T cells on rectal cancer lines was determined by the LDH method.

RESULTS: The percentage of Vδ1 T cells in rectal tumor

tissues from rectal cancer patients was significantly increased, and positively correlated with the T stage. The percentage of Vδ2 T cells in rectal tumor tissues from rectal cancer patients was significantly decreased, and negatively correlated with the T stage. After culture for 14 d with 1 μg/mL anti-TCR γδ antibodies, the percentage of Vδ1 T cells from para-carcinoma tissues was 21.45% ± 4.64%, and the percentage of Vδ2 T cells was 38.64% ± 8.05%. After culture for 14 d, the percentage of Vδ1 T cells from rectal cancer tissues was 67.45% ± 11.75% and the percentage of Vδ2 T cells was 8.94% ± 2.85%. Tumor-infiltrating Vδ1 T cells had strong inhibitory effects, and tumor-infiltrating Vδ2 T cells showed strong cytolytic activity. The inhibitory effects of Vδ1 T cells from para-carcinoma tissues and from rectal cancer tissue were not significantly different. In addition, the cytolytic activities of Vδ2 T cells from para-carcinoma tissues and from rectal cancer tissues were not significantly different.

CONCLUSION: A percentage imbalance in Vδ1 and Vδ2 T cells in rectal cancer patients may contribute to the development of rectal cancer.

Key words: Rectal cancer; T cells; Vδ1 T cells; Vδ2 T cells; Foxp3; Cytotoxicity

Core tip: The percentage of tumor-infiltrating Vδ1 T cells in rectal cancer patients increased when T stage increased, whereas the percentage of tumor-infiltrating Vδ2 T cells in rectal cancer patients decreased as T stage increased. Vδ1 T cells from rectal cancer tissues had strong regulatory effects, and in rectal cancer tissues the main infiltrating γδ T cells were Vδ1 T cells. Although Vδ2 T cells from rectal cancer tissues have strong cytotoxic effects, there was little infiltration of Vδ2 T cells in rectal cancer tissues. Thus, an immunosuppressant microenvironment was formed in rectal cancer tissues, which may limit antitumor immunity and allow tumors in rectal cancer patients to evade immune surveillance.

INTRODUCTION

T cells can be divided into two major subsets according to their expression of rearranged adaptive T cell receptors (TCRs, γδ T cells and αβ T cells)[1]. γδ T cells, which represent a small subset (1%-10%) of CD3+ cells[2], can be divided into two subsets: Vδ1 T cells in the epithelial-associated lymphoid tissue and Vδ2 T cells in the peripheral blood[3,4]. They differ in their cytokine production and receptor expression, Vδ2 T cells being more inflammatory[5], and Vδ1 T cells having more of a regulatory phenotype[6]. It has been demonstrated that Vδ1 T cells express Foxp3, and their number is substantially decreased in the peripheral blood of patients with new-onset systemic lupus erythematosus (SLE)[6]. Vδ2 T cells have predominantly been investigated in the context of tumor immunosurveillance and host defense against viral invasion[7-10].

Rectal cancer is one of the most common causes of cancer deaths worldwide[11]. In recent years, combined chemoradiotherapy followed by total mesorectal excision (TME) has become the standard treatment for patients with locally advanced rectal cancer[12]. Local excision is often considered a curative treatment alternative to TME in early rectal cancer[13], however, one of the limitations of this approach is that it is impossible to determine the pN-category[13]. Lymph node involvement in rectal cancer is known to correlate with T stage[14,15]. There is increasing evidence that immune-profiling may help to predict clinical outcomes in rectal cancer, possibly more reliably than TNM classification or grading. There is evidence that a low number of tumor-infiltrating lymphocytes (TILs) predicts lymph node involvement in melanoma, gastric cancer, breast cancer, and cervical cancer[13,16-18]. The precise role of γδ T cells in the development of rectal cancer remains elusive.

In this study, we found that the percentage of Vδ1 T cells in rectal tumor tissues from rectal cancer patients was significantly increased, whereas the percentage of Vδ2 T cells in these tissues was significantly decreased. The percentages of Vδ1 and Vδ2 T cells correlated with the T stage of rectal cancer patients. To obtain Vδ1 and Vδ2 T cells, tumor tissues and para-carcinoma tissues were minced into small pieces, digested with a triple enzyme mixture containing collagenase type Ⅳ, hyaluronidase, and deoxyribonuclease and stimulated by anti-TCR γδ antibodies. After 14 d culture, the percentage of Vδ1 T cells from para-carcinoma tissues was 21.45% ± 4.64% and the percentage of Vδ2 T cells was 38.64% ± 8.05%. The percentage of Vδ1 T cells from rectal cancer tissues was 67.45% ± 11.75% and the percentage of Vδ2 T cells was 8.94% ± 2.85%. Functional assays demonstrated that tumor-infiltrating Vδ1 T cells in rectal cancer patients have strong inhibitory effects, and tumor-infiltrating Vδ2 T cells displayed strong cytolytic activity. The inhibitory effects of Vδ1 T cells and the cytolytic activity of Vδ2 T cells from para-carcinoma tissues and from rectal cancer tissues were not significantly different. Collectively, these data suggest that an imbalance in the Vδ1 and Vδ2 T cell percentages creates an immunosuppressant microenvironment in rectal cancer tissues, which may allow tumors to limit antitumor immunity and evade

immune surveillance in rectal cancer patients.

MATERIALS AND METHODS

Patients

Twenty patients with rectal cancer were enrolled in this study. The study was approved by the Fifth Affiliated Hospital of Xinjiang Medical University (Xinjiang, China) and written informed consent was obtained from each participating patient. Peripheral blood, tumor tissues, and para-carcinoma tissues were collected from the patients.

Antibodies and reagents

RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco; FITC-conjugated anti-human TCRγδ (IMMU510) was purchased from Beckman Coulter Immunotech; APC-conjugated anti-human CD3 (HIT3a) and FITC-conjugated anti-human TCR Vδ2 (B6) were purchased from Biolegend; FITC-conjugated anti-human TCR Vδ1 (TS8.2) was obtained from Pierce; a CellTrace™ CFSE Cell Proliferation Kit was purchased from Invitrogen; the CytoTox 96® Non-Radioactive Cytotoxicity Assay was purchased from Promega; interleukin 2 was purchased from Read United Cross Pharmaceutical Co., Ltd.

Cells

To expand Vδ1 T cells and Vδ2 T cells *in vitro*, tumor tissues and para-carcinoma tissues were minced into small pieces and then digested with a triple enzyme mixture containing collagenase type Ⅳ, hyaluronidase, and deoxyribonuclease for 2 h at room temperature. After digestion, the cells were washed twice in RPMI-1640 and cultured in RPMI-1640 medium with 10% FBS and 200 IU/mL interleukin 2 in 24-well culture plates coated with 1 μg/mL anti-pan-TCRγδ mAb. The HR8348 (human rectal carcinoma) cell line was obtained from the Cell Culture Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences. HR8348 cells were cultured in complete RPMI-1640 medium with 10% FBS.

Flow cytometric analysis

Cells were washed with PBS containing 1% bovine serum albumin (BSA) and incubated with surface-staining antibodies for 30 min at 4 ℃. The cells were then washed and resuspended in PBS. Cytometry data were acquired using a BD Accuri C6 flow cytometer (Becton Dickinson). Data analysis was carried out with FlowJo software (Tree Star Inc.).

CFSE proliferation assay

Naïve CD4 T cells were labeled with CFSE and used as the responder cells. The cells were cultured with Vδ1 T cells in the dark at a ratio of 1:2. After 5 d in culture, the cells were collected and washed twice with PBS containing 1% BSA. The cells were analyzed using a BD Accuri C6 flow cytometer (Becton Dickinson). Data analysis was performed using FlowJo software (Tree Star Inc.).

LDH assay

To determine specific cytotoxicity, we used the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) based on the calorimetric detection of the released enzyme lactate dehydrogenase (LDH). HR8348 and Vδ2 T cells were co-cultured at the ratios of 10:1, 20:1 and 30:1. Assays were performed in triplicate. After 6 h at 37 ℃, 50 μL supernatant was assayed for LDH activity following the manufacturer's protocol. Controls for spontaneous LDH release in effector and target cells, as well as target maximum release, were prepared. The percentage of cytotoxicity was calculated as follows:

%Cytotoxicity = ([Experimental - Effector spontaneous - Target spontaneous]/[Target maximum - Target spontaneous]) × 100

Statistical analysis

The results are expressed as mean ± SD. Data were analyzed by *t*-test or one-way analysis of variance (ANOVA) (SPSS version 16.0), followed by Tukey-Kramer multiple comparisons. In all analyses, the minimum acceptable level of significance was $P < 0.05$.

RESULTS

Percentage of Vδ1 and Vδ2 T cells in tumor tissue and para-carcinoma tissue from rectal cancer patients

We first compared the percentages of total γδ T cells and the Vδ1 and Vδ2 T subsets in tumor tissues and para-carcinoma tissues from rectal cancer patients. There was no significant difference in the percentage of total γδ T cells in the tumor tissues and para-carcinoma tissues of rectal cancer patients (4.32% ± 0.026% *vs* 4.30% ± 0.037%, $P > 0.05$) (Figure 1A). The percentage of Vδ1 T cells in tumor tissues was significantly greater than in para-carcinoma tissues (2.58% ± 0.017% *vs* 1.03% ± 0.008%, $P < 0.01$) (Figure 1B), and the percentage of Vδ2 T cells was significantly lower in tumor tissue than in para-carcinoma tissue (1.75% ± 0.012% *vs* 3.27% ± 0.032%, $P < 0.05$) (Figure 1C).

Correlation of Vδ1 and Vδ2 T cells with TNM stage in rectal cancer patients

The percentage of peripheral Vδ1 T cells in rectal cancer patients increased as T stage increased (Figure 2A), whereas the percentage of peripheral Vδ2 T cells decreased as T stage increased (Figure 2B). However, there was no significant correlation between N category or M category and the percentage of Vδ1 or Vδ2 T cells (data not shown).

Figure 1 Percentage of infiltrating γδT cells in 20 rectal cancer patients. Cells were stained with an anti-γδ TCR mAb, anti-Vδ1 mAb or anti-Vδ2 mAb and analyzed by flow cytometry. The left panels show representative histogram results from flow cytometry for γδ T cells (A), Vδ1 T cells (B), and Vδ2 T cells (C). The right panels show bar graphs of the percentage of positively stained cells from the patients.

Percentage of Vδ1 and Vδ2 T cells after 14 d amplification with anti-TCRγδ antibody

After culture in RPMI-1640 medium containing 10% FBS in 24-well culture plates coated with 1 μg/mL anti-TCR γδ antibody for 14 d, the percentage of Vδ1 T cells from para-carcinoma tissues was 21.45% ± 4.64%, and the percentage of Vδ2 T cells was 38.64% ± 8.05% (Figure 3A). After culture for 14 d, the percentage of Vδ1 T cells from rectal cancer tissues was 67.45% ± 11.75%, and the percentage of Vδ2 T cells was 8.94% ± 2.85% (Figure 3B).

Regulatory effects of tumor-infiltrating Vδ1 T cells and cytolytic activity of tumor-infiltrating Vδ2 T cells

The proliferation rate of naïve CD4T cells from the blood of rectal cancer patients was 80.23% ± 11.86%; when co-cultured with Vδ1 T cells from para-carcinoma

tissues, the proliferation rate was 53.45% ± 7.95%, and when co-cultured with Vδ1 T cells from rectal cancer tissues, the proliferation rate was 52.53% ± 8.52% (Figure 4A). The inhibitory effects of Vδ1 T cells from para-carcinoma tissues and from rectal cancer tissues were not significantly different (Figure 4B). In addition, the cytolytic activities of Vδ2 T cells from para-carcinoma tissues and from rectal cancer tissues were not significantly different (Figure 4C).

DISCUSSION

The major finding of this study is that the percentage of Vδ1 T cells in the rectal tumor tissues of rectal cancer patients significantly increased, and the percentage of Vδ2 T cells in the rectal tumor tissues of rectal cancer patients significantly decreased, when

Figure 2 Percentage of tumor-infiltrating Vδ1 and Vδ2 T cells correlated with disease T stage. A: Tumor-infiltrating Vδ1 T cells positively correlated with disease T stage; B: Tumor-infiltrating Vδ2 T cells negatively correlated with disease T stage.

Figure 3 Percentage of Vδ1 and Vδ2 T cells from cancer tissues or para-carcinoma tissues after expansion. Cells were stained with anti-CD3 mAb, anti-Vδ1 mAb or anti-Vδ2 mAb and analyzed by flow cytometry. The left panels show representative dot plots from flow cytometry for Vδ1 and Vδ2 T cells from para-carcinoma tissues (A) and rectal cancer tissues (B). The right panels show bar graphs of the percentage of positively stained cells from 20 rectal cancer patients.

compared with para-carcinoma tissues. γδ T cells can be divided into two subsets: Vδ1 T cells found in the epithelial-associated lymphoid tissue and Vδ2 T cells in the peripheral blood. Our results showed that after culture for 14 d with 1 μg/mL anti-TCR γδ antibody, the major subset from para-carcinoma tissues was Vδ1 T cells, and the major subset from rectal cancer tissues was Vδ2 T cells. Tumor-infiltrating Vδ1 T cells had strong inhibitory effects, and tumor-infiltrating Vδ2 T cells showed strong cytolytic activity. Although there were no significant differences in the cytolytic activities of Vδ2 T cells from para-carcinoma tissues

and from rectal cancer tissues, the predominant subset in rectal cancer tissues was Vδ1 T cells. Thus, tumors may limit antitumor immunity and evade immune surveillance in rectal cancer patients by forming an immunosuppressant microenvironment.

The MHC-independent antigen recognition and strong cytotoxicity to tumor cells make γδ T cells attractive candidate effector cells for cancer immunotherapy[19-25]. Administration of Vδ2 T cells at suitable intervals after chemotherapy and zoledronate may substantially increase antitumor activity in a range of malignancies[26], whereas tumor-infiltrating Vδ1 T

A

Figure 4 **Functional analysis of Vδ1 and Vδ2 T cells from cancer tissues or para-carcinoma tissues.** A: Fresh naïve CD4 T cells were labeled with CFSE and co-cultured with Vδ1 T cells from cancer tissues or para-carcinoma tissues; B: Lysis of HR8348 cells by Vδ2 T cells at effector to target cell ratios (E:T) of 10:1, 20:1 and 40:1.

cells mainly have an immunosuppressive function and promote cancer development[27,28]. The infiltration of γδ T cells in cancer tissues has been reported in some tumors[29-32]. However, to date, there has been no research on the percentages of Vδ1 and Vδ2 T cells in rectal cancer tissues. In this study, we used the FACS method to analyze the percentage of Vδ1 T cells and Vδ2 T cells in tumor tissues and para-carcinoma tissues

from 20 rectal cancer patients. The results showed that the percentage of Vδ1 T cells in the rectal tumor tissues of these patients was significantly increased and positively correlated with the T stage, whereas the percentage of Vδ2 T cells in the rectal tumor tissues was significantly decreased and negatively correlated with the T stage.

We also discovered that after culture for 14 d with

1 μg/mL anti-TCR γδ antibody, the major subset of γδ T cells from para-carcinoma tissues was Vδ1, and the major subset from rectal cancer tissues was Vδ2. This result is consistent with the predominant subset in rectal cancer tissues being Vδ1 T cells, and the predominant subset in para-carcinoma tissues being Vδ2 T cells, and with the findings of a previous study which showed that the major γδ T cells infiltrating breast cancer tissues were Vδ1 T cells[28]. Vδ2 T cells have a more inflammatory phenotype; Vδ1 T cells have a more regulatory phenotype and have been shown to express Foxp3[6]. Vδ2 T cells have a major cytotoxicity function, and have predominantly been investigated in tumor immunosurveillance and host defense against viral invasion[7-10]. Our results demonstrate that tumor-infiltrating Vδ1 T cells have strong inhibitory effects, and tumor-infiltrating Vδ2 T cells have strong cytolytic activity, consistent with previous studies on the function of Vδ1 T cells and Vδ2 T cells.

The findings in this study suggest that a percentage imbalance in Vδ1 and Vδ2 T cells creates an immunosuppressant microenvironment in rectal cancer tissues, which may enable tumors to limit antitumor immunity and evade immune surveillance in rectal cancer patients. This is the first report on the percentages of Vδ1 and Vδ2 T cells in rectal cancer tissues. We demonstrate that an imbalance in Vδ1 and Vδ2 T cell percentages in cancer tissues may facilitate the development of rectal cancer. The results of this study provide a new insight into immunotherapy for rectal cancer.

COMMENTS

Background

γδT cells can be divided into two subsets: Vδ1 T cells and Vδ2 T cells. Vδ1 T cells, which have been shown to express Foxp3, have a regulatory function. Vδ2 T cells have largely been investigated in the context of tumor immunosurveillance and host defense against viral invasion. An imbalance of Vδ1 and Vδ2 T cell percentages in cancer tissues may facilitate the development of rectal cancer.

Research frontiers

γδT cells have been shown to be useful in cancer immunotherapy. They can be divided into two subsets: Vδ1 and Vδ2. Administration of Vδ2 T cells at suitable intervals after chemotherapy and zoledronate may substantially increase antitumor activity in a range of malignancies, whereas tumor-infiltrating Vδ1 T cells mainly have an immune repression function and promote cancer development. However, there has been no research on the percentages of Vδ1 and Vδ2 T cells in rectal cancer tissues.

Innovations and breakthroughs

This is the first study to report the percentages of Vδ1 and Vδ2 T cells in rectal cancer tissues. The authors demonstrate that an imbalance in the percentages of Vδ1 and Vδ2 T cells in cancer tissues may facilitate the development of rectal cancer.

Applications

This study provides new insight into immunotherapy for rectal cancer.

Peer-review

This study is meaningful and the findings that the imbalance of Vδ1 and Vδ2 T cell percentages in rectal cancer tissues are interesting, and provide new insight into immunotherapy for rectal cancer.

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ORIGINAL ARTICLE

Case Control Study

Serum vitamin D and colonic vitamin D receptor in inflammatory bowel disease

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Abstract

AIM: To determine serum vitamin D levels and colonic vitamin D receptor (VDR) expression in inflammatory bowel disease (IBD) and non-IBD patients and correlate these with histopathology.

METHODS: Puerto Rican IBD (n = 10) and non-

IBD (n = 10) patients $\geqslant$ 21 years old scheduled for colonoscopy were recruited. Each patient completed a questionnaire and provided a serum sample and a colonic biopsy of normal-appearing mucosa. For IBD patients, an additional biopsy was collected from visually diseased mucosa. Serum vitamin D levels were measured by ultra-performance liquid chromatography and mass spectrometry. Hematoxylin and eosin stained tissue sections from colonic biopsies were classified histologically as normal or colitis (active/inactive), and scored for the degree of inflammation present (0-3, inactive/absent to severe). Tissue sections from colonic biopsies were also stained by immunohistochemistry for VDR, for which representative diagnostic areas were photographed and scored for staining intensity using a 4-point scale.

RESULTS: The IBD cohort was significantly younger (40.40 ± 5.27, $P < 0.05$) than the non-IBD cohort (56.70 ± 1.64) with a higher prevalence of vitamin D deficiency (40% vs 20%, respectively) and insufficiency (70% vs 50%, respectively). Histologic inflammation was significantly higher in visually diseased mucosa from IBD patients (1.95 ± 0.25) than in normal-appearing mucosa from control patients (0.25 ± 0.08, $P < 0.01$) and from IBD patients (0.65 ± 0.36, $P < 0.05$) and correlated inversely with VDR expression in visually diseased colonic tissue from IBD patients (r = -0.44, $P < 0.05$) and from IBD patients with Crohn's disease (r = -0.69, $P < 0.05$), but not in normal-appearing colonic tissue from control patients or IBD patients. Control and IBD patient serum vitamin D levels correlated positively with VDR expression in normal colon from control and IBD patients (r = 0.38, $P < 0.05$) and with patient age (r = 0.54, $P < 0.01$).

CONCLUSION: Levels of serum vitamin D correlate positively with colonic VDR expression in visually normal mucosa whereas inflammation correlates negatively with colonic VDR expression in visually diseased mucosa in Puerto Rican patients.

Key words: Colitis; Inflammation; Vitamin D; Vitamin D receptor; Inflammatory bowel disease

Core tip: Our study examines for the first time the relationship between serum vitamin D levels, colonic vitamin D receptor (VDR) expression, and histologic disease activity. We show in Puerto Rican patients that colonic VDR expression and inflammation are negatively correlated in endoscopically and histologically diseased colon and that serum vitamin D levels positively correlate with VDR expression in endoscopically and histologically normal colon. These findings contribute to our understanding of the role of vitamin D and VDR in patients with inflammatory bowel disease and could affect the current care of these patients.

INTRODUCTION

Although the incidence and prevalence of inflammatory bowel disease (IBD) is stable in first world countries, IBD is becoming more frequent in other geographic areas and in minority populations such as blacks and Hispanics, where they were rarely seen[1]. Puerto Rico is no exception, and the rate at which ulcerative colitis and Crohn's disease are diagnosed is increasing[2,3]. Vendrell et al[4] found that the prevalence of IBD among insured Puerto Ricans in 2005 is among the highest described for Hispanic populations. Vitamin D deficiency is common among patients with IBD, yet evaluation of vitamin D status is not currently considered standard of care in these patients, and guidelines for treating vitamin D deficiency in these patients are nonexistent[5]. Increasing evidence suggests that vitamin D is an environmental factor linked to the pathogenesis and severity of IBD, including Crohn's disease and ulcerative colitis[6,7].

Vitamin D is a hormone precursor present in two forms: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Cholecalciferol is synthesized in the skin upon sunlight exposure and is subsequently converted to 25-hydroxyvitamin D, the major inactive circulating vitamin D metabolite. 25-hydroxyvitamin D has a long half-life of about 3 wk, making it the best biomarker to evaluate vitamin D status[8,9]. The active form of vitamin D, $1,25(OH)_2D_3$ (calcitriol), exerts its biological functions *via* the vitamin D receptor (VDR), a member of a superfamily of nuclear hormone receptors. One of the major roles of vitamin D and VDR is to regulate intestinal absorption and serum levels of calcium and phosphate. Many tissues and cells in the body, including immune cells and colonocytes, express VDR and possess the enzymes necessary to produce local $1,25(OH)_2D_3$[6]. The main source of local $1,25(OH)_2D_3$ is the intestinal autocrine/paracrine vitamin D system, which plays a critical role in maintaining both mucosal immunity and normal growth of epithelial cells[9]. Vitamin D-VDR signaling results in anti-inflammatory, immune-modulating, anti-mitotic, pro-differentiating, and pro-apoptotic effects, which are attributable to the hundreds of genes that contain vitamin D responsive elements (VDRE)[9-11]. These properties are critical in the context of immune diseases, such as IBD, and in cancer prevention; however, the mechanisms by which vitamin D exerts these properties are not well understood.

Importantly, the mechanisms by which circulating vitamin D levels regulate colonic VDR expression in homeostasis and disease states are also incompletely understood. Studies in bone cells have revealed that the *VDR* gene contains VDRE, suggesting that vitamin D can increase colonic VDR expression[12]. However, treatment of Caco-2 colon cancer cells with calcitriol resulted in increased transcript levels of CYP24A1, the major 1,25(OH)2D3 inactivating enzyme[13], and xenografts of HT29 colon cancer cells overexpressing CYP24A1 are more proliferative and invasive[14]. Additionally, SNAIL1 has been shown to repress VDR expression in colon cancer cells[15-17]. Any situation that impairs the 1,25(OH)$_2$D$_3$/VDR system at the intestinal mucosal level, such as vitamin D deficiency or increased *CYP24A1* or *SNAIL* expression, may increase risk for development or progression of IBD and colorectal cancer (CRC)[9], one of the most severe complications for patients with long-standing IBD. In fact, IBD colitis patients are six times more likely to develop CRC than the general population[9], and this risk increases with disease severity, duration, and extent[18]. Interestingly, we recently found differential expression of VDR in Puerto Rican patients with colitis-associated and sporadic colorectal neoplasia, with a significant decrease in VDR expression in sporadic dysplasia and CRC compared with normal and colitis-associated CRC tissue[19].

Recent animal studies have demonstrated that vitamin D supplementation ameliorates, whereas vitamin D or VDR deficiency worsens, inflammation in murine models of IBD[20,21], providing strong evidence for vitamin D as an anti-inflammatory immuno-modulator in IBD. Furthermore, *in vivo* and *in vitro* studies have shown that combining vitamin D with steroids, immunomodulators, or biologics produces synergistic effects[22-24]. In vitro studies have suggested that VDR plays a critical role in preserving intestinal mucosal barrier integrity[10,25]. It has also been demonstrated that 1,25(OH)$_2$D$_3$ signaling through VDR is a direct and important inducer of *NOD2* expression[26]. A key downstream signaling consequence of NOD2 activation by the agonist muramyl dipeptide is stimulation of NF-κB transcription factor function, which induces expression of the gene encoding defensin β2 (*DEFβ2/HBD2*), a gut antimicrobial peptide that plays an important role in the mucosal immune barrier and whose deficiency has been linked to colonic Crohn's disease[27].

There is a paucity of data on the role of vitamin D in IBD disease activity preventing firm conclusions regarding its use as a predictor of disease severity. Nevertheless, Joseph et al found that 79% of patients with Crohn's disease in India were vitamin D deficient and that the clinical disease activity correlated negatively with serum 25-hydroxyvitamin D levels[28]. Ulitsky et al. reported a high rate (49.8%) of vitamin D deficiency in a cohort of 504 IBD patients in the north-central United States and an independent association of this deficiency with lower quality of life and greater disease activity of Crohn's disease[29]. There is also some evidence that vitamin D supplementation may help in maintaining remission in patients with Crohn's disease[30].

In light of the protective and regulatory effects that vitamin D has on the colonic epithelial barrier and immune system, respectively, we postulate that patients with IBD who have low levels of vitamin D are susceptible to progression to severe disease and perpetuation of chronic mucosal inflammation, proven risk factors for CRC in these patients. Elucidating the role of vitamin D and VDR in patients with IBD could therefore affect the current care of these patients. In the present study, we examine the relationship between serum vitamin D levels, colonic VDR expression, and histologic disease activity. To our knowledge, this relationship has not previously been studied in either a Puerto Rican or any other IBD population.

MATERIALS AND METHODS

Use of human subjects and internal review board approval
This study was carried out in compliance with all NIH regulations concerning the Protection of Human Subjects. The study was approved by the University of Puerto Rico Medical Sciences Campus Institutional Review Board under protocol number 1250313.

Patients and collection of samples
Puerto Rican patients (both sexes) over 21 years old with a documented diagnosis of ulcerative colitis or Crohn's disease by standard endoscopic, histologic, radiologic, and/or clinical criteria who were seen at the University of Puerto Rico Center for IBD and were undergoing colonoscopy and biopsy for clinical indications were offered participation in this study. Patients were informed about the purpose of the study, benefits and risks involved, and their rights and confidentiality. Patients over 21 years old undergoing a colonoscopy for other reasons (but not due to suspected IBD, chronic diarrhea, malabsorption syndrome, or malignancy) were recruited as controls. Patients or controls who had used vitamin D, vitamin D plus calcium, or multivitamin supplementation during the previous 3 mo were excluded from study participation. Patients who agreed to participate signed the IRB-approved informed consent form and completed a questionnaire to gather demographic, lifestyle, and disease-related information. Our study included 10 controls and 10 patients with IBD (Table 1, also see Table 2 for additional disease and demographic information on individual participants). Two colon biopsies were collected from IBD patients, one from visually diseased mucosa and one from

	Control (*n* = 10)	IBD (*n* = 10)
Sex		
Male	4	6
Female	6	4
Mean age (yr)	56.70 ± 1.64	40.40 ± 5.27[a]
IBD diagnosis		
CD		7
UC		3

[a]*P* < 0.05. CD: Crohn's disease; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

normal appearing mucosa. Controls provided one colonic biopsy, which was subsequently confirmed as histologically normal. A serum blood sample was collected from all patients and stored at -80 ℃ until time of analysis.

Histological analysis
Tissue sections (2-4 μm) were stained with hematoxylin and eosin and analyzed independently by two pathologists (CIG and AAI). All biopsies were classified histologically as normal, inactive colitis, or active colitis, based on Geboes *et al*[31]. The degree of inflammation for each biopsy site was recorded as follows: 0 = inactive/absent, 1 = mild, 2 = moderate, or 3 = severe.

Vitamin D levels
Serum samples were analyzed for 25-hydroxyvitamin D levels by ultra-performance liquid chromatography and mass spectrometry at the De Diego Research Foundation Bioanalytical Laboratory (Agilent; detection limit 7.5 ng/mL). Samples were classified as deficient (< 20 ng/mL) or not deficient (≥ 20 ng/mL) and as sufficient (≥ 30 ng/mL) or insufficient (< 30 ng/mL).

Vitamin D receptor immunohistochemical staining
Tissue sections were stained for VDR by immunohistochemistry, and VDR expression was scored as previously described[19]. Briefly, formalin-fixed, paraffin-embedded tissue sections mounted on charged glass slides were deparaffinized with Hemo-De xylene-substitute and rehydrated with graded ethanol dilutions and distilled water. Endogenous peroxidase activity was quenched with hydrogen peroxide (3%, aqueous). Heat-induced antigen retrieval was performed using citrate-EDTA buffer (10 mmol/L; 2 mmol/L EDTA, 0.05% Tween 20, pH 6.2) at 95 ℃ to 99 ℃ for 40 min and at room temperature for 20 min. After samples were blocked with normal goat serum (Biogenex, San Ramon, CA), tissues were incubated overnight with the anti-VDR antibody (Ab3508; Abcam Inc, dilution 1:2000). Secondary detection of the primary antibody was achieved using the peroxidase-based Super Sensitive Link-Label IHC Detection System

(Biogenex) and 3,3-diaminobenzidine chromogen solution (Biogenex). Nuclei were counterstained with hematoxylin before dehydration, clearing, and coverslipping. Representative areas containing the diagnosis of interest (normal, inactive colitis, active colitis) were photographed and scored in triplicate by observers blinded to the patients' diagnoses. Staining intensity was scored using a four-point scale (0-3), where 0 indicates the weakest staining and 3 indicates the strongest staining[19].

Statistical analysis
The statistical significance of differences in inflammation scores and VDR expression between control mucosa, normal-appearing mucosa from IBD patients, and visually diseased mucosa from IBD patients was determined using the Kruskal-Wallis test followed by the Dunn multiple comparisons test. Differences in age and serum 25-hydroxyvitamin D levels between control and IBD patients were assessed using the Mann-Whitney test. Spearman correlation coefficient was used for analyzing correlations between VDR expression, inflammation scores, serum 25-hydroxyvitamin D levels, and age. All statistical analyses were performed with GraphPad Prism v6.0a (GraphPad Software, San Diego, CA, United States). Data are presented as mean ± SE, and statistical significance was established at *P* < 0.05.

RESULTS
The control and IBD groups had similar numbers of patients from both sexes; however, as might be expected, IBD patients were significantly younger than controls (*P* < 0.05, Table 1). Most IBD patients (7/10) had a diagnosis of Crohn's disease.

More IBD patients have vitamin D insufficiency and deficiency than control patients
Mean serum 25-hydroxyvitamin D levels were not significantly or substantially different between control (27.61 ± 3.36 ng/mL) and IBD patients (25.18 ± 2.36 ng/mL; Figure 1A). Nevertheless, twice as many IBD patients (4/10) had vitamin D deficiency (< 20 ng/mL) compared to controls (2/10; Figure 1B). Furthermore, a higher percentage of IBD patients (7/10) had insufficient levels of vitamin D (< 30 ng/mL) compared to controls (5/10; Figure 1C), half of which had vitamin D insufficiency. Serum vitamin D levels did not significantly correlate with colonic inflammation scores.

Inflammation scores, but not VDR expression levels, are increased in diseased colon from IBD patients
As expected, visually diseased mucosa from IBD patients had significantly higher inflammation scores (1.95 ± 0.25) than normal-appearing mucosa from IBD (0.65 ± 0.36, *P* < 0.05) or controls (0.25 ± 0.08, *P* < 0.01; Figure 2A). Inflammation scores were consistently

Table 2 Demographic and disease information for control and inflammatory bowel disease patients

Patient ID	Age (yr)	Sex	Diagnosis	IBD duration (yr)	Medications	Colonoscopy findings
UC1	63	F	Ulcerative colitis	15	Mesalamine, Azathioprine	Proctosigmoiditis
CD1	29	F	Crohn's disease	2	Adalimumab	Active mild colitis
CD2	41	M	Crohn's disease[1]	16	Enalapril	Active Crohn's disease at ileocolonic anastomosis
UC2	25	M	Ulcerative colitis	2	Sulfazalazine	Mild proctosigmoiditis
CD3	23	M	Crohn's disease	3	Adalimumab, Azathioprine, folic acid	Active Crohn's disease with ileocecal valve involvement
CD4	55	M	Crohn's disease[1]	10	Mesalamine	Crohn's ileocolitis with small external hemorrhoids
CD5	38	M	Crohn's disease[1]	8	Infliximab	Active disease at ileocolonic anastomosis
CD6	32	F	Crohn's disease[1]	8	Infliximab	Mild sigmoiditis with internal/ external hemorrhoids
CD7	28	M	Crohn's disease[1]	2	Adalimumab	Friable ileocecal valve anastomosis with small aphtous ulcer; mild distal proctitis
UC3	70	F	Ulcerative colitis	8	Mesalamine	Mild proctitis
C1	50	F	HTN, dyslipidemia, DM-2	-	Irbesartan, Simvastatine, Glipizide, Insulin, Clonazepam	Normal study
C2	55	F	HTN	-	Losartan	Single diminutive sessile polyp
C3	67	M	DM-2, post-liver transplant secondary to ETOH	-	Folic acid, Glipizide, Tacrolimus	Hemorrhoids internal/external
C4	54	M	HIV	-	Efavirnez, Tenofovir, Lamivudine	External hemorrhoids
C5	63	M	HTN, hypothyroidism, CKD, post-liver transplant secondary to ETOH	-	Tacrolimus, Propranolol, Pepcid, Simvastatin	Normal study
C6	57	F	Hypothyroidism, GERD	-	Ranitidine, Omeprazole, Levothyroxine	
C7	55	M	HTN, dyslipidemia, anxiety, depression	-	Losartan, Clopidrogel, Amlodipine, Simvastatin, Clonazepam, Trazodone, Venlafaxine	Two polyps < 1 cm, both tubular adenomas
C8	59	F	Hypothyroidism, depression, anxiety	-	Levothyroxine, Gabapentin, Sertraline, Clonazepam, Estazolam	Moderate pandiverticulosis
C9	56	F	DM-2, dyslipidemia, NASH	-	Simvastatin, Aspirin, Gliburide	Normal sigmoidoscopy/normal virtual colonoscopy
C10	51	F	None reported	-	none	Two small sessile polyps-hyperplastic polyps; left side diverticulosis

[1]IBD-related surgery. F: Female; M: Male; HTN: Hypertension; DM-2: Type 2 diabetes mellitus; HIV: Human immunodeficiency virus; CKD: Chronic kidney disease; GERD: Gastroesophageal reflux disease; NASH: Nonalcoholic steatohepatitis; IBD: Inflammatory bowel disease.

higher in visually diseased than in normal appearing mucosa for all but two IBD patients. Statistically significant differences in VDR immunohistochemistry scores were not observed in comparisons between control mucosa (2.03 ± 0.20), normal appearing mucosa from IBD patients (1.93 ± 0.24), and visually diseased mucosa from IBD patients (2.30 ± 0.21; Figure 2B). VDR immunostaining was mainly found within glandular epithelial cells and lamina propria cells and was observed in both the nuclear and the cytoplasmic compartments.

VDR expression negatively correlates with inflammatory status in the colon of IBD patients
VDR immunohistochemistry and inflammation scores did not correlate significantly in colonic tissue from controls ($r = -0.36$, $P > 0.05$; Figure 3A), but

correlated inversely in visually diseased tissue from IBD patients ($r = -0.44$, $P < 0.05$, Figure 3B). This inverse correlation was stronger in IBD patients who had Crohn's disease ($r = -0.69$, $P < 0.05$; Figure 3C). Statistically significant correlations between VDR immunohistochemistry and inflammation scores were not observed for normal-appearing colonic tissue from IBD patients.

VDR expression in normal appearing mucosa from control and IBD patients positively correlates with serum vitamin D levels
Colonic VDR immunohistochemistry scores did not correlate with serum vitamin D levels in control and IBD patients when VDR immunohistochemistry scores from visually diseased mucosa from IBD patients were used ($r = 0.13$, $P > 0.05$; Figure 4A). When VDR

Figure 1 Vitamin D status in control patients and patients with inflammatory bowel disease. A: Serum levels of 25-hydroxyvitamin D3 in control and inflammatory bowel disease (IBD) patients (*n* = 10 patients/group). B: Proportion of control and IBD patients with vitamin D deficiency (< 20 ng/mL). C: Proportion of control and IBD patients with vitamin D insufficiency (< 30 ng/mL).

immunohistochemistry scores from normal-appearing mucosa from IBD patients were used, colonic VDR immunohistochemistry scores positively correlated with serum vitamin D levels in controls and IBD patients (*r* = 0.38, *P* < 0.05; Figure 4B).

Colonic inflammatory status negatively correlates with patient age

Colonic inflammation scores were inversely correlated with patient age in both control and IBD patients when inflammation scores from visually diseased mucosa were used for patients with IBD (*r* = -0.57, *P* < 0.01; Figure 5).

Serum vitamin D levels positively correlate with patient age

Serum vitamin D levels correlated positively with patient age in IBD patients alone (*r* = 0.81, *P* < 0.01; Figure 6A) and in control and IBD patients (*r* = 0.53, *P* < 0.01; Figure 6B).

DISCUSSION

In the present study, we describe vitamin D status in IBD and non-IBD patients (controls) from Puerto Rico and examine how this relates to colonic inflammation and VDR expression. The prevalence of vitamin D deficiency determined for our Puerto Rican IBD cohort was 40% compared with a prevalence of 20% for the control group. Interestingly, although IBD patients have lower vitamin D levels, we found that both Hispanic cohorts have vitamin D insufficiency, despite living on a sunny tropical island. Vitamin D deficiency has been shown to be fairly common in the United States (41.6%), especially among blacks (82.1%) and Hispanics (69.2%)[32]. In a genome-wide association study examining 25-hydroxyvitamin D concentrations in 33996 individuals of European descent, Wang et al. found that variants near genes involved in cholesterol synthesis, hydroxylation, and vitamin D transport affect vitamin D status[33]. They concluded that these genetic variations identify individuals who have a substantially raised risk of vitamin D insufficiency. It is therefore possible that environmental factors as well as genetic polymorphisms might be affecting vitamin D metabolism in our study population.

Although the expression of VDR has been reported to be decreased in patients with ulcerative colitis, dysplasia, and colitis-associated CRC[34], we have previously shown that in Puerto Rican patients there is a significant decrease in colonic VDR expression in sporadic dysplasia and CRC but not in colitis-associated cancer or in IBD, when compared to normal tissue[19]. In the present study, no significant differences were found in VDR expression between cohorts, which is consistent with our previous results, or between sources of colonic samples in IBD patients, despite finding significantly higher inflammation scores in visually diseased mucosa when compared to normal-appearing mucosa from IBD patients. Although $1,25(OH)_2D_3$ is thought to regulate VDR expression in target tissues[35], the mechanism for regulation of colonic VDR expression in relation to vitamin D is currently unknown (*i.e.*, positive or negative feedback).

In our study, decreased colonic VDR expression was found to be associated with higher histologic inflammation scores, which reflect disease activity, in diseased mucosa. Our study also found a positive

Figure 2 Colonic inflammatory and vitamin D receptor status in control and inflammatory bowel disease patients. Inflammation score (A) and VDR expression (B) for colonic mucosa from control patients, normal appearing mucosa from IBD patients, and visually diseased mucosa from IBD patients (n = 10 per group). Each IBD patient contributed a biopsy from normal appearing mucosa and another biopsy from visually diseased mucosa. VDR: Vitamin D receptor; IBD: Inflammatory bowel disease.

correlation between VDR scores from normal appearing mucosa (controls and IBD patients) and serum vitamin D levels but not with VDR scores

from mucosa that appeared diseased, suggesting the following points. First, colonic VDR expression is regulated *via* a positive-feedback mechanism. Second,

Figure 3 Correlation between inflammation and vitamin D receptor. Immunohistochemistry (IHC) scores for vitamin D receptor and inflammation scores in colonic mucosa from control patients (A), inflammatory bowel disease (IBD) patients (B), and patients with Crohn's disease (CD) (C). In B and C, inflammation and VDR scores from visually diseased mucosa were used for IBD and CD patients (red circles).

Figure 4 Correlation between serum 25-hydroxyvitamin D3 levels and vitamin D receptor immunohistochemistry scores in control and inflammatory bowel disease patients. A: VDR scores from visually diseased mucosa were used for IBD patients (red circles); B: VDR scores from normal appearing mucosa were used for IBD patients (pink circles). VDR: Vitamin D receptor; CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; IHC: Immunohistochemistry.

the disease process disrupts the vitamin D-VDR system in diseased areas of the colon. These points concur with those reported by Ham *et al*[36], who found lower serum vitamin D levels in patients with active IBD than in patients in remission. Low serum vitamin D levels may truly reflect insufficiency/deficiency or could result from 25-hydroxyvitamin D being converted to the active form of vitamin D_3. Moreover, differences in IBD presentation between Hispanics and

non-Hispanic whites have been reported[37]; however, there is a paucity of data on the disease course in these populations. Similarly, the metabolism of vitamin D may differ among ethnic groups, where other environmental factors, such as geographical location, and comorbidities, such as obesity, could influence the prevalence of vitamin D deficiency[38]. In addition to the intricacies of vitamin D pathways, the amount of active vitamin D necessary at a molecular level to exert its diverse properties in the intestines, especially in regard to patients with IBD, is unknown.

Potential limitations of this study include the small sample size and the fact that patients in the control group were older than IBD patients, which prevented age matching. This age difference between the groups is likely responsible for the correlation between age and colonic inflammation that we observed in this study. It is unknown how the age of the patient, comorbidities, and current medication use alter the mechanisms of vitamin D in the body. Interestingly, our findings suggest that age directly correlates with higher serum levels of vitamin D in IBD patients, but

Figure 5 Correlation between age and inflammation scores for control and inflammatory bowel disease patients. A: inflammation scores from visually diseased mucosa were used for IBD patients (red circles); B: inflammation scores from both normal appearing mucosa (pink circles) and visually diseased mucosa (red circles) were used for IBD patients. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Figure 6 Correlation between age and serum 25-hydroxyvitamin D₃ levels for inflammatory bowel disease patients (A) and all patients (B).

conditions, decrease the risk of developing CRC, and improve quality of life.

other studies suggest a decline in vitamin D levels with aging due to decreased absorption. Our control group was found to have vitamin D insufficiency and not sufficiency as expected; therefore, it was not a suitable "control cohort" for the purposes of this study.

In conclusion, the natural history of IBD in Puerto Ricans and Hispanics in general warrants further investigation. We previously found that the incidence and prevalence of this disease were increased in our population, with a particular aggressive disease behavior among younger patients[3,4]. Our current investigation showed a positive correlation between serum vitamin D levels and VDR expression in normal appearing colonic mucosa and a negative correlation between VDR expression and inflammation in diseased mucosa, particularly among patients with Crohn's disease from the IBD cohort. Future studies should examine whether these relationships also exist in other populations, Hispanic or otherwise. The identification of potentially correctible risk factors, such as vitamin D deficiency, may result in disease modification strategies that reduce the severity of these chronic

COMMENTS

Background
Increasing evidence suggests that vitamin D is an environmental factor linked to the pathogenesis and severity of inflammatory bowel disease (IBD). Of concern, patients with IBD are more likely to develop colorectal cancer than the general population, and low levels of vitamin D are associated with an elevated risk of this malignancy.

Research frontiers
The relationship between serum vitamin D levels and colonic vitamin D receptor (VDR) expression remains unclear. Furthermore, the effect of inflammation or

disease activity on this relationship is incompletely understood.

Innovations and breakthroughs

To the authors knowledge, the relationship between serum vitamin D levels, colonic VDR expression, and histologic disease activity has not previously been studied in either a Puerto Rican or any other IBD population. The authors show, for the first time, that in normal mucosa serum vitamin D levels correlate with colonic VDR expression and that in diseased IBD mucosa VDR expression negatively correlates with inflammation. Curiously, they also show that serum vitamin D levels correlate with age in IBD patients from Puerto Rico.

Applications

This study suggests that vitamin D insufficiency and deficiency could be a potentially correctible risk factor in the Puerto Rican population. Targeting the vitamin D-VDR axis together with disease modification strategies could reduce the severity of these chronic conditions, decrease the risk of developing CRC, and improve quality of life. Additionally, colonic VDR expression in conjunction with histological analysis could serve the dual role of indicating relative vitamin D status in normal mucosa and disease activity in mucosa affected by IBD.

Terminology

IBD is a chronic and debilitating condition of the intestines that manifests as Crohn's disease or ulcerative colitis. Crohn's disease can affect any part of the gastrointestinal tract, including the colon or large intestine, in a discontinuous manner, whereas ulcerative colitis usually affects the colon in a continuous manner, starting at the rectum and spreading proximally. Cholecalciferol, or vitamin D, is a lipid-soluble vitamin that is involved in regulating calcium and phosphate balance and in many other processes. The major inactive, circulating metabolite of vitamin D is 25-hydroxyvitamin D, and the active form of vitamin D is known as calcitriol, or 1,25-dihydroxyvitamin D. The effects of calcitriol are mediated by the vitamin D receptor (VDR), a nuclear hormone receptor.

Peer-review

This work focused on a small group of subjects with and without IBD, and elucidated relationships between gut inflammation, vitamin D and VDR staining, and the study raises some interesting findings that are worthy of publication/circulation.

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ORIGINAL ARTICLE

Case Control Study

Relationship between indoleamine 2,3-dioxygenase activity and lymphatic invasion propensity of colorectal carcinoma

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Abstract

AIM: To evaluate whether serum and tumor indoleamine 2,3-dioxygenase activities can predict lymphatic invasion (LI) or lymph node metastasis in colorectal carcinoma.

METHODS: The study group consisted of 44 colorectal carcinoma patients. The patients were re-grouped according to the presence or absence of LI and lymph node metastasis. Forty-three cancer-free subjects without any metabolic disturbances were included into the control group. Serum neopterin was measured by enzyme linked immunosorbent assay. Urinary neopterin and biopterin, serum tryptophan (Trp) and kynurenine (Kyn) concentrations of all patients were determined by high performance liquid chromatography. Kyn/Trp was calculated and its correlation with serum neopterin was determined to estimate the serum indoleamine 2,3-dioxygenase activity. Tissue sections from the studied tumors were re-examined histopathologically

and were stained by immunohistochemistry with indoleamine-2,3-dioxygenase antibodies.

RESULTS: Neither serum nor urinary neopterin was significantly different between the patient and control groups (both $P > 0.05$). However, colorectal carcinoma patients showed a significant positive correlation between the serum neopterin levels and Kyn/Trp ($r = 0.450$, $P < 0.01$). Urinary biopterin was significantly higher in cancer cases ($P < 0.05$). Serum Kyn/Trp was significantly higher in colorectal carcinoma patients ($P < 0.01$). Lymphatic invasion was present in 23 of 44 patients, of which only 12 patients had lymph node metastasis. Eleven patients with LI had no lymph node metastasis. Indoleamine-2,3-dioxygenase intensity score was significantly higher in LI positive cancer group ($44.56\% \pm 6.11\%$) than negative colorectal cancer patients ($24.04\% \pm 6.90\%$), ($P < 0.05$). Indoleamine 2,3-dioxygenase expression correlated both with the presence of LI and lymph node metastasis ($P < 0.01$ and $P < 0.05$, respectively). A significant difference between the accuracy of diagnosis by using either total indoleamine-2,3-dioxygenase immunostaining score or of lymph node metastasis was found during the evaluation of cancer patients.

CONCLUSION: Indoleamine-2,3-dioxygenase expression may predict the presence of unrecognized LI and lymph node metastasis and may be included in the histopathological evaluation of colorectal carcinoma cases.

Key words: Colorectal carcinoma; Tryptophan; Indoleamine-2,3-dioxygen; Lymphovascular invasion; Lymph node metastasis

Core tip: Colorectal cancer (CRC) is one of the major public health problems in the world. Clinicopathological findings of patients who died of recurrent CRC after resection revealed that approximately 14% of patients with lymph node negative CRC die because of the presence of unrecognizable tumor cells that are categorized as micrometastases. Tryptophan degrading enzyme, indoleamine-2,3-dioxygenase expression by tumor cells has been shown to be correlated with a poor clinical prognosis of colon cancer. Our data indicated that high total indoleamine-2,3-dioxygenase immunostaining score is a strong predictor for immune tolerance, lymphatic invasion and subsequent lymph node metastasis. Therefore, indoleamine-2,3-dioxygenase immunostaining might be recommended for histopathological evaluation of CRC cases.

INTRODUCTION

Colorectal cancer (CRC) is the foremost one among the major public health problems in the world. Its incidence is approximately 7 per 100000, with approximately 5000 new cases and 3200 deaths annually[1]. Staging of CRC has prognostic value in terms of taking decisions about adjuvant therapy and follow up. American Joint Committee on Cancer (AJCC), the TNM staging system[2] places patients into one of four stages regarding tumor size and invasion depth of bowel wall, lymph node metastasis and distant metastasis. However current prognostic criteria have been debated for their precision in stratification of patients according to these rules. The character of the invasive margin and the number of metastatic lymph nodes are already accepted as more reliable prognostic criteria in CRC[3]. Recent studies demonstrated that significant risk factors for lymph node metastasis are level of submucosal spreading and the presence of lymphovascular invasion[4,5]. Clinicopathological findings of patients who died of recurrent CRC after resection revealed that the mean survival time has been significantly influenced by the histological grade of tumor, the depth of wall invasion, the presence of lymphatic or vascular invasion and the lymph node metastasis[6].

It has been asserted that despite having removed whole macroscopic disease with curative intent, one of five CRC patients with stages one or two would develop recurrence[7]. Likewise, approximately 14% of patients with lymph node negative CRC die of tumor recurrence, which can be related to the presence of unrecognizable tumor cells that are categorized as micrometastases[8]. Micrometastases could not be detected by conventional histopathologic analysis and none of the clinicopathologic parameters examined is correlated with the presence of occult cancer cells in lymph nodes[8]. On the other hand, failure to examine enough lymph nodes may result in a failure to identify patients whose lymph nodes are affected by cancer and thus may result in mislabeling[9]. The use of techniques to increase the number of lymph nodes harvested and identify all micrometastases is a subject of debate since 1998[10]. The ultra-staging of colon cancer leads to detection of occult metastases in patient's deemed node negative by conventional techniques of pathologic staging[11]. Nevertheless, because ultra staging involves extensive nodal sectioning and immunohistochemistry, this method remains investigational rather than routine examination[11]. However, according to others, the

presence of micrometastases in patients with stages one and two CRC seems not to have any impact on cancer-specific survival[7,12,13].

These evidences reflect the unusual biologic behavior of the tumor and/or host. Therefore, other cellular criteria should be included in the evaluation of patients' outcome. The type, density, and expression of immunomodulator substances of immune and/or tumor cells in CRC have an important prognostic value that may be superior to and independent of those of the cancer-staging systems[14]. Moreover, the pathologic interactions between tumor and host immune cells within the tumor microenvironment create an immunosuppressive network that promotes tumor growth and protects the tumor from immune attack[15]. In body fluids, information about T helper cell 1 (Th1)-derived cellular immune activation can be provided by neopterin, which is mainly synthesized by the activated monocytes/macrophages in response to induction by interferon-gamma (IFN-gamma)[16]. IFN-gamma selectively stimulates the early steps of pteridine biosynthesis in macrophages, thereby leading to accumulation and excretion of dihydroneopterin and neopterin[17]. Actually neopterin is a by-product in the biopterin biosynthesis[18]. Chronic stimulation of Th1-mediated immunity may also cause enhanced indoelamine 2,3-dioxygenase (IDO) activity in malignant diseases[19]. In cancer patients, significantly accelerated degradation of tryptophan (Trp) due to higher IDO activity with lowered serum concentrations of Trp and increased kynurenine (Kyn) as well as an increased Kyn to Trp ratio has been recognized[20,21]. It appears that decreased Trp levels or increased concentrations of its degradation products may be directly involved in diminished T-cell responsiveness[19]. This phenomenon could be best explained by IDO expression within the tumors[15]. The enzyme IDO has recently attracted special attention[22] and may be expressed constitutively by tumor cells as part of the genetic changes involved in malignant transformation[23,24]. IDO-expressing cells have been considered to create a state of immunologic unresponsiveness towards tumor-derived antigens[25]. Although IDO expression by tumor cells has been shown to correlate with a poor clinical prognosis of colon cancer[15], assessment of tumor IDO activity has not took place currently in routine histopathological evaluation concept of CRCs. "Is the evaluation of IDO activity of tumor tissue an effective method to predict the invasiveness of tumor cells?" This is a rational question considering the host immune tolerance against CRC cells. In this respect, estimation of total tumor tissue IDO immunostaining score during the routine histopathological examination of CRC specimens appears to be an effective strategy to predict the suppression of anti-tumor immune response and the enhancement of tumor invasion.

Aim of this study was to evaluate whether serum and tumor IDO activities can predict the lymphatic invasion or lymph node metastasis propensity in CRC patients.

MATERIALS AND METHODS

Patients

A total of 87 patients with the diagnosis of primary CRC or with uncomplicated cholelithiasis which were referred to Gazi University, Faculty of Medicine, Department of General Surgery for surgical evaluation accepted as candidates for this prospective randomized study. All participants' rights were protected and informed consents were obtained according to the Helsinki Declaration. Local Ethic Committee also approved the study protocol.

The exclusion criteria for this study were to have immune system disorders, obstacle for surgical intervention due to cardio-pulmonary or metabolic risks, to receive neoadjuvant chemotherapy due to the late stage carcinoma, having malnutrition, chronic granulomatosis, and collagen tissue or neurodegenerative diseases.

Control group (Group 1) consisted of 43 cancer-free subjects, aged 62.06 ± 2.07 (mean $\pm$ SEM) years, body mass index (BMI): 26.1 ± 0.6 kg/m^2 and was programmed for elective laparoscopic cholecystectomy for cholelithiasis without acute inflammation. These patients had undergone diagnostic endoscopic evaluation whenever their complaints suggestive for digestive diseases. Patients in whom no pathology being found in either the upper or lower gastrointestinal system were included in cancer-free control group.

Group 2 constituted of 44 CRC patients, aged 60.95 ± 2.11 years, BMI: 25.4 ± 0.5 kg/m^2, and underwent elective abdominal surgery for CRC. Diagnosis was made by endoscopic survey and histological examination of tumor biopsies in all CRC patients that were stratified as stage 1, 2 or 3 according to the TNM classification of the AJCC staging[2]. The presence of distant metastases was ruled out by pre-operative upper abdominal ultrasonography, computed tomography-based evaluation, chest X-ray and intra-operative exploration. All patients underwent surgery with curative intent and with histologically confirmed disease-free resection margins were included in Group 2. During the study period, uniform surgical management protocol was carried out. In all cases, curative resection had included the main lymphovascular supply to the bowel. For proximal colon tumors, lymphadenectomy was extended to the origin of the ileocolic, right colic and middle colic arteries. For distal colon tumors and rectal tumors, lymphadenectomy was extended to the origin of the lower mesenteric artery. Total mesorectal excision was performed in all patients with tumors of the middle and lower rectum. Cases with subsequently identified

Figure 1 Hematoxylin and eosin stained (A, C and E) and indoleamine-2,3-dioxygenase immunostained (B, D and F) materials for the histopathological evaluation of colorectal carcinoma patients (Magnification × 100). The presence of lymphatic invasion, indoleamine-2,3-dioxygenase (IDO) intensity, IDO proportion and total IDO immunostaining scores were determined by antibodies against IDO (Tumor cells show strong positive staining while normal mucosa shows negative or very weak staining). A and B: Lymphatic invasion negative, IDO intensity, 3: IDO proportion score, 5%: total IDO immunostaining score, 4; C and D: Lymphatic invasion positive, IDO intensity, 3: IDO proportion score, 70%: total IDO immunostaining score, 6; E and F: Lymphatic invasion positive, IDO intensity, 3: IDO proportion score, 90%: total IDO immunostaining score, 7.

distant metastases or incomplete clearance of the tumor were excluded.

Postoperative confirmation of preoperative diagnosis in Group 2 was made by routine histopathological examination of postoperative specimens. The patients were subsequently followed-up for a mean of 28.3 ± 1.2 mo. The patients' age and gender and localization, histological type and the differentiation of tumors were noted as standard clinical variables. It has been suggested that 12 lymph nodes could be considered as the minimum acceptable harvest from a careful specimen dissection[26]. Conventionally, lymph nodes were identified by palpation. Whenever, fewer than 12 nodes were found after careful gross examination, additional enhancement techniques were employed[27].

The same team of pathologists examined surgical specimens for tumor and lymph nodes, and the same technique for lymph node assessment was utilized during the entire period of the study. Three or more consecutive sections were examined from each lymph node[28] and routine histological examination of all specimens was performed using triple levelling techniques and the sections were stained with haematoxylin and eosin (HE) (Figure 1A, C and E).

Histopathological evaluation

The surgical resection materials of the primary CRC cases were re-evaluated histopathologically for the presence of lymphatic invasion, intratumoral and peritumoral lymphoid cell infiltration (lymphocytic

tumor response), pathological stage and tumor grade. Lymphatic invasion was noted as present or absent. Suspicious cases for the presence of lymphatic invasion were evaluated immunohistochemically with podoplanin antibody. Lymphocytic tumor response was semi-quantitatively estimated (from point 0 to point 3) according to the intensity of infiltrating lymphocytes. Tumors were graded according to the degree of glandular differentiation: grade 1, grade 2 and grade 3. The number of metastatic and reactive lymph nodes was also noted.

Immunohistochemical staining was performed on paraffin-embedded blocks of each case. 4 μm thick sections of the representative tumor blocks were studied by indirect immunoperoxidase staining. The sections were re-hydrated in a graded series of alcohol, blocked with non-immune blocking solution. Antigen unmasking was performed by microwaving the sections in a 10mM citrate buffer, pH 6.0 for 20 min. The sections then were incubated with primary antibody of "anti-indolamine 2,3-dioxygenase" for 2 h at 37 ℃ (dilution; 1:300, mouse monoclonal antibody, Clone 10.1, Millipore, Billerica, MA, United States). After washing, they were incubated with secondary antibody (multi-species ultra streptavidine detection system-horse radish peroxidase, Zymed, MA, United States) and streptavidine-biotin complex (Zymed, MA, United States) for 20 min per each at room temperature, respectively. Immunoreactions were developed with diaminobenzidine (diaminobenzidinetetrachloride, Zymed, United States) as chromogen. The percentage of the tumor cells showing cytoplasmic staining and its intensity were semi-quantitatively estimated (Figure 1B, D and F).

Quantification of total IDO score
Immunoreactivity was semi-quantitatively estimated as previously described[29]. The total IDO immuno-staining score was calculated as the sum of a proportion score and an intensity score. The proportion score reflects the estimated fraction of positively stained infiltrating cells (score 0, none; score 1, < 10%; score 2, 10%-50%; score 3, 51%-80%; score 4, > 80%). The intensity score represents the estimated staining intensity (score 0, no staining; score 1, weak; score 2, moderate; score 3, strong) giving a total score ranging from 0 to 12. We defined IDO overexpression as a total score 4 or > 4. The frequency of increased total IDO immunostaining score of CRC group was estimated by comparing each patient with the cutoff value; 4 (Figure 1A-F).

Serum IDO activity and serum neopterin determination
After centrifugation, serum specimens were stored at -20 ℃ until assayed. Serum Trp and Kyn concentrations were determined by high-performance liquid chromatography as described, the ratio of Kyn to Trp was calculated to estimate the activity of IDO[30].

Serum neopterin concentrations were determined according to the manufacturer's instructions by a commercially available enzyme immunoassay kit (ELISA, Demeditec Diagnostics, Kiel, Germany). In order to test the serum specific IDO activities of patients, correlations between the Kyn/Trp ratio and neopterin values were computed.

Urinary neopterin and biopterin determination
Neopterin, biopterin and creatinine levels in urine were analyzed by high performance liquid chromatography. Neopterin, biopterin and creatinine isocratically eluted and quantified in the same chromatographic run[31].

Statistical analysis
Data were expressed as mean ± SEM. After checking the data by the Kolmogorov-Smirnov test, normally distributed data were analyzed using independent sample *t*-test. Nonparametric data were compared with Mann-Whitney *U* test. Correlations were assessed using Spearman's rank or Pearson tests. For two or more categorical, independent groups, Pearson's χ^2 and linear-by-linear association tests were made and *P* values less than 0.05 were considered to indicate statistical significance.

RESULTS
A pronounced rise in serum neopterin concentration above the standard cut off value (10 nmol/L) and increased urinary neopterin excretion[32] were observed in cancer patients, but they did not reach the statistical significance (*P* = 0.634 and *P* = 0.090 *vs* cancer-free patients, respectively) (Table 1). Both in CRC (Group 2) and cancer-free groups (Group 1) a significant correlation was observed between the serum and urinary neopterin concentrations (*P* = 0.00001, *r* =0.450 and *P* = 0.002, *r* =0.481, respectively). Although CRC patients showed a mild decrease in serum Trp levels, we could not find a statistical difference when compared with the cancer-free patients (*P* = 0.254). However, cancer patients had a considerable rise in serum Kyn, a toxic metabolite of Trp degradation pathway (*P* = 0.042 *vs* cancer-free patients). Therefore, a remarkable increase in Kyn/Trp which reflects the serum IDO activity of CRC group was observed when compared to controls (*P* = 0.006) (Table 1). CRC cases also showed a significant positive correlation between the serum neopterin levels and Kyn/Trp (*r* = 0.450, *P* = 0.003). Evaluation of relationship between serum Trp and Kyn/Trp disclosed a significant negative correlation in both groups (*P* = 0.012, *r* = -0.383 for Group 1; *P* = 0.0001, *r* = -0.670 for Group 2). Thus, significant negative correlations were calculated between the serum Trp and Kyn values of cancer cases (*r* = 0.332, *P* = 0.003). Even neopterin levels were not found statistically significant in Group 2, sum of urinary biopterin and urinary neopterin

Table 1 Serum tryptophan, kynurenine and neopterin, urinary neopterin and biopterin of cancer-free group (Group 1) and colorectal cancer patients (Group 2)

Group	Group 1 (n = 43)	Group 2 (n = 44)	P value
Age (yr)[1]	62.06 ± 2.07	60.95 ± 2.11	0.708
Tryptophan (μmol/L)[1]	21.95 ± 1.03	19.91 ± 1.33	0.254
Kynurenine (μmol/L)[2]	1.03 ± 0.19	1.20 ± 0.13	0.042[a]
Kynurenine/tryptophan[1]	41.31 ± 3.62	89.47 ± 14.32	0.006[a]
Serum neopterin (nmol/L)[2]	13.28 ± 1.08	16.93 ± 2.84	0.634
Urinary neopterin (μmol neopterin/mol creatinine)	63.54 ± 5.64	80.45 ± 8.09	0.090
Urinary biopterin (μmol biopterin/mol creatinine)[1]	67.33 ± 6.22	91.41 ± 12.00	0.038[a]
Urinary neopterin (μmol neopterin/mol creatinine) + urinary biopterin (μmol biopterin/mol creatinine)[1]	129.83 ± 9.42	172.60 ± 17.17	0.048[a]

[a]$P < 0.05$, Group 2 *vs* Group 1 considered statistically significant; [1]Independent sample *t*-test; [2]Mann-Whitney *U* test. Colorectal cancer patients showed more than two times increase in overall serum kynurenine-tryptophan ratio and a non-significant rise in the activity of pteridine pathway when compared with the cancer-free patients (Group 1).

Table 2 Evaluation of serum pteridine and tryptophan metabolites of lymphatic invasion negative (Group 2/c) and positive (Group 2/d) colorectal cancer patients

Group	Group 2/c (n = 21)	Group 2/d (n = 23)	P value
Tryptophan (μmol/L)[1]	19.14 ± 1.68	20.62 ± 2.06	0.586
Kynurenine (μmol/L)[2]	1.45 ± 0.24	0.96 ± 0.11	0.080
Kynurenine/tryptophan	95.85 ± 17.40	83.08 ± 23.13	0.662
Serum neopterin (nmol/L)[2]	18.43 ± 5.18	15.56 ± 2.78	0.613
Urinary neopterin (μmol neopterin/mol creatinine)[1]	85.79 ± 11.76	75.68 ± 11.34	0.541
Urinary biopterin (μmol biopterin/mol creatinine)[1]	76.56 ± 16.44	105.43 ± 17.20	0.234
Urinary neopterin (μmol neopterin/mol creatinine) + urinary biopterin (μmol biopterin/mol creatinine)[1]	167.42 ± 21.83	177.50 ± 26.82	0.774
IDO (%)	24.04 ± 6.90	44.56 ± 6.11	0.031[a]

[a]$P < 0.05$, group 2/c *vs* group 2/d, considered statistically significant; [1]Independent sample *t*-test; [2]Mann-Whitney *U* test. The comparison displayed that serum indoleamine-2,3-dioxygenase (IDO) activities were strongly predictive for lymphatic invasion.

concentrations indicated significant increase in cell-mediated immune response in CRC patients compared to Group 1. While sum of urinary biopterin and urinary neopterin concentrations were calculated lower in lymph node-positive patients, they were higher in lymphatic invasion-positive CRC patients.

We examined 1398 HE stained serial sections of 466 lymph nodes from 44 patients with CRC. The number of harvested lymph nodes per patient ranged 10 to 16 (median: 10 and mean ± SEM: 12.59 ± 1.56). The average number of serial sections from tumor tissue for each patient was 16. These were examined after staining with H-E and monoclonal antibody anti-

IDO. Lymphovascular invasion was found in 23 of 44 patients (52.3%). However, we could not demonstrate lymphatic invasion in remaining 21 patients. Only 12 patients (52%) with lymphatic invasion had lymph node metastasis. Although 11 patients (25%) displayed lymphatic invasion, they had no lymph node metastasis. Taking into consideration of all patients, 32 had negative nodes whereas, 12 had evidences of nodal metastasis. We could not observe at all an association between the serum neopterin levels and presence versus absence of lymph node metastases ($P = 0.368$). Evaluation of serum pteridine and Trp metabolites of lymphatic invasion negative (Group 2/c) and positive (Group 2/d) CRC patients displayed serum IDO activities that were significantly higher in lymphatic invasion (Table 2). On the other hand, assessment of serum pteridine and Trp metabolites of lymph node metastasis negative (Group 2/a) and positive (Group 2/b) CRC patients displayed serum IDO activities that were not predictive for lymph node dissemination alone (Table 3).

The tumor grade was higher in the lymph node-positive group (40%). While, IDO intensity score was 24.04% ± 6.90% in lymphatic invasion negative group, it was 44.56% ± 6.11% in lymphatic invasion positive patients ($P = 0.031$). The frequency of increased total IDO immunostaining score of CRC patients with lymphatic invasion was 78 % (18 of 23 patients). While 92 % of the frequency of increased total IDO immunostaining score ($\geq$ 4) was found for patients with lymphatic invasion-lymph node metastasis positive (11 of 12 patients), 38 % was found for lymphatic invasion-lymph node metastasis negative group (8 of 21 patients). There was a significant association between the lymphatic invasion and total IDO immunostaining scores ($P = 0.014$; $r = +0.366$). Weak correlation between the lymph node metastasis and total IDO immunostaining score ($P = 0.043$; $r = +0.310$) confirmed that conventional histopathologic techniques were inefficient to reveal the lymph node invasion adequately. However, lymphatic invasion seemed the most valuable parameter indicating the lymph node metastasis ($P = 0.001$; $r = +0.476$) (Table 4). Although lymphatic invasion showed significant linear-by-linear association with total IDO immunostaining score of tumor tissue ($P = 0.016$) and lymph node metastasis ($P = 0.002$), we could not find an association neither with lymphocytic infiltration score ($P = 0.211$) nor tumor grade ($P = 0.358$) (Table 5). Regarding the evaluation of CRC cases, no statistical difference was displayed either by using total IDO immunostaining score or lymphatic invasion ($P = 0.581$). Positive predictive value (PPV) or the proportion of patients with the higher total IDO immunostaining scores ($\geq$ 4) who have lymphatic invasion was 70% and negative predictive value (NPV) was calculated as 72% for the same group. The specificity and sensitivity of presence of lymphatic

Table 3 Evaluation of serum pteridine and tryptophan metabolites of lymph node metastasis negative (Group 2/a) and positive (Group 2/b) colorectal cancer patients

Group	Group 2/a (n = 32)	Group 2/b (n = 12)	P value
Tryptophan (μmol/L)[1]	18.04 ± 1.39	23.98 ± 2.98	0.047[a]
Kynurenine (μmol/L)[1]	1.33 ± 0.17	0.87 ± 0.14	0.139
Kynurenine/tryptophan[1]	103.20 ± 18.13	53.27 ± 17.36	0.121
Serum neopterin (nmol/L)[2]	17.78 ± 3.68	14.67 ± 3.94	0.368
Urinary neopterin (μmol neopterin/mol creatinine)[1]	83.07 ± 9.76	71.29 ± 12.96	0.552
Urinary biopterin (μmol biopterin/mol creatinine)[1]	96.87 ± 13.83	69.56 ± 23.13	0.371
Urinary neopterin (μmol neopterin/mol creatinine) + Urinary biopterin (μmol biopterin/mol creatinine)[1]	179.95 ± 20.48	143.23 ± 24.95	0.400
IDO (%)[1]	30.32 ± 5.74	48.75 ± 8.30	0.089

[a]P < 0.05; group 2/a *vs* group 2/b, considered statistically significant; [1]Independent sample *t*-test; [2]Mann-Whitney *U* test. The comparison displayed that serum indoleamine-2,3-dioxygenase (IDO) activities were not predictive for lymph node dissemination alone.

Table 4 Correlation between lymphatic invasion and lymph node metastasis, total serum indoleamine-2,3-dioxygenase score and serum tryptophan in colorectal cancer patients

	Pearson correlation	
Lymphatic invasion	Lymph node metastasis $P = 0.001^a$, $r = +0.476$	Tumor total IDO score $P = 0.014^a$, $r = +0.366$
Lymph node metastasis	Serum tryptophan $P = 0.047^a$, $r = +0.305$	Tumor total IDO score $P = 0.043^a$, $r = +0.310$

[a]P < 0.05 considered statistically significant. IDO: Indoleamine-2,3-dioxygenase.

Table 5 Evaluation of the association between lymphatic invasion, lymph node metastasis and tumor total indoleamine-2,3-dioxygenase score in colorectal cancer patients

Lymphocytic infiltration- lymphatic invasion	$P = 0.211$
Lymph node metastasis- lymphatic invasion	$P = 0.002^a$
Tumor differentiation- lymphatic invasion	$P = 0.358$
Tumor total IDO score- lymphatic invasion	$P = 0.016^a$

[a]P < 0.05 considered statistically significant. Lymphatic invasion showed linear-by-linear association with lymph node metastasis and tumor total IDO score in colorectal cancer patients. IDO: Indoleamine-2,3-dioxygenase.

invasion plus lymph node metastasis in patient with higher total IDO immunostaining score was 62% and 85%, respectively, whereas, we found a significant difference between the accuracy of diagnosis by using either total IDO immunostaining score or lymph node metastasis during the evaluation of cancer patients (P = 0.0013; PPV: 38.46, NPV: 89%).

DISCUSSION

In our study, CRC patients had increased systemic immune activation. The elevated neopterin production is also associated with Trp degradation. Trp depletion as well as the accumulation of its cytotoxic metabolites results in a strong inhibitory effect on the development of immune responses[33]. Increases in both the urinary biopterin excretion and Kyn/Trp of CRC patients compared to the matched controls suggested that IFN-gamma-mediated immune response may be enhanced by the malignant growth. Actually, IFN-γ-induced guanosine triphosphate (GTP) cyclohydrolase I activity correlates with the sum of neopterin plus biopterin rather than with neopterin or biopterin alone[34]. Thus, lower values of neopterin plus biopterin in lymph node-positive patients considering the higher values of lymphatic invasion-positive cancer cases may be suggestive for insufficient immune response in these patients.

It is well known that IDO activity is characterized best by the Kyn/Trp which correlates with concentrations of immune activation marker neopterin[35]. Indeed, in our study highly significant correlation between neopterin concentrations and the increased Kyn/Trp clearly indicated that the formation of Kyn is related to IDO activity by IFN-gamma stimulation. IDO rapidly degrades Trp that is crucial to sustain proliferation of T lymphocytes[36]. Despite the phenomena of immune activation, low Trp levels or increased concentrations of its degradation products may be directly involved in diminished T-cell responsiveness to antigenic stimulation in cancer patients[37]. Actually, physiological levels of IDO may be needed for maintenance of T cells function, which is necessary for antigen presentation, but increased levels of IDO and accumulation of IDO-mediated cytotoxic metabolites from the Kyn pathway will hamper cell function in activated T cells[38]. On the other hand, IDO-induced Trp depletion from the tumor microenvironment could be the results of elevated levels of the enzyme and augmented Trp consumption by both tumor cells and antigen presenting cells of the host[22]. However, CRCs with a high density of infiltrating memory and effector T cells are less likely to disseminate to lymphovascular and perineural structures and to regional lymph nodes and, present a less advanced pathological stage, and increased survival[39].

In this study we analyzed the expression of IDO proteins in human colorectal tumor samples. Our results are also in line with previous studies, indicating that human tumors frequently express IDO[38]. 52.4% of tumor specimens showed IDO-high expression, whereas in 47.6% of tumor specimens, the staining was scored as IDO-low expression. These findings indicated that certain colon cancer subsets are different in their ability to express IDO. The finding, IDO activity significantly correlated with immunostaining scores *in vitro* strongly suggests that the enzyme is active and might exert its

immunosuppressive activity in patients classified as IDO-high expression. However, further prospective studies by using fresh tumor tissues are required to confirm this hypothesis. In this study, we verified that lymphovascular invasion is a strong predictor of lymph node metastasis. All lymphatic invasion negative patients were also lymph node negative (21 patients; 47.7%). When compared with the lymphatic invasion-lymph node positive CRC patients (27.3%), lymphatic invasion-lymph node negative patients had significantly lower expression of IDO (4.7% ± 0.4% and 2.6% ± 0.4%, respectively, P = 0.012). The increased frequency of IDO activity could enhance immune tolerance against the cancer cells. While 23 patients with positive lymphatic invasion showed 78% increased frequency of IDO activity (IDO score $\geqslant$ 4), lymphatic invasion-lymph node positive CRC patients and lymphatic invasion-lymph node negative patients had 92% and 38%, respectively.

In this study, in order to compare with the tumor IDO activity, lymphatic invasion was demonstrated with advanced immunohistochemical techniques. However, histological evaluation of lymph nodes was made by routine conventional methods. Despite of the positive lymphatic invasion, lymph node metastases were found negative in 11 patients.

Likewise, multivariate analysis of CRC showed that lymphatic invasion is an independent predictor factor for lymph node metastasis[5]. Very recently, it has been indicated that to the patients with T1 or T2 CRC, with positive lymphovascular invasion, radical surgery should be recommended because of the greater risk for lymph node metastasis[40]. This surgical strategy was supported by our data. Additionally, in routine conventional histopathological examinations, lymphatic invasion and lymph node metastases cannot always be defined due to limited number of histological sections.

As a conclusion, these evidences coupled with our data showed that high total IDO immunostaining score is a strong predictor for immune tolerance, lymphatic invasion and subsequent lymph node metastasis. Therefore, IDO immunostaining might be recommended for histopathological evaluation of CRC cases.

COMMENTS

Background
Lymphatic invasion and the depth of submucosal invasion of tumor cells are risk factors for lymph node metastasis, which is the strongest prognostic determinant for colorectal cancer (CRC) patients. It was suggested that indoleamine-2,3-dioxygenase (IDO) high-expressing colorectal tumor cells enable cancer subsets to avoid immune attack. However, still a satisfactory tool does not exist for the determination of unrecognizable tumor cells that may cause recurrent CRC.

Research frontiers
IDO high-expressing colorectal tumor cells enable cancer subsets to avoid

immune attack. Although IDO expression by tumor cells has been shown to correlate with a poor clinical prognosis of colon cancer, assessment of tumor IDO activity has not took place currently in routine histopathological evaluation concept of CRCs. Thus the current research hotspot is to evaluate the adequate retrieval and assessment of colorectal mesenteric lymph nodes to ensure that the lymph nodes do not contain unrecognizable tumor cells.

Innovations and breakthroughs
In CRC patients, significantly accelerated degradation of tryptophan (Trp) with lowered serum concentrations of Trp and increased kynurenine as well as an increased Kyn/Trp has been recognized. Although IDO expression by tumor cells has been shown to correlate with a poor clinical prognosis of colon cancer, assessment of tumor IDO activity does not take place currently in routine histopathological evaluation of CRCs. This data revealed that high total IDO immunostaining score is a strong predictor for immune tolerance, lymphatic invasion and subsequent lymph node metastasis. Therefore, IDO immunostaining might be recommended for histopathological evaluation of CRC cases.

Applications
High total IDO immunostaining score is a strong predictor for immune tolerance, lymphatic invasion and subsequent lymph node metastasis. Thus, during the histopathological evaluation of CRC, IDO immunostaining might be used.

Terminology
CRC is one of the major public health problems in the world. Lymphatic invasion and the depth of submucosal invasion of tumor cells are risk factors for lymph node metastasis, which are strongest prognostic determinants for colorectal cancer patients. Trp degrading enzyme, IDO expression by tumor cells has been shown to correlate with a poor clinical prognosis of colon cancer. Indicator of cellular immune response, neopterin is produced by the activated macrophages and associated with Trp degradation. Actually, IFN-gamma-induced guanosine triphosphate (GTP) cyclohydrolase I activity results in synthesis of neopterin, as well as biopterin.

Peer-review
This is an interesting study, a lot of work behind many thanks for authors.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Total mesorectal excision for mid and low rectal cancer: Laparoscopic *vs* robotic surgery

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Santo Stefano Hospital, Prato, Italy and of the Misericordia Hospital, Grosseto, Italy.

Informed consent statement: The IRB allowed us to waive the requirement for obtaining informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to have imaging study by written consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: Statistical analysis and dataset are available from the corresponding author at fferoci@yahoo.it.

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Abstract

AIM: To compare the short- and long-term outcomes of laparoscopic and robotic surgery for middle and low rectal cancer.

METHODS: This is a retrospective study on a pro-spectively collected database containing 111 patients who underwent minimally invasive rectal resection with total mesorectal excision (TME) with curative intent between January 2008 and December 2014 (robot, n = 53; laparoscopy, n = 58). The patients all had a diagnosis of middle and low rectal adenocarcinoma with stage I-III disease. The median follow-up period was 37.4 mo. Perioperative results, morbidity a pathological data were evaluated and compared. The 3-year overall survival and disease-free survival rates were calculated and compared.

RESULTS: Patients were comparable in terms of preoperative and demographic parameters. The median surgery time was 192 min for laparoscopic TME (L-TME) and 342 min for robotic TME (R-TME) (P < 0.001). There were no differences found in the rates of conversion to open surgery and morbidity. The

patients who underwent laparoscopic surgery stayed in the hospital two days longer than the robotic group patients (8 d for L-TME and 6 d for R-TME, $P < 0.001$). The pathologic evaluation showed a higher number of harvested lymph nodes in the robotic group (18 for R-TME, 11 for L-TME, $P < 0.001$) and a shorter distal resection margin for laparoscopic patients (1.5 cm for L-TME, 2.5 cm for R-TME, $P < 0.001$). The three-year overall survival and disease-free survival rates were similar between groups.

CONCLUSION: Both L-TME and R-TME achieved acceptable clinical and oncologic outcomes. The robotic technique showed some advantages in rectal surgery that should be validated by further studies.

Key words: Robotic surgery; Laparoscopic surgery; Rectal cancer; Total mesorectal excision; Minimally invasive surgery

Core tip: The aim of this retrospective study was to compare the short- and long-term outcomes of 111 patients who underwent minimally invasive rectal resection with total mesorectal excision (TME) with curative intent. The median surgery time was shorter for laparoscopic TME while there were no differences found in the rates of conversion to open surgery and morbidity. The pathologic evaluation showed a higher number of harvested lymph nodes in the robotic group and a shorter distal resection margin for laparoscopic patients but the three-year overall survival and disease-free survival rates were similar between groups.

INTRODUCTION

Several randomized clinical trials have shown the long-term oncologic results of laparoscopic versus open surgery for colorectal cancer[1-3]. The recently published long-term results of the COLOR II trial state that laparoscopic surgery in patients with rectal cancer produces similar rates of locoregional recurrence, 3-year disease-free survival and 3-year overall survival as open surgery[4]. However, technical difficulties associated with laparoscopic resection and the extensive training required to perform the operation have limited its dissemination outside specialized centres[5-7]. A robot-assisted approach could potentially

overcome some of the limitations of conventional laparoscopic rectal surgery. A robotic system enables the surgeon to control a three dimensional, high-definition, 10-fold magnification vision steady camera. It provides wrist motion for endoscopic instruments (7 degrees of freedom, 180 degrees of articulation and 540 degrees of rotation). The motion scaling feature reduces physiological tremors, provides superior dexterity, and increases ergonomic comfort[8]. Therefore, robotic systems can overcome several of the technical difficulties associated with traditional laparoscopic surgery and allow high-quality manoeuvres to be performed in narrow spaces such as the pelvic cavity. The additional third arm instrument is a fixed retractor used to improve vision and stability in restricted spaces. Robot-assisted operations have been used for years in other surgical specialties. However, it was not until 2002 that Weber *et al*[9] reported the first two cases of robot-assisted colectomies. Several meta-analyses have been published and they demonstrate the scientific community's interest in this surgery[10-14]. The most relevant data resulting from these studies was that robotic surgery had reduced conversion to open surgery compared to the laparoscopic group. Additionally, the short-term clinical and oncologic outcomes were not significantly different between groups. The recently published 5-year results demonstrate that there are similar rates for overall survival, disease-free survival, and local recurrence between robotic and laparoscopic surgical procedures[15]. There are currently two ongoing multicentre randomized controlled trials comparing robotic versus laparoscopic surgery for rectal cancer: the ROLARR[16] and COLRAR[17] trials.

The aim of this study was to compare the feasibility and short-term and long-term outcomes of robotic surgery for middle and low rectal cancer with the results of conventional laparoscopic surgery in two different centres with high volume of colorectal minimally invasive surgery.

MATERIALS AND METHODS

Study population and patient selection

This is a retrospective study on a prospectively collected database containing 111 patients who underwent minimally invasive rectal resection with total mesorectal excision (TME) for curative intent. The patients all had a diagnosis of middle and low rectal adenocarcinoma (tumour located within 12 cm from the anal verge). The enrolled patients were from two Italian institutions; the Unit of General and Oncologic Surgery, Santo Stefano Hospital, Prato and the Unit of Minimally Invasive Surgery, Division of General and Laparoscopic Surgery, European Institute of Oncology, University of Milan. The Institutional Review Boards of both hospitals approved the study.

Patients considered for minimally invasive surgery were enrolled between January 1, 2008 and December

31, 2014. The exclusion criteria included emergency cases, patients with clinical T4 or metastatic disease, and those with contraindication for prolonged pneumoperitoneum. All surgeries were performed by the expert surgeons MS (Santo Stefano Hospital, Prato) and PPB (European Institute of Oncology, University of Milan). Both surgeons have performed more than 100 laparoscopic colorectal resections. The robotic resections were executed by a single surgeon (PPB).

Data collection and evaluation parameters

The preoperative data included the following: general patient characteristics, American Society of Anaesthesiologists (ASA) score, body mass index (BMI), previous neoadjuvant treatment, distance of the lesion from the anal verge, and tumour biomarkers.

The intraoperative data consisted of the following: surgical time including the docking of the robot, adjunct procedures, intraoperative complications, blood loss, ileostomy, and conversions to laparotomy.

The postoperative results included first bowel movement, hospital length of stay, postoperative surgical and non-surgical complications, and need for revision surgery. The postoperative complications were defined as adverse events that occurred within 30 d after surgery. All of the complications were diagnosed and categorized according to patient's symptoms with the aid of laboratory and radiological evaluation to confirm clinical suspicions. The diagnosis of anastomotic leakage was based on clinical suspicion and required contrast radiography (radiograph or computed tomographic scan) or surgery to confirm the diagnosis. The signs of clinical leakage included abdominal pain or fever, discharge of pus or bowel contents through the indwelling drain, and local or generalized peritonitis. The total numbers of postoperative complications were counted for all cases related to morbidity. The postoperative surgical complications were also stratified by the Clavien-Dindo classification[18].

The tumour-node-metastasis (TNM) stage, resection margins, numbers of harvested lymph nodes, lymphovascular invasion, and circumferential resection margin (CRM) were evaluated for analysis of the pathologic outcomes. The involved CRM was defined when the tumour was located 1 mm or less from the CRM[19]. The pathologic analyses conducted after 2010 used the criteria of the American Joint Committee on Cancer seventh edition[20]. TNM stages evaluated before 2010[21] have been reviewed according to the newest edition.

All patients undergoing surgery were registered in our database and received close follow-up. We calculated the 3-year overall survival and disease-free survival rates. A local recurrence was defined as the relapse of the tumour at the primary site confirmed by radiological or histological evidence. Simultaneous local and systemic recurrences were counted as a local recurrence. A distant metastasis was considered a metastatic lesion diagnosed in other organs beyond the primary site.

Clinical management

The preoperative patient work-up included a colonoscopy with biopsy, standard blood testing, thoracic-abdominal computed tomography (CT), transrectal ultrasonography (US), and pelvic magnetic resonance imaging (MRI) if necessary. For locally advanced disease (clinical stage T3 N0 or any T N+) that was confirmed by MRI and/or US we considered neoadjuvant CRT. After neoadjuvant therapy, the patients received a thoracic-abdominal CT for restaging. A radical surgical treatment was proposed in all cases, including the patients with a pathological complete response[22]. Surgery was performed 8 wk after the completion of RT, when tumour regression was maximal[23].

Total mesorectal excision was the standard procedure for middle and low rectal cancer. The surgical techniques were performed as described in previous reports[24,25]. The tumour height and the absence of direct tumour invasion into the levator ani muscle or sphincter muscle were the primary considerations for sphincter-preserving procedures. Both institutions applied similar fast-track protocols and similar discharge criteria for the perioperative management of colorectal surgical patients[26]. Neoadjuvant and adjuvant therapies were administered according to the Italian National Institute of Health recommendations and the most current NCCN guideline for rectal cancer[27]. The discharged patients received a physical examination and tumour marker analysis at 1 mo, 3 mo, and then every 3 mo for the first 3 years. The patients were then evaluated every 6 mo until 5 years after surgery. Each patient was evaluated by colonoscopy at 1 year and 3 years after surgery and then every 5 years. We obtained chest and abdominopelvic computed tomography scans every 6 mo for the first 3 years. We then obtained scans every 12 mo until 5 years after surgery to detect local recurrence or systemic metastasis during the follow-up period.

Statistical analysis

The differences in clinically important baseline characteristics, intraoperative outcomes, short-term (30-d) postoperative outcomes and long-term (3-year) outcomes were compared between the laparoscopic and robotic cohorts. A univariate analysis was performed using the Mann-Whitney U test for continuous variables. The χ^2 test was used for categorical variables. A P values < 0.05 was considered statistically significance for all analyses and all tests were two-sided. The univariate results are reported as median (interquartile range) or frequency (percent).

Table 1 Patient characteristics *n* (%)

	L-TME (*n* = 58)	R-TME (*n* = 53)	*P* value
Age[1] (yr)	66 (33-80)	66 (42-84)	0.597
Sex			0.031
Male	42 (72.4)	27 (50.9)	
Female	16 (27.6)	26 (49.1)	
BMI[1] (kg/m^2)	24.6 (19-37)	24.6 (18-31)	0.512
ASA score			0.082
1	7 (12.1)	11 (20.8)	
2	31 (53.4)	33 (62.3)	
3	20 (34.5)	9 (16.9)	
Neoadjuvant therapy	25 (43.1)	26 (49.1)	0.571
Tumour location from anal verge[1] (cm)	8 (3-12)	8 (4-12)	0.607
CEA[1]	1.55 (0.6-51.6)	1.65 (0.5-11.1)	0.803
CA 19.9[1]	7.85 (0.8-241)	7.5 (2-905)	0.896

[1]Median (range). L-TME: Laparoscopic total mesorectal excision; R-TME: Robotic total mesorectal excision; BMI: Body mass index; ASA: American Society of Anesthesiologists.

The patient overall survival and disease free survival were calculated using the Kaplan-Meier method and were compared with the log-rank test. All data were analysed on an intention-to-treat basis. The data were tabulated using a Microsoft© Excel spreadsheet (Excel for Windows©; Microsoft Corporation, Redmond, WA, United States) and were processed with SPSS© 16.0 for Windows (SPSS, Chicago, IL, United States).

The overall survival for both groups was calculated as the interval from surgery to death and disease free survival was calculated as the interval from surgery to the first diagnosis of recurrence. Due to the current lack of a uniform consensus regarding the definition of conversion to laparotomic surgery, we defined a converted rectal resection as any interruption of the minimally invasive approach (laparoscopic or robotic) and subsequent use of a conventional abdominal incision for completion of the operation.

RESULTS

Patient characteristics

There were 58 laparoscopic rectal resections with TME (L-TME) and 53 robotic rectal resections with TME (R-TME).

There were no significant differences between groups for age and Body Mass Index. There were more males in the laparoscopic group (*P* = 0.031). The ASA score showed no significant differences between the laparoscopic and robotic patients and a score of 2 was the most common value in both groups. The groups were similar with respect to tumour location, preoperative presence of tumour markers, and rate of patients who underwent preoperative CRT (Table 1).

Perioperative clinical outcomes

The median surgical time was 342 min (range 249-536 min) in the R-TME group, which includes time spent for the docking of the robot. The median surgical time was 192 min (range 90-335 min) in the L-TME group (*P* < 0.001). A transanal mechanical end to end anastomosis was performed in all the robotic procedures and in 54 laparoscopic patients. There was a manual coloanal anastomosis executed in the remaining four L-TME cases. There were eight adjunct procedures in the laparoscopic group that included the following: one prolonged lysis of visceral adhesions, one cholecystectomy, two urologic procedures, one left adrenalectomy, two liver biopsies, and one resection of jejunal gastro intestinal stromal tumour. There were the following five adjunct procedures performed in the robotic surgery group: two hysterectomy and salpingo-oopherectomy, two cholecystectomies and one urologic procedure. There were no intraoperative complications in either group. There were no significant differences for intraoperative bleeding or diverting ileostomy. There was one conversion to laparotomy in the laparoscopic due to the presence of extensive visceral adhesions and there were two conversions in the robotic group (*P* = 0.605). The cause of conversion to laparotomy in both robotic procedures was the need to resect the anastomotic colon after the intraoperative identification of ischemia. The other robotic case was converted to conventional laparoscopy for the same reason. The day of first bowel movement, perioperative morbidity, and rate of revision surgery were similar between groups. However, patients who underwent laparoscopic surgery stayed at the hospital two days longer than the robotic group patients (8 d for L-TME and 6 d for R-TME, *P* < 0.001). There were no 30-d mortalities (Table 2).

Postoperative pathologic assessment

The tumour stage distribution and lymphovascular invasion did not differ between groups. The factors indicating the mesorectal excision quality such as invasion of distal resection margin (DRM) and positivity of CRM were not significantly different. The CRM was less than 1 mm in one laparoscopic patient (*P* = 0.523) and the DRM was involved in one laparoscopic and one robotic patient (*P* = 0.729). The median number of harvested nodes was 11 (range 3-27) in the laparoscopic group and 18 (range 4-49) in the robotic group (*P* < 0.001). The median length of DRM was 1.5 cm (range 0.5-5 cm) for the L-TME and 2.5 cm (range 0.5-10 cm) cm for the R-TME (*P* < 0.001). A pathological complete response after neoadjuvant therapy was observed in 6 (10.3%) laparoscopic patients and in 5 (9.4%) robotic cases (*P* = 0.381). Our results are comparable with data reported in a recent meta-analysis[22] (Table 3).

Oncologic long-term outcomes

The median follow-up period for all cases was 37.4 mo (range 2-85 mo). There were no patients lost to follow-up. There was no significant difference in the administration of adjuvant chemotherapy between

Table 2 Perioperative outcomes *n* (%)			
	L-TME	**R-TME**	*P* value
	(*n* = 58)	(*n* = 53)	
Operative time[1] (min)	192 (90-335)	342 (249-536)	< 0.001
Anastomosis			0.120
Mechanical transanal	54 (93.1)	53 (100)	
Manual coloanal	4 (6.9)	0	
Adjunctive procedure	8 (13.8)	5 (9.4)	0.562
Diverting ileostomy	43 (74.1)	41 (77.4)	0.825
Intraoperative blood loss[2] (mL)	47.4 (0-400)	60.8 (0-400)	0.510
Conversion to laparotomy	1 (1.7)	2 (3.8)	0.605
Hospital stay[1] (d)	8 (5-53)	6 (3-17)	< 0.001
First bowel movement[1]	1 (1-6)	1 (1-6)	0.904
(postoperative day)			
Total morbidity	26 (44.8)	17 (32.1)	0.122
Surgical morbidity			
Anastomotic leak	8 (13.8)	3 (5.7)	0.208
Peritoneal haemorrhage	2 (3.4)	1 (1.9)	0.534
Stomal stricture	3 (5.2)	1 (1.9)	0.620
Wound infection	2 (3.4)	0	0.496
Ileus	4 (6.8)	3 (5.7)	0.551
Abdominal pain[3]	2 (3.4)	3 (5.7)	0.457
Other surgical complications	5 (8.6)	3 (5.7)	0.410
Non surgical morbidity	11 (19.0)	6 (11.4)	0.302
Reoperation	8 (13.8)	3 (5.7)	0.208
Clavien-Dindo Classification			0.297
0	32 (55.2)	36 (67.9)	
1	9 (15.5)	5 (9.4)	
2	7 (12.1)	8 (15.1)	
3a	2 (3.4)	1 (1.9)	
3b	4 (6.8)	3 (5.7)	
4a	2 (3.4)	0	
4b	2 (3.4)	0	

[1]Median (range); [2]Mean (range); [3]Without other causes. L-TME: Laparoscopic total mesorectal excision; R-TME: Robotic total mesorectal excision.

Table 3 Pathologic evaluation *n* (%)			
	L-TME	**R-TME**	*P* value
	(*n* = 58)	(*n* = 53)	
TNM stage			0.716
Stage Ⅰ	28 (48.3)	22 (41.5)	
Stage Ⅱ	11 (19.0)	8 (15.1)	
Stage Ⅲ	13 (22.4)	18 (34.0)	
Pathological complete response	6 (10.3)	5 (9.4)	0.381
Total harvested lymph nodes[1]	11 (3-27)	18 (4-49)	< 0.001
DRM[1] (cm)	1.5 (0.5-5)	2.5 (0.5-10)	< 0.001
DRM			0.729
Involved	1 (1.7)	1 (1.9)	
Non involved	57 (98.3)	52 (98.1)	
CRM			0.523
Involved	1 (1.7)	0	
Non involved	57 (98.3)	53 (100)	
Lymphovascular invasion	10 (17.2)	5 (9.4)	0.087

[1]Median (range). L-TME: Laparoscopic total mesorectal excision; R-TME: Robotic total mesorectal excision; DRM: Distal resection margin; CRM: Circumferential resection margin.

Table 4 Long-term outcomes *n* (%)			
	L-TME	**R-TME**	*P* value
	(*n* = 58)	(*n* = 53)	
Adjuvant chemotherapy	25 (43.1)	27 (50.9)	0.700
Distant recurrence	5 (8.6)	9 (17.0)	0.265
Local recurrence	3 (5.2)	1 (1.9)	0.618
Overall mortalit	6 (10.3)	5 (9.4)	0.564
Mortality for rectal cancer	4 (6.9)	4 (7.5)	0.491
3-yr overall survival (%)	90.0	90.2	0.956
3-yr disease-free survival (%)	83.4	79.2	0.268

L-TME: Laparoscopic total mesorectal excision; R-TME: Robotic total mesorectal excision.

groups. There were local recurrences observed in three laparoscopic patients (5.2%) and one robotic case (1.9%, *P* = 0.618). There were distant metastasis in nine R-TME cases (17%) and five L-TME cases (8.6%, *P* = 0.265). The overall patient mortality rate was 10.3% (6 patients) for the laparoscopic group and 9.4% (5 patients) for the robotic group (*P* = 0.564). There were four patient deaths in each group due to the primary diagnosis of rectal cancer. The remaining deaths occurred for other reasons (Table 4).

The 3-year overall survival rate (Figure 1A) was 90.2% in R-TME group and 90.0% in L-TME group (*P* = 0.956). The 3-year disease-free survival rate (Figure 1B) was 79.2% in R-TME and 83.4% in L-TME (*P* = 0.268). There was no mortality or tumour recurrence in patients achieving a pathological complete response after neoadjuvant therapy in either group.

DISCUSSION

In this study, the robotic and laparoscopic patients were comparable with respect to intraoperative, short-term, and long-term results.

Robotic resections required a longer median surgical time, as reported in other series[28]. However,

the similar rates of diverting ileostomy reflect the confidence of the robotic surgeon.

Although there were no differences in postoperative morbidity, the length of hospital stay was longer in the laparoscopic group for unclear reasons. This result is consistent with data from a pilot randomized trial comparing laparoscopic and robotic TME[29].

Our evaluation of CRM positivity and DRM involvement parameters accessed the quality of mesorectal excision and showed no significant differences between robotic and laparoscopic procedures. These results are oncologically acceptable and comparable to other reports[30-32]. The evidence of a longer median DRM in the robotic group (2.5 cm for R-TME; 1.5 cm for L-TME; *P* < 0.001) may be the result of technical advantages of the robotic approach because it allows the surgeon to perform high-quality manoeuvres in narrow spaces such as the pelvic cavity. Despite this consideration, a median DRM of 1.5 cm in the laparoscopic group was adequate and did not compromise the oncological outcome[33-35].

The total number of harvested lymph nodes was higher for the robotic group and this finding contrasts previously reported data[10-13,36]. The lower median

A

B

Figure 1 **The 3-year overall (A) and disease-free (B) survival rate between robotic and laparoscopic total mesorectal excision surgical procedures.** DTS: Disease-free survival; OS: Overall survival; L-TME: Laparoscopic total mesorectal excision; R-TME: Robotic total mesorectal excision.

number of resected lymph nodes for L-TME did not translate into higher rates of recurrence or mortality. This finding demonstrates the lymphadenectomy was accurate in both the laparoscopic and robotic procedures.

The 3-year survival rates of this study did not differ significantly between groups and is comparable with previously reported 3-year and 5-year outcomes[14,37-39].

The advantages of laparoscopic TME compared to the open approach have been examined in several studies[40-43]. The procedure has been described as oncologically safe and is associated with the standard benefits of minimally invasive techniques. Recent trials have reported long-term oncologic outcomes of laparoscopic TME and have shown survival rates similar to those obtained with open surgery[31,44-47].

The 3-year analysis of the CLASICC trial suggested there are improved outcomes for early stage rectal cancer excised laparoscopically compared with open surgery[48]. Despite these advantages, laparoscopic surgery for middle and low rectal cancers can be very challenging due to technical difficulties. Thus, the MRC CLASICC trial revealed high conversion rates, CRM involvement, and an increased incidence of urinary and sexual dysfunctions[49]. Although higher CRM infiltration did not result in increased local recurrence rates, the concerns regarding laparoscopic rectal cancer surgery led to decreased use in United Kingdom/ United States for rectal cancer[50,51]. The rate of conversion to open surgery is critical in minimally invasive rectal cancer surgery because the converted patients had higher complication rates than non- converted cases[45]. Additionally, one series reported the conversion patients had the worst oncological outcomes[52]. The COREAN trial revealed outcomes of the laparoscopic approach were comparable to open resection in middle and low rectal cancer after neoadjuvant therapy[53]. However, the low conversion rate of 1.5% and the excellent oncological outcomes achieved by a high volume skilled surgeon in low BMI patients may not be reproducible. All of the procedures in this trial have been performed by seven highly skilled laparoscopic specialists (each one performed more than 200 laparoscopic rectal resections). This suggests that excellent results in laparoscopic rectal cancer surgery can be achieved in expert surgeons. A recent study assessing the learning period for laparoscopic TME stated that 90 operations were required to achieve adequate oncological safety. However, fewer surgeries were needed to achieve operative safety[35].

Robot-assisted surgery may overcome several technical limitations of conventional laparoscopy such as a stable and high-definition 3D image, finer dissection with articulated tools, and better ergonomics for the surgeon. Several meta-analyses comparing robotic and laparoscopic TME[10-14] showed there was a lower percentage of conversion for robotic surgery. However, intraoperative reports indicate there are no significant differences in short-term and oncologic outcomes between the two approaches.

We did not evaluate the costs and preservation of genitourinary function in this study. One of the main concerns regarding robotic technology is the expense and maintenance of the equipment. Baek showed there are increased costs in robotic rectal resection compared to the standard laparoscopic procedure[54]. Conversely, recent studies have demonstrated a superiority of robotic rectal resection in recovery of urinary voiding and sexual function[55,56] due to improved visualization of the autonomic plexii in the pelvis. We are waiting for the results of the ROLARR and COLRAR trials to better define the optimal surgical approach in patients with advanced middle and low rectal cancer.

There are several aspects of our study that me-

rit discussion. First, the patients were assigned to robotic surgery or laparoscopy in an uncontrolled and nonrandomized manner, which is a limitation. However, to reduce the margin of error the data were obtained independently by two authors. Additionally, the retrospective nature of this study is a limitation. However, both surgical centres followed similar perioperative and oncological protocols. Therefore, clinical differences were reduced. Furthermore, this study was limited by its small sample size.

In conclusion, our observations suggest that L-TME and R-TME can be safely performed at high volume centres for rectal cancer. Both procedures achieve acceptable clinical and oncologic outcomes. Moreover, the robotic technique shows some advantages in rectal surgery that should be validated by further studies.

COMMENTS

Background

The short- and long-term outcomes of laparoscopic total mesorectal excision (L-TME) were found to be acceptable in previous reports. However, the benefits of the robotic approach for treatment of rectal cancer (R-TME) have not been clearly described.

Research frontiers

There were no differences found in the rates of conversion to open surgery and morbidity. The patients who underwent laparoscopic surgery stayed in the hospital two days longer than the robotic group patients (8 d or L-TME and 6 d for R-TME, $P < 0.001$). The pathologic evaluation showed a higher number of harvested lymph nodes in the robotic group (18 for R-TME; 11 for L-TME; $P < 0.001$) and a shorter distal resection margin for laparoscopic patients (1.5 cm for L-TME; 2.5 cm for R-TME; $P < 0.001$). The three-year overall survival and disease-free survival rates were similar between groups.

Innovations and breakthroughs

Both procedures achieved acceptable clinical and oncologic outcomes. Moreover, the robotic technique showed some advantages in rectal surgery that should be validated by further studies.

Applications

Author's observations suggested that L-TME and R-TME can be safely performed at high volume centres for rectal cancer.

Peer-review

This is a retrospective comparative study of a prospectively collected data of 111 patients who underwent minimally invasive TME (53 patients robotic assisted *vs* 58 patients laparoscopic assisted TME). The authors concluded that both techniques achieved acceptable similar clinical and oncologic outcomes. The manuscript is well written in clear English, and the authors addressed the study limitation such as the small study sample size, selection bias, and the retrospective nature of the study.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Different risk factors for advanced colorectal neoplasm in young adults

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Author contributions: Kim JY analyzed the data and wrote the manuscript as a first author; Park DI organized and supervised the study as a corresponding author; Jung YS, Park JH and Kim HJ contributed to collect and arrange the data; Cho YK and Sohn CI attended to analyze the data; Jeon WK supported the statistical analysis; Kim BI attended to revise the manuscript and Choi KY created the study concept and design.

Institutional review board statement: The study was reviewed and approved by the Kangbuk Samsung Hospital Institutional Review Board.

Informed consent statement: This study can prejudice the study participants no more than minimal risk. Data which were used in this study were already acquired for report of the result to subjects who had examination and administration of the result. The present study could contribute to preventing the disease through interpretation and application of the results of health care examination. There will be no risk to participants because this study will be analyzed retrospectively using only obtained data without additional administration of medicine, treatment or examination. Kangbuk Samsung Hospital institutional review board exempted the written informed consent of the present study.

Conflict-of-interest statement: None of the authors have any conflicts of interest or financial arrangements that could potentially influence the described research.

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Abstract

AIM: To compare the risk of developing advanced colorectal neoplasm (ACRN) according to age in Koreans.

METHODS: A total of 70428 Koreans from an occupational cohort who underwent a colonoscopy between 2003 and 2012 at Kangbuk Samsung Hospital were retrospectively selected. We evaluated and compared odds ratios (OR) for ACRN between the young-adults (YA < 50 years) and in the older-adults (OA $\geq$ 50 years). ACRN was defined as an adenoma $\geq$ 10 mm in diameter, adenoma with any component of villous histology, high-grade dysplasia, or invasive cancer.

RESULTS: In the YA group, age (OR = 1.08, 95%CI: 1.06-1.09), male sex (OR = 1.26, 95%CI: 1.02-1.55), current smoking (OR = 1.37, 95%CI: 1.15-1.63), family history of colorectal cancer (OR = 1.46, 95%CI: 1.01-2.10), diabetes mellitus related factors (OR = 1.27, 95%CI: 1.06-1.54), obesity (OR = 1.23, 95%CI: 1.03-1.47), CEA (OR = 1.04, 95%CI: 1.01-1.09) and low-density lipoprotein-cholesterol (OR = 1.01, 95%CI: 1.01-1.02) were related with an increased risk of ACRN. However, age (OR = 1.08, 95%CI: 1.06-1.09), male sex (OR = 2.12, 95%CI: 1.68-2.68), current smoking (OR = 1.38, 95%CI: 1.12-1.71), obesity (OR = 1.34, 95%CI: 1.09-1.65) and CEA (OR = 1.05, 95%CI: 1.01-1.09) also increased the risk of ACRN in the OA group.

CONCLUSION: The risks of ACRN differed based on age group. Different colonoscopic screening strategies are appropriate for particular subjects with risk factors for ACRN, even in subjects younger than 50 years.

Key words: Young-adult; Advanced colorectal neoplasm; Risk factors; Age; Metabolic abnormality

Core tip: The development of colorectal cancer can be prevented through screening colonoscopy with detection and removal of advanced colorectal adenomas. Age is an important risk factor for development of advanced colorectal neoplasm (ACRN). Risk factors for the development of ACRN differ between young adults (YA, < 50 years) and older adults (OA, ≥ 50 years). Metabolic abnormalities including diabetes mellitus related factors and serum level of low-density lipoprotein-cholesterol were more related with increased risk of ACRN in the YA group. Different colonoscopic screening strategies would be appropriate to the particular subjects with risk factors for ACRN, even those younger than 50 years.

INTRODUCTION

Colorectal cancer (CRC) has primarily been considered a Western disease, however, the incidence and mortality of CRC has been increasing in Asia, especially in South Korea[1]. The estimated age-standardized rate of CRC was high in world-wide of 45.0 per 100000 in South Korea[2,3]. CRC has become an important clinical burden in South Korea. It has been well documented that most CRC arises from colorectal adenomas (CRA)[4]. Especially, advanced CRA is a definite precancerous lesion and the development of CRC can be prevented with a screening colonoscopy that detects and removes advanced CRA[5].

Unlike other cancers, no single risk factor accounts for most cases of CRC[6]. It has been reported that old age, male sex, family history, diabetes mellitus (DM), smoking, alcohol, obesity and inflammatory bowel disease were related with the development of advanced colorectal neoplasm (ACRN) and modifiable factors including dietary factor and exercise affect ACRN as well[6].

Recently, screening using colonoscopy has decreased the incidence of CRC[7,8]. However, the prevalence of ACRN is still around 3.5% in subject younger than 50 years[9] and rectal cancer is also increased in this group[10]. In many countries including South Korea, fecal occult blood testing or colonoscopy is used for CRC screening starting at the age of 50[11,12]. Few studies investigate the risk factors for ACRN in large cohorts of subjects younger than 50 years. In these backgrounds, the aim of the present study was to compare the differences for risk factors of ACRN between the subjects younger or older than 50 years in a large population of Korean subjects who underwent screening a colonoscopy.

MATERIALS AND METHODS

Study population

The study population consisted of subjects who underwent a comprehensive health examination between 2003 and 2012 at Kangbuk Samsung Hospital, College of Medicine, Sungkyunkwan University. We excluded (1) patients with a history of other cancers; (2) patients with a history of inflammatory bowel disease; (3) patients who had taken a previous colonoscopy; (4) patients who had undergone colon surgery; (5) patients who had an incomplete colonoscopy; and (6) patients with missing data. The flow chart of subject inclusion and exclusion of subjects in analysis is described in Figure 1. The study population was classified into two groups according to age. Patients who were younger than 50 were defined as the young-adult (YA) group and while those who were older than 50 were assigned to the older-adult (OA) group. This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital.

Measurements

Data on medical history, medication use, and health-related behaviors were collected through a self-administered questionnaire under the supervision of a well-trained interviewer. Alcohol consumption and cigarette smoking were identified. A heavy drinker was defined as a subject who drinks more than 4 times per week. Family history of CRC was defined as CRC in one

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┌─────────────────────────────────┐
│  Potential participants identified  │
│        (n = 121794)             │
└─────────────────────────────────┘
                │
                │        ┌──────────────────────────────────────────┐
                │───────▶│ Exclusions (n = 35247)                   │
                │        │     Other cancer patients: 1304          │
                │        │     Inflammatory bowel disease patients: 499 │
                │        │     Previous colonoscopy: 27160          │
                │        │     Previous history of colon surgery: 68 │
                │        │     incomplete study: 6266               │
                │        └──────────────────────────────────────────┘
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│  Potential participants identified  │
│        (n = 86547)              │
└─────────────────────────────────┘
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                │        ┌──────────────────────────────────────────┐
                │───────▶│ Exclusions (n = 16119)                   │
                │        │     Missing data: 16119                  │
                │        └──────────────────────────────────────────┘
                ▼
┌─────────────────────────────────┐
│  Participants identified for analyses │
│        (n = 70428)              │
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Figure 1 Flow diagram of subject enrollment.

or more first-degree relatives at any age. The weekly frequency of moderate to vigorous physical activity was also assessed.

Physical characteristics and serum biochemical parameters were measured by a trained nurse. Body weight was measured with subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. The Asia-Pacific criteria for obesity based on BMI guidelines were used to diagnose obesity[13]. Subjects with a BMI $\geq$ 25 kg/m^2 were defined as obese. Trained nurses measured blood pressure using standard mercury sphygmomanometers with subjects seated after at least 10 min of rest.

Blood samples were taken from the antecubital vein after at least 10 h of fasting. Serum triglyceride and total cholesterol levels were determined using an enzymatic colorimetric assay; low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) levels were determined using a homogeneous enzymatic colorimetric assay. Serum carcinoembryonic antigen (CEA) and hemoglobin A1c (HbA1c) levels were measured by immunoassay. Serum insulin level was measured using an electrochemiluminescence immunoassay on the Modular Analytics E170 apparatus (Roche Diagnostics). Serum fasting glucose level was measured using the hexokinase method on the Cobas Integra 800 apparatus (Roche Diagnostics). The Laboratory Medicine Department at Kangbuk Samsung Hospital

in Seoul, South Korea has been accredited by the Korean Society of Laboratory Medicine and the Korean Association of Quality Assurance for Clinical Laboratories.

In the present study, the authors evaluated the effect of metabolic abnormalities (MetA) on the development of ACRN. MetA was defined according to the modified National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III criteria[14] as follows: (1) triglycerides $\geq$ 150 mg/dL or specific treatment for this lipid abnormality; (2) high-density lipoprotein cholesterol < 40 mg/dL for men and < 50 mg/dL for women; (3) elevated blood pressure $\geq$ 130/85 mmHg or use of antihypertensive medications as hypertension (HTN)-related factors; and (4) fasting plasma glucose $\geq$ 100 mg/dL, hemoglobin A1c $\geq$ 6.5%, or use of diabetes medications as DM-related factors.

Diagnosis of colorectal neoplasm
Colonoscopies were performed by experienced colonoscopists unaware of the present study. Bowel preparations were carried out using 4 L of polyethylene glycol solution. Most subjects were consciously sedated with midazolam and pethidine. Histological assessment of all polyps was performed by experienced pathologists unaware of the subjects' clinical data. ACRN was defined as an adenoma $\geq$ 10 mm in diameter, adenoma with any component of villous histology, high-grade dysplasia, or invasive cancer[15].

Table 1 Baseline characteristics of 70428 subjects *n* (%)

	Total (*n* = 70428)	< 50 yr (*n* = 59782)	≥ 50 yr (*n* = 10646)	*P* value
Age (year, mean ± SD)	41.6 ± 8.3	38.9 ± 5.3	56.5 ± 5.8	< 0.001
Sex				< 0.001
Male	48868 (69.4)	42644 (71.3)	6244 (58.5)	
Female	21560 (30.6)	17138 (28.7)	4422 (41.5)	
BMI (kg/m², mean ± SD)	23.8 ± 3.1	23.8 ± 3.2	24.1 ± 2.8	< 0.001
Obesity[1]	23658 (33.6)	19955 (33.4)	3703 (34.8)	0.005
Smoking				< 0.001
Never	38955 (55.3)	33071 (55.3)	5884 (55.3)	
Former	11480 (16.3)	9610 (16.1)	1870 (17.6)	
Current	19993 (28.4)	17101 (28.6)	2892 (27.2)	
Alcohol intake				< 0.001
No	22090 (31.4)	18517 (31.0)	3573 (33.6)	
Moderate	46026 (65.4)	39404 (65.9)	6622 (62.2)	
Heavy[2]	2312 (3.3)	1861 (3.1)	451 (4.2)	
CRC family history	2779 (3.9)	2111 (3.5)	668 (6.3)	< 0.001
SBP (mmHg)	113.3 ± 13.0	113.0 ± 12.9	115.0 ± 13.7	< 0.001
DBP (mmHg)	72.6 ± 9.6	72.3 ± 9.5	74.1 ± 9.8	< 0.001
HTN-R[3]	23871 (33.9)	19227 (32.2)	4644 (43.6)	< 0.001
DM-R[4]	16886 (24.0)	11983 (20.0)	3718 (34.9)	< 0.001
Regular exercise[5]	38379 (54.5)	33398 (55.9)	4981 (46.8)	0.000
Fasting glucose (mg/dL)	93.6 ± 14.8	92.9 ± 13.7	97.7 ± 19.2	< 0.001
Insulin (μIU/mL)	5.3 ± 5.1	5.1 ± 3.3	6.2 ± 10.5	< 0.001
HbA1c (%)	5.7 ± 0.5	5.6 ± 0.5	5.8 ± 0.7	< 0.001
Total-C (mg/dL)	199.8 ± 34.7	198.6 ± 34.3	206.4 ± 36.2	< 0.001
Triglyceride (mg/dL)	116.0 ± 76.3	115.3 ± 76.7	119.5 ± 73.8	< 0.001
Triglyceride ≥ 150 mg/dL	15456 (21.9)	12957 (21.7)	2499 (23.5)	< 0.001
HDL-C (mg/dL)	55.1 ± 13.8	55.2 ± 13.8	54.8 ± 13.8	0.003
HDL-C abnormality[6]	10901 (15.5)	8727 (14.6)	2174 (20.4)	< 0.001
LDL-C (mg/dL)	124.9 ± 32.0	124.1 ± 31.7	129.0 ± 33.1	< 0.001
CEA (ng/mL)	1.62 ± 1.61	1.58 ± 1.61	1.83 ± 1.58	< 0.001
ACRN[7]	960 (1.4)	564 (0.9)	396 (3.7)	< 0.001
CRC	54 (0.1)	25 (0.1)	29 (0.3)	< 0.001

[1]BMI ≥ 25 kg/m²; [2]More than 4 times per week as heavy; [3]Elevated blood pressure ≥ 130/85 mmHg or use of antihypertensive medications; [4]Fasting plasma glucose ≥ 100 mg/dL or hemoglobin A1c ≥ 6.5%, or use of diabetes medications; [5]More than once per week; [6]HDL-C < 40 mg/dL for men or < 50 mg/dL for women; [7]ACRN was defined as a polyp size of ≥ 10 mm in diameter, polyp with any component of villous histology, high-grade dysplasia, or invasive cancer. SD: Standard deviation; BMI: Body mass index; CRC: Colorectal cancer; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HTN-R: Hypertension-related factors; DM-R: Diabetes mellitus-related factors; HbA1c: Hemoglobin A1c; Total-C: Total cholesterol; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; CEA: Carcinoembryonic antigen; ACRN: Advanced colorectal neoplasm.

Statistical analysis

Continuous variables including age, BMI, blood pressure and laboratory values were expressed as mean ± SD and compared using the Student's *t*-test. Categorical variables including sex, alcohol consumption, smoking status, CRC family history, underlying metabolic condition, regular exercise and development of CRC were expressed as percentages and analyzed using the χ^2 test. We used multiple logistic regression analysis to determine the odds ratios (OR) for developing ACRN. *P* values < 0.05 were considered statistically significant. All data analysis was performed using SPSS for Windows (Version 18.0; SPSS, Chicago, IL, United States). The present study was reviewed by our expert biostatistician, Lee MY.

RESULTS

A total of 121794 subjects underwent a screening colonoscopy and a comprehensive health examination at Kangbuk Samsung Hospital between 2003 and 2012. We excluded 1304 subjects with other cancers, 499 patients with inflammatory bowel disease, 27160 subjects with a previous colonoscopy, 68 subjects with a history of colon surgery, 6266 subjects with an incomplete colonoscopy and 16119 subjects with missing data. Finally, 70428 subjects were included in this analysis (Figure 1).

There were 59782 subjects younger than 50 and 10646 subjects older than 50. Baseline characteristics of YA and OA subjects are described in Table 1. The mean age of the YA group was 38.9 ± 5.3 years and that of the OA group was 56.5 ± 5.8 years. There were more males in the YA group (71.3%) than in the OA group (58.5%, *P* < 0.001). There were 960 total cases of ACRN (1.4%), with 564 cases (0.9%) in the YA group and 396 cases (3.7%) in the OA group. The prevalence of CRC was also more frequent in the OA group (0.3%) than in the YA group (0.1%). Baseline characteristics of male and female subjects are

Table 2 Multivariate analysis of risk factors for the development of advanced colorectal neoplasm in subjects under 50

	n (%)	OR	95%CI	*P* value
Age	564/59782 (0.9)	1.08	1.07-1.10	< 0.001
Sex				
Female	123/17138 (0.7)		Reference	
Male	441/42644 (1.0)	1.26	1.02-1.55	0.036
Smoking				
Never/former	358/42681 (0.8)		Reference	
Current	206/17101 (1.2)	1.37	1.15-1.63	< 0.001
CRC family history				
No	533/57671 (0.9)		Reference	
Yes	31/2111 (1.5)	1.46	1.01-2.10	0.044
DM-R				
No	405/47799 (0.8)		Reference	
Yes	159/11983 (1.3)	1.27	1.06-1.54	0.012
Obesity				
No	327/39827 (0.8)		Reference	
Yes	237/19955 (1.2)	1.23	1.03-1.47	0.021
LDL-C	564/59782 (0.9)	1.01	1.01-1.02	0.006
CEA	564/59782 (0.9)	1.04	1.01-1.09	0.031

Adjusted for age, sex, smoking, alcohol, obesity (body mass index $\geq$ 25 kg/m^2), triglyceride $\geq$ 150 mg/dL, HDL-C abnormality (HDL-C < 40 mg/dL for men or < 50 mg/dL for women), LDL-C, DM-R (fasting plasma glucose $\geq$ 100 mg/dL or hemoglobin A1c $\geq$ 6.5%, or use of diabetes medications), HTN-R (elevated blood pressure $\geq$ 130/85 mmHg or use of antihypertensive medications), insulin, regular exercise, CRC family history and CEA. OR: Odds ratio; CI: Confidence interval; CRC: Colorectal cancer; DM-R: Diabetes mellitus-related factors; HTN-R: Hypertension-related factors; CEA: Carcinoembryonic antigen; LDL-C: Low-density lipoprotein-cholesterol; HDL-C: High-density lipoprotein-cholesterol.

described in Supplementary Table 1.

ORs were calculated for each risk factors for ACRN in YA subjects (Table 2). Even in the YA group, age (OR = 1.08, 95%CI: 1.07-1.10, *P* < 0.001) was related with increased risk of ACRN. Risk was also increased in males (OR = 1.26, 95%CI: 1.02-1.55, *P* < 0.001) and current smokers (OR = 1.37, 95%CI: 1.15-1.63, *P* < 0.001). A family history of CRC (OR = 1.46, 95%CI: 1.01-2.10, *P* = 0.044) was also related with ACRN. Metabolic factors significantly affected ACRN development. Subject with DM-related factors (OR = 1.27, 95%CI: 1.06-1.54, *P* = 0.012), obesity (OR = 1.23, 95%CI: 1.03-1.47, *P* = 0.021) and serum LDL-C levels (OR = 1.01, 95%CI: 1.01-1.02, *P* = 0.006) increased risk of ACRN. Serum CEA level (OR = 1.04, 95%CI: 1.01-1.09, *P* = 0.031) was also related with the development for ACRN. The risk factors of ACRN in the YA group were also analyzed by sex (Table 3). In females, only age (OR = 1.08, 95%CI: 1.04-1.11, *P* < 0.001) and serum CEA levels (OR = 1.12, 95%CI: 1.02-1.23, *P* = 0.023) were related with ACRN. However, in males, age (OR = 1.09, 95%CI: 1.07-1.10, *P* < 0.001), current smoking (OR = 1.54, 95%CI: 1.27-1.87, *P* < 0.001), CRC family history (OR = 1.58, 95%CI: 1.06-2.36, *P* = 0.026), DM-related factors (OR = 1.34, 95%CI: 1.09-1.65, *P* = 0.005), obesity (OR = 1.24, 95%CI: 1.02-1.50, *P* = 0.028), serum LDL-C level (OR = 1.01, 95%CI: 1.01-1.02, *P* = 0.008) and serum CEA level (OR = 1.03, 95%CI: 1.01-1.05, *P* = 0.014) related with ACRN.

In the OA group, age (OR = 1.08, 95%CI: 1.06-1.09, *P* < 0.001) and male sex (OR = 2.12, 95%CI: 1.68-2.68, *P* < 0.001) were also significantly related with ACRN. ACRN was increased in smokers

(OR = 1.38, 95%CI: 1.12-1.71, *P* = 0.003) and obese subjects (OR = 1.34, 95%CI: 1.09-1.65, *P* = 0.005). Serum CEA levels (OR = 1.05, 95%CI: 1.01-1.09, *P* = 0.022) also related with ACRN (Table 4). Risk factors for ACRN in the OA group by sex were also assessed (Table 5). In females, only age (OR = 1.04, 95%CI: 1.01-1.08, *P* = 0.006) was related with ACRN. In males, age (OR = 1.09, 95%CI: 1.07-1.11, *P* < 0.001), current smoking (OR = 1.44, 95%CI: 1.12-1.79, *P* = 0.004), obesity (OR = 1.41, 95%CI: 1.11-1.79, *P* = 0.005) and serum CEA level (OR = 1.04, 95%CI: 1.01-1.08, *P* = 0.023) related with ACRN. Whereas, family history of CRC showed a protective effect (OR = 0.55, 95%CI: 0.31-0.96, *P* = 0.037).

DISCUSSION

The present study evaluated different risk factors for ACRN according to age and sex groups. To the best of our knowledge, this is the first study to compare laboratory factors, anthropometric data and life habits between the age groups in a large population when evaluating the risk of ACRN.

Many studies have evaluated age as an important risk factor for development of CRA and CRC[16-18]. In the present study, the prevalence of ACRN and CRC were significantly greater in the OA group (3.7% and 0.3%, respectively) than in the YA group (0.9% and 0.1%, respectively). Age was also an important risk factor for ACRN in the OA and YA groups and in the male and female subgroups. In particular, it was the only risk factor for ACRN in the female subgroup of the OA group. Therefore, age should be regarded as a critical factor in screening colonoscopy. The authors

Table 3 Multivariate analysis of risk factors for the development of advanced colorectal neoplasm in subjects under 50 according to sex

	n (%)	OR	95%CI	*P* value
Female (*n* = 17138)				
Age	123/17138 (0.7)	1.08	1.04-1.11	< 0.001
CEA	123/17138 (0.7)	1.12	1.02-1.23	0.023
Male (*n* = 42644)				
Age	441/42644 (1.0)	1.09	1.07-1.10	< 0.001
Smoking				
Never/former	255/29005 (0.9)		Reference	
Current	186/13639 (1.4)	1.54	1.27-1.87	< 0.001
CRC family history				
No	415/41174 (1.0)		Reference	
Yes	26/1470 (1.8)	1.58	1.06-2.36	0.026
DM-R				
No	300/32967 (0.9)		Reference	
Yes	141/9677 (1.5)	1.34	1.09-1.65	0.005
Obesity				
No	226/25027 (0.9)		Reference	
Yes	215/17617 (1.2)	1.24	1.02-1.50	0.028
LDL-C	441/42644 (1.0)	1.01	1.01-1.02	0.008
CEA	441/42644 (1.0)	1.03	1.01-1.05	0.014

Adjusted for age, sex, smoking, alcohol, obesity (body mass index $\geq$ 25 kg/m^2), abdominal obesity (waist circumference $\geq$ 90 cm for men or $\geq$ 85 cm for women), triglyceride $\geq$ 150 mg/dL, HDL-C abnormality (HDL-C < 40 mg/dL for men or < 50 mg/dL for women), LDL-C, DM-R (fasting plasma glucose $\geq$ 100 mg/dL or hemoglobin A1c $\geq$ 6.5%, or use of diabetes medications), HTN-R (elevated blood pressure $\geq$ 130/85 mmHg or use of antihypertensive medications), insulin, regular exercise, CRC family history and CEA. OR: Odds ratio; CI: Confidence interval; CRC: Colorectal cancer; DM-R: Diabetes mellitus-related factors; HTN-R: Hypertension-related factors; CEA: Carcinoembryonic antigen; LDL-C: Low-density lipoprotein-cholesterol; HDL-C: High-density lipoprotein-cholesterol.

Table 4 Multivariate analysis of risk factors for the development of advanced colorectal neoplasm in subjects over 50

	n (%)	OR	95%CI	*P* value
Age	396/10646 (3.7)	1.08	1.06-1.09	< 0.001
Sex				
Female	102/4422 (2.3)		Reference	
Male	294/6224 (4.7)	2.12	1.68-2.68	< 0.001
Smoking				
Never/former	261/7754 (3.4)		Reference	
Current	135/2892 (4.7)	1.38	1.12-1.71	0.003
Obesity				
No	229/6943 (3.3)		Reference	
Yes	167/3703 (4.5)	1.34	1.09-1.65	0.005
CEA	396/10646 (3.7)	1.05	1.01-1.09	0.022

Adjusted for age, sex, smoking, alcohol, obesity (body mass index $\geq$ 25 kg/m^2), triglyceride $\geq$ 150 mg/dL, HDL-C abnormality (HDL-C < 40 mg/dL for men or < 50 mg/dL for women), LDL-C, DM-R (fasting plasma glucose $\geq$ 100 mg/dL or hemoglobin A1c $\geq$ 6.5%, or use of diabetes medications), HTN-R (elevated blood pressure $\geq$ 130/85 mmHg or use of antihypertensive medications), insulin, regular exercise, CRC family history and CEA. OR: Odds ratio; CI: Confidence interval; CRC: Colorectal cancer; DM-R: Diabetes mellitus-related factors; HTN-R: Hypertension-related factors; CEA: Carcinoembryonic antigen; LDL-C: Low-density lipoprotein-cholesterol; HDL-C: High-density lipoprotein-cholesterol.

considered screening colonoscopies beginning at 50 years of age to be appropriate and acceptable.

In the present study, male sex was revealed as a risk factor for ACRN regardless of age group, with an adjusted OR with 2.12 in the OA group. Previous studies reported that male sex increased the risk of CRA[17,19,20]. and this association was also found for ACRN[21]. Smoking was associated with significantly increased risk of ACRN in the modifiable factors through life style modification and this result was consistent with previous studies[22,23]. In the present study, this relationship was evaluated regardless of age groups, especially in males. The effect of smoking on

ACRN according to sex was not clear. A previous study reported that smoking affected females more[24], while other studies reported the effect of smoking on CRC was similar in both sexes[25] or greater in males[18]. Whereas, alcohol consumption was not associated with ACRN in our study. The effect of alcohol on the development of ACRN is controversial[26,27]. Further study is required.

A family history of CRC within first degree relatives is a well-known risk factor for ACRN[28,29]. In the present study, CRC family history was evaluated as a risk factor of ACRN in the YA group, whereas it showed a protective effect in the OA group. This was

Table 5 Multivariate analysis of risk factors for the development of advanced colorectal neoplasm in subjects over 50 according to sex

	n (%)	OR	95%CI	*P* value
Female (*n* = 4422)				
Age	102/4422 (2.3)	1.04	1.01-1.08	0.006
Male (*n* = 6224)				
Age	294/6224 (4.7)	1.09	1.07-1.11	< 0.001
Smoking				
Never/former	188/4410 (4.3)		Reference	
Current	106/1814 (5.8)	1.44	1.12-1.79	0.004
Obesity				
No	164/3833 (4.3)		Reference	
Yes	130/2391 (5.4)	1.41	1.11-1.79	0.005
CRC family history				
No	281/5796 (4.8)		Reference	
Yes	13/438 (3.0)	0.55	0.31-0.96	0.037
CEA	294/6224 (4.7)	1.04	1.01-1.08	0.023

Adjusted for age, smoking, alcohol, obesity (body mass index $\geq$ 25 kg/m^2), triglyceride $\geq$ 150 mg/dL, HDL-C abnormality (HDL-C < 40 mg/dL for men or < 50 mg/dL for women), LDL-C, DM-R (fasting plasma glucose $\geq$ 100 mg/dL or hemoglobin A1c $\geq$ 6.5%, or use of diabetes medications), HTN-R (elevated blood pressure $\geq$ 130/85 mmHg or use of antihypertensive medications), insulin, regular exercise, CRC family history and CEA. OR: Odds ratio; CI: Confidence interval; CRC: Colorectal cancer; DM-R: Diabetes mellitus-related factors; HTN-R: Hypertension-related factors; CEA: Carcinoembryonic antigen; LDL-C: Low-density lipoprotein-cholesterol; HDL-C: High-density lipoprotein-cholesterol.

due to the fact that this study targeted subjects at their first screening colonoscopy. In the OA group, high risk subjects with a familial history of CRC were likely excluded because they had started screening colonoscopies in their forties. The authors do not conclude that familial CRC history is protective in older adults; however, early screening colonoscopies may be protective in patients younger than 50 with a family history of CRC.

There are clear associations between ACRN and metabolic syndromes and obesity[30-33]. Obesity was related with an increased risk of ACRN in both the OA and YA groups in the present study. However, in cases of MetA, DM-related factors and LDL-C increased the risk of ACRN only in the YA group. These significant associations were not evaluated in the OA group. Especially, the authors evaluated the risks of ACRN in subjects with documented DM or dyslipidemia as well in subjects with early-stage MatA using NCEP-ATP Ⅲ criteria. There were no associations between hypertriglyceridemia or HDL-C abnormalities and ACRN in the YA group. Subjects under 50 with metabolic syndrome or any glucose or LDL-C metabolism abnormality should be receive special attention. It was reported that metabolic syndrome and smoking significantly impacted both the prevalence of colorectal neoplasms and the diagnostic yield of screening tests in men aged 40 to 49 years[34]. The reasons why younger subjects were more susceptible to abnormal glucose or lipid metabolism were not clearly evaluated. It was reported that obesity and metabolic syndrome affected on CRA in the young subjects[35], however, a study which was performed in South Korea was also evaluated that the effect of obesity and metabolic syndrome disappeared in the subjects with older than 50 years[36]. The reasons for the greater effect of obesity and MetA on the YA group require further

evaluation.

The prevalence of ACRN was 1.0% in females, which was lower than in males (1.5%). In the OA group, 2.3% of females and 4.7% of males had ACRNs, while in the YA group, 0.7% of females and 1.0% of males had ACRN. In the present study, the effects of obesity and MetA on ACRN were evaluated only in males, however, there was no such relationship in females. The mean BMIs of the present study were much lower in females (21.9 ± 3.0 kg/m^2 in the YA group and 23.7 ± 3.0 kg/m^2 in the OA group) than in males (24.6 ± 2.9 kg/m^2 in the YA group and 24.3 ± 2.6 kg/m^2 in the OA group). Most serum markers were higher in males than in females. These absolute values of BMI and serum markers might cause differences between females and males, although the details of these effects remain unclear. Previous studies reported that BMI or MetA was associated with increased risk of CRC or CRA in males, whereas this association was generally weaker in females[37-41]. Generally, these differences are considered to be related to the hormonal status of premenopausal women. Preclinical data support a role for estrogen and its receptors in the initiation and progression of CRC and estrogen can exert protective effects through estrogen receptor β[42]. CRC risk increased in subjects with MetA[43], and adipokines can link obesity with CRC risk in postmenopausal women[44]. Whereas, recent studies found significant associations with the risk of CRC development even in postmenopausal women[45]. Further studies are needed to clarify the differences in risk by sex.

The present study had several limitations. Around 20% of initial subject in YA group and around 30% of initial subject in OA group were excluded because they had previous colonoscopy. The authors targeted subjects at their first screening colonoscopy, so high

risk subjects with a family history of CRC in the OA group might be missed. These could effect on the study as a selection bias. However, the present study aimed to assess the average risk of ACRN. Subjects who had previous colonoscopy is heterogenous group including subjects with ACRN, subjects with low risk adenoma or subject with normal colonoscopy. It is also difficult to assess the result of previous colonoscopy relying on subject's memory. Therefore, the authors excluded the subjects who had previous colonoscopy to prevent the heterogeneity of study subjects. Adenoma detection rate or the present study was 14.2% and the rate was slightly low when it was compared with recommended adenoma detection rate. However, the present study targeted subject who took first colonoscopy and the majority of subject were YA with under the age of 50. This could make adenoma detection rate relatively low. The cross-sectional study with a single ethnic group precludes our ability to assess causation. Data on smoking, alcohol intake and exercise were evaluated by simple questionnaires, not quantitatively. However, data collection was difficult because this was a large-scale population-based study. However, we collected various anthropometric measurements and metabolic laboratory factors which were frequently used in clinical practice for all study participants. This provides valuable risk factors, which are crucial for determining screening strategies.

The prevalence of ACRN is 5%-10% in Western countries[26,28], however, it is only 1%-5% in Asia[17,36]. Therefore, clear targets for screening colonoscopy and ACRN evaluation are required in Asian populations. In the present study, age, male, current smoking, obesity and CEA were important risk factors for ACRN, despite the low prevalence. Furthermore, all study subjects were asymptomatic, of average risk, enrolled in a screening setting and were representative of our general screening target population. Especially, in the YA group, subject with family history of CRC or MetA including DM-related factor or increased LDL-C significantly increased the risk of ACRN. The authors enrolled almost 60000 subjects younger than 50 with a mean age of 38.9 ± 5.3 years, which was much younger than subjects in the previous studies. The authors recommend screening colonoscopies in subjects under 50 with specific risk factors. These results are meaningful because of the large YA population in this study.

In conclusion, risk factors for ACRN differed between the OA and YA groups. This study provides a valuable link between risk factors and colonoscopic findings according to age, which are crucial for determining screening strategies. Our findings suggest that more careful and aggressive screening should be considered for subjects with specific considerations, even younger than 50 years.

COMMENTS

Background
Development of advanced colorectal neoplasm (ACRN) is related with several risk factors. Evaluating the risk of ACRN is important for preventing the colorectal cancer. However, few studies evaluated different risk factor between young adults (YA, < 50 years) and older adults (OA, ≥ 50 years).

Research frontiers
To compare the risk of developing ACRN according to age in Koreans

Innovations and break through
The risks of ACRN differed based on age group. Age was an important risk factor for ACRN in both YA and OA groups. Metabolic abnormalities including diabetes mellitus (DM) related factors and serum level of low-density lipoprotein-cholesterol (LDL-C) were more related with increased risk of ACRN in the YA group.

Applications
Colorectal cancer (CRC) screening using colonoscopy was recommended after 50 years. The risks of ACRN differed based on age group. Different colonoscopic screening strategies are appropriate for particular subjects with risk factors for ACRN, even in subjects younger than 50 years.

Terminology
ACRN was defined as an adenoma ≥ 10 mm in diameter, adenoma with any component of villous histology, high-grade dysplasia, or invasive cancer.

Peer-review
The manuscript described the risk factors for ACRN in young Korean adults included age, male gender, current smoking, family history of CRC, DM, obesity, LDL-C and carcinoembryonic antigen. The sheer size of this study including 70000 patients who underwent colonoscopy probably warrants publication.

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ORIGINAL ARTICLE

Retrospective Study

Proposal of a computed tomography classification for hepatic alveolar echinococcosis

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Abstract

AIM: To establish a computed tomography (CT)-morphological classification for hepatic alveolar echinococcosis was the aim of the study.

METHODS: The CT morphology of hepatic lesions in 228 patients with confirmed alveolar echinococcosis (AE) drawn from the Echinococcus Databank of the University Hospital of Ulm was reviewed retrospectively. For this reason, CT datasets of combined positron emission tomography (PET)-CT examinations were evaluated. The diagnosis of AE was made in patients

with unequivocal seropositivity; positive histological findings following diagnostic puncture or partial resection of the liver; and/or findings typical for AE at either ultrasonography, CT, magnetic resonance imaging or PET-CT. The CT-morphological findings were grouped into the new classification scheme.

RESULTS: Within the classification a lesion was dedicated to one out of five "primary morphologies" as well as to one out of six "patterns of calcification". "primary morphology" and "pattern of calcification" are primarily focussed on separately from each other and combined, whereas the "primary morphology" V is not further characterized by a "pattern of calcification". Based on the five primary morphologies, further descriptive sub-criteria were appended to types Ⅰ-Ⅲ. An analysis of the calcification pattern in relation to the primary morphology revealed the exclusive association of the central calcification with type Ⅳ primary morphology. Similarly, certain calcification patterns exhibited a clear predominance for other primary morphologies, which underscores the delimitation of the individual primary morphological types from each other. These relationships in terms of calcification patterns extend into the primary morphological sub-criteria, demonstrating the clear subordination of those criteria.

CONCLUSION: The proposed CT-morphological classification (EMUC-CT) is intended to facilitate the recognition and interpretation of lesions in hepatic alveolar echinococcosis. This could help to interpret different clinical courses better and shall assist in the context of scientific studies to improve the comparability of CT findings.

Key words: Hepatic alveolar echinococcosis; Diagnosis; Echinococcus multilocularis; Classification; Computed tomography; Alveolar echinococcosis

Core tip: Computed tomography (CT), mostly combined with positron emission tomography, provides one of the most important diagnostic tools in suspected alveolar echinococcosis. Aim of the study was to establish a new CT-classification based on a large patient collective with confirmed hepatic alveolar echinococcosis. The Echinococcosis Multilocularis Ulm Classification-CT presented in this paper is intended to facilitate the recognition and interpretation of hepatic lesions in alveolar echinococcosis based on CT-morphological criteria. It can also be used to more objectively interpret different clinical courses and enhance the comparability of CT findings in the context of scientific studies.

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INTRODUCTION

Alveolar echinococcosis (AE) is caused by the larval (metacestode) stage of the cyclophyllid tapeworm, *Echinococcus multilocularis.* It is considered the most dangerous parasitic disease of Europe and has to be distinguished from the worldwide more common cystic echinococcosis (CE) that is caused by a related tapeworm species, *E. granulosus*[1]. The species' very different patterns of growth in humans result in two distinct disease entities[2]. Thus, in about 98% of cases, AE presents as a malignant-appearing hepatic lesion with a tendency to infiltrative growth and the potential for metastasis; by comparison, CE most often presents as a smooth, clearly demarcated cyst and affects the liver in only about 60% of cases[2]. Humans are infected through the accidental ingestion of the helminth ova. Formation of protoscolices from the larval germinal epithelium occurs only rarely in humans, hence their designation as a dead-end or incidental host of *E. multilocularis*[1].

As a parasitic disease of humans, AE is rare; left untreated, however, it is associated with a high mortality. Southwestern Germany, together with other regions, mostly in neighboring countries, represents one of the largest endemic areas for this parasitosis in Europe[3,4]. Further endemic areas in North America and Asia lie exclusively in temperate climatic zones of the Northern Hemisphere[5,6]. As a result of this regional accumulation of cases, numerous patients with AE have been treated at the University Hospital of Ulm, which has for many years maintained a databank of AE cases.

In addition to serodiagnostics[1,7-10], diagnostic imaging plays a crucial role in the work-up of suspected AE. Ultrasonography (US) is often the initial imaging method[7,11]. Computed tomography (CT), usually in combination with 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET), represents one of the most important imaging modalities, both for initial diagnosis and for monitoring patients' subsequent disease course[12-14]. Calcifications are especially well-visualized at CT while magnetic resonance imaging (MRI) most clearly shows the small vesicular structures that are typical for the disease[15].

Because AE mimics the biological behavior of malignant tumors, the World Health Organization (WHO) Informal Working Group in Echinococcosis developed a classification for staging this disease that is analogous to the TNM system for malignant diseases. The PNM classification (P = parasitic liver lesion; N = infiltration of neighboring organs; M = metastases,

yes/no) uses the findings of diagnostic imaging and provides a standardized assessment of disease stage and stage-adapted therapy recommendations[16].

Whereas a classification of hepatic AE based on its morphological characteristics at MRI had been proposed as early as 2003[17], no systematic characterization of the CT morphology of this disease has been published. Thus, the objective of the present study was to propose a new CT classification for alveolar echinococcosis based on a large patient collective with confirmed hepatic AE. The CT morphological classification proposed in the present study seeks to facilitate the recognition and interpretation of lesions in hepatic AE and to aid in the frequently challenging differential diagnosis that includes neoplasms of the liver such as cholangiocellular carcinoma, biliary cystadenoma and cystadenocarcinoma, or metastases of other tumors[17,18]. The new classification should also assist in the interpretation of patients' clinical course and improve the comparability of CT findings in the context of scientific studies.

As an acronym for the new CT classification, we propose EMUC-CT (Echinococcosis Multilocularis Ulm Classification - CT).

MATERIALS AND METHODS

The CT morphology of hepatic lesions in 228 patients (*n* = 106 males; 122 females; mean age at diagnosis: 50.8 ± 17.1 years; mean age at time of PET-CT examination: 55.6 ± 17.3 years) with confirmed AE drawn from the Echinococcus Databank of the University Hospital of Ulm was reviewed retrospectively. The diagnosis of AE was made in patients with unequivocal seropositivity; positive histological findings following diagnostic puncture or partial resection of the liver; and/or findings typical for AE at either US, CT, MRI or PET-CT. According to the modified WHO criteria of Brunetti *et al*[1] 116 cases (50.9%) were confirmed by positive histopathology and proven specific enzyme linked immunosorbent assay (ELISA) from tissue samples. An additional 97 patients (42.5%) were considered probable cases with positive serology in two different methods and positive imaging for AE with two respective imaging techniques, while 15 patients (6.6%) were considered possible cases with a positive medical history and a positive result for imaging and serology in one test each.

Based on the reviewer's many years' experience together with reports in the literature of CT findings in patients with hepatic AE, individual CT-morphological findings were grouped according to a new classification scheme. For this reason, CT datasets of combined PET-CT examinations were evaluated: these were, in most cases, contrast-enhanced images acquired during the venous phase, though some were native images. All examinations were performed at the University Hospital of Ulm between March 2006 and December

2014 using the following CT scanners: Siemens, Biograph mCT-S(40) (CT: Somatom Definition AS 40; collimation 16 mm × 1.2 mm; - images viewed at 1.5 to 5.0 mm slice thickness with reconstruction intervals equal to or less to slice thickness). General Electrics, Discovery LS (CT: Lightspeed plus; - collimation 4 mm × 5.0 mm; reconstruction slice thickness is 5 mm and the slice interval is 4.25 mm). Prior imaging from other centers was not included. Subsequent assessment of patients' clinical course (data not shown) was based on both the initial and final (as of the date of the study) CT examinations. The initial dataset, which was used for creation of the classification and the present analyses, was not necessarily the first of the entire examination series but the first to be electronically documented in the PACS system.

Review of CT scans revealed broad variability in the morphological appearance of lesions in patients with hepatic AE. Lesions were grouped into five primary morphologies. In cases with multiple hepatic lesions, the largest lesion was generally selected and described according to the classification system. In these cases, it was generally possible to recognize a single primary morphological pattern. Less frequently, lesions in the same liver exhibited different primary morphologies: these must be studied separately during the clinical course.

Assessment of a lesion's calcification pattern separate from its primary morphology was found to be useful since the extent and morphology of calcifications may vary widely over the course of the disease or as a result of therapy and may, in some cases, contribute to a markedly changed appearance, both of the lesions and their primary morphology. Thus, in the context of the classification, lesions were assigned to both a "primary morphology" and a "calcification pattern".

Based on the five primary morphologies, further descriptive sub-criteria were appended to types Ⅰ - Ⅲ. Thus, the presence of cystic components in types Ⅰ and Ⅱ and the presence of more solid portions at the edges in type Ⅲa and Ⅲb, lead to further characterization of the lesion.

The study design complies with the requirements of the Helsinki Declaration and was approved by the Ethics Commission of Ulm University.

Statistical analysis
Statistical analyses were performed using the SAS statistical software package (version 9.2; SAS Institute Inc., Cary, NC, United States). Data were analyzed descriptively with regard to absolute and relative frequencies, means and standard deviation.

RESULTS
The primary morphologies and their respective sub-criteria established for the present study were as follows: Ⅰ. Diffuse infiltrating (with/without cystoid portion); Ⅱ. Primarily circumscribed tumor-like

Figure 1 Schematic representation of the calcification patterns. A: Without calcifications; B: With feathery calcifications; C: With focal calcifications; D: With a central calcification; E: With diffuse calcifications; F: With calcifications primarily at the edge.

Figure 2 Diffuse infiltrating with cystoid portion (A); without cystoid portion (B).

Table 1 Primary morphologies with their respective additional sub-criteria (insofar as sub-criteria are available for a given primary morphology)

Primary morphology
Ⅰ Diffuse infiltrating
With cystoid portion
Without cystoid portion
Ⅱ Primarily circumscribed tumor-like
With cystoid portion
Without cystoid portion
Ⅲ (a) Primarily cystoid - intermediate (approximately 3-8 cm)
With more solid portions at the edge
Without more solid portions at the edge
(b) Primarily cystoid - widespread (approximately > 8 cm)
With more solid portions at the edge
Without more solid portions at the edge
Ⅳ Small-cystoid/metastatic* (approximately < 3 cm)
Ⅴ Mainly calcified

Table 2 Patterns of calcification. The pattern of calcification has to be considered separately from the Primary morphology and then combined. Pattern of calcification group (*) only occurs with Primary morphology IV*; Primary morphology V is not further characterized by a Pattern of calcification

Pattern of calcification
Without calcifications (Figure 1A)
With feathery calcifications (Figure 1B)
With focal calcifications (Figure 1C)
With a central calcification* (Figure 1D)
With diffuse calcifications (Figure 1E)
With calcifications primarily at the edge (Figure 1F)

(with/without cystoid portion); Ⅲa. Primarily cystoid - intermediate (with/without more solid portions at the edge) and Ⅲb. Primarily cystoid - widespread (with/without more solid portions at the edge); Ⅳ. Small-cystoid/metastatic*; and Ⅴ. Mainly calcified (Tables 1 and 2).

With the exception of primary morphology type Ⅴ, the following six calcification patterns were assigned: without calcifications; with feathery calcifications; with focal calcifications; with a central calcification* (possible only with type Ⅳ *); with diffuse calcifications; with calcifications primarily at the edge (Tables 1 and 2; Figure 1).

The different primary morphologies Ⅰ-Ⅴ (together with their respective sub-criteria) are demonstrated from corresponding CT images (Figures 2-7). For primary morphologies Ⅰ-Ⅳ, preferably non- or rather

less-calcified lesions were selected in order to illustrate the relevant morphological features unobscured by significant calcification overlay.

Notwithstanding the separate initial consideration of primary morphology and calcification pattern, certain associations between the two classification criteria were observed, as will be discussed below. For example, the specific calcification pattern of a central calcification was noted to be a characteristic occurrence with type Ⅳ small-cystoid/metastatic disease and can serve in these cases as a crucial diagnostic clue for assignment of unclear CT findings of this manifestation type. Based on this observation, this primary morphology was established as a separate type Ⅳ, distinct from the primary cystoid intermediate or widespread disease described by types Ⅲa and Ⅲb, respectively. By contrast, these latter types are often associated with a primarily marginal calcification at the edges. The diffusely infiltrating type Ⅰ often exhibits initially feathery, less often focal, calcifications that tend to become more diffuse as the disease progresses. With primarily circumscribed,

Figure 3 Primarily circumscribed tumor-like with cystoid portion (A); without cystoid portion (B).

Figure 4 Primarily cystoid - intermediate with more solid portions at the edge (A); without more solid portions at the edge (B).

Figure 5 Primarily cystoid - widespread with more solid portions at the edge (A); without more solid portions at the edge (B).

tumor-like type Ⅱ lesions, there may often be either focal or diffuse calcification. However, in the present classification scheme, the various calcification patterns can, in principle, be associated with any of the primary morphologies (with the exception of type V, "mainly calcified"). Furthermore the special case of a central calcification can be observed, which is, when it occurs, exclusively linked to type Ⅳ disease (shown below, Figure 8). The primay morphologies type Ⅰ-Ⅴ are defined as follows:

Type Ⅰ, diffuse infiltrating
The diffuse infiltrating type does not show any defined central focus of growth but instead is characterized primarily by a diffuse, at times fan-shaped, sometimes longitudinally extending spread of the lesion into surrounding tissue. Frequently, tiny vesicular structures may be recognized as the fundamental structure of the lesion. These small vesicular elements, which are well-known from MRI imaging and often better visualized with that imaging modality, represent a characteristic feature of the development of this disease. Distinct from these vesicular elements are the intrahepatic bile ducts which in many cases are observed peripheral to the lesion: these may be focally congested and appear to converge on the lesion. With larger type Ⅰ lesions, or over the course of the disease or therapy, larger cystoid structures may develop, either centrally or

Figure 6 Small-cystoid/metastatic* (A, B): exclusive occurrence of the central calcification* (B).

Figure 7 Mainly calcified.

decentralized in the lesion: these are distinct from the small vesicular elements described above. Unlike the small vesicular structures, these may represent necrotic areas of the lesion and may be significant as a parameter indicative of the course of the disease. In some cases, these large cystoid areas may also represent a conglomerate of multiple small vesicular elements.

Type II, primarily circumscribed tumor-like

Of the five primary morphological types, the primarily circumscribed tumor-like type presents with the broadest variety of morphological manifestations and its identification represents the greatest challenge. Not only is it difficult to distinguish from type I but also from type III, especially when the latter, over the course of the disease or therapy, changes the shape of its solid and cystoid components. Presumably, type II lesions showing an affinity with disease type I would more frequently exhibit the small vesicular elements that are better visualized at MRI.

While it could be argued that, based on this high morphological variability, type II represents a mixed type that comprises special cases of type I and type III disease, we nevertheless consider this primary morphological pattern to represent a distinct disease type: its primarily circumscribed appearance could, for example, provide information about the patient's

immunological response, especially in cases in which an affinity to type I is suspected. In other type II cases, as already noted above, conclusions may be drawn regarding therapy response or regarding the activity of the lesion or course of the disease in the association of an type III lesion. Corresponding to the morphological variability of type II, assignment of a given lesion to this group depends, to a greater degree than with the other groups, on the examiner's subjective judgment. In general, the lesions subsumed under type II include those in which a primarily circumscribed, central and predominantly solid lesion could be defined. Some type II lesions may exhibit short offshoots into the surrounding hepatic tissue. In contrast to type II disease, the related type I is not characterized by a central, circumscribed tumor body and, in general, presents with a larger number of diffuse, often longitudinally extending offshoots (see above). As with type I, type II lesions may also contain larger cystoid portions: these may be located centrally or, again distinct from type III a/b "primary cystoid" disease, may be decentralized. Cystoid portions in type II lesions can in many cases be interpreted as necrotic regions but sometimes may as well represent a conglomerate of multiple small vesicular elements (see also above/description of type I lesions).

Type III a, primarily cystoid - intermediate/Type III b, primarily cystoid - widespread

Primarily cystoid manifestations define the third group of primary morphologies. Lesions in this group may reach intermediate size or spread to such an extent that they occupy large areas of a hepatic lobe. An exact size boundary between the two subgroups (III a - intermediate; III b - widespread) was not drawn and, as will be explained below, is not useful. However, intermediate-sized lesions tend to fall within a range of 3-8 cm in diameter, while widespread lesions generally exhibit larger diameters. Although the present classification does not establish an exact cut-off, the two subgroups were established based on the observation that the intermediate cystoid lesions mostly occur as multiple, disseminated le-

Figure 8 Frequency of primary morphologies, appended sub-criteria and individual calcification patterns, respectively.

sions, whereas widespread cystoid lesions often consist of one single lesion. An exact analysis of this phenomenon remains to be performed. There appears to be a correlation between uni-/multifocality and lesion size, which may depend on differences in the behavior of the parasite or, more likely, on differences in the host's immune response. The phenomenon may also be a reflection of the number of larvae reaching the liver, which then, in turn, could exert an influence on the growth of the individual lesions. Further studies to elucidate this question are planned.

Primarily cystoid lesions in group Ⅲ may manifest as purely cystoid in form or exhibit a more or less thick, solid marginal rim, while the cystoid portion remains relatively central. It is possible that, during the course of the disease, a centrifugal decrease of the more solid portions of the lesion might give the tumor a more and more cystoid appearance (this fact might represent an initial overlap with type Ⅱ). It remains for future studies to assess whether the observation of such changes in those lesions can be confirmed and whether an decreasing solid component corresponds with progressing inactivity of the lesion or with patients' response to therapy. In this case, as has already been mentioned above, the cystoid portion in type Ⅲ lesions should be interpreted as a necrotizing area in the sense of a potentially increasing inactivation, as presumably occurs in type Ⅰ and in some type Ⅱ lesions.

Type Ⅳ, small-cystoid/metastatic*

For several reasons, the small-cystoid/metastatic* morphology of type Ⅳ was not designated as a subgroup of the cystoid types in group Ⅲ. Although the observation made with type Ⅲ lesions of an inverse relationship between the size and number of lesions would appear to apply to type Ⅳ and extend to a typical pattern of often very disseminated disease involvement, type Ⅳ lesions exhibit a number

of characteristics that are not observed in type Ⅲ disease. For example, a very specific calcification pattern, characterized by a tiny, centrally located calcification, may occur in type Ⅳ lesions. As with other disease types, these calcifications may become more pronounced as the disease progresses. In addition, type Ⅳ lesions do not exhibit the more or less extensive solid portions at their margins that are frequently encountered in type Ⅲ lesions, which typically calcify at their edges. In light of these significant morphological differences, we deferred a strict delineation of lesion diameters between type Ⅳ and type Ⅲ disease. Most type Ⅳ lesions are less than 3 cm in diameter. In the absence, at least initially, of a central calcification* in type Ⅳ disease, the correlation of liver pathology to the diagnosis of AE may be very difficult, as the lesions tend to resemble protein-rich cysts or hypodense metastases. Knowledge of this manifestation form should prompt the clinician to consider a diagnosis of AE in patients with corresponding clinical suspicion.

Type Ⅴ, mainly calcified

Subsumed under primary morphology type Ⅴ - "mainly calcified" are those lesions consisting predominantly of a calcified component. Although smaller in extent, parenchymal/solid or cystoid portions are occasionally observed: they are usually marginal and quite insignificant within the overall extent of the lesion. Type Ⅴ is the sole primary morphological group in which the calcification is an obligatory part of the main description and thus the only group among the primary morphologies with no further separate calcification pattern associated with the lesion description. Type Ⅴ lesions are, for the most part, rather small. They often show a longitudinal or oval configuration, and may be solitary or multiple in number. Whether these represent older, inactive foci that have evolved from other primary morphologies, or belong to a distinct,

fully or partially active disease form characterized by rapid and pronounced ossification, remains to be determined in further studies. The former hypothesis is supported by the relatively small size of the lesions, their frequently longitudinal configuration, and the fact that there is little or no dissemination; all of these being important criteria for differentiating these lesions from other disease types. It is possible that a specific immune response by the host is responsible for this rapid, pronounced calcification.

In addition, predominantly calcified, longitudinally configured lesions observed in the context of recurrent disease at resection margins, representing either active lesions or post-operative cicatricial changes, are in part subsumed under type Ⅴ. Other lesions, especially belonging to type Ⅰ, may also occur at resection margins and may also be calcified. Under these considerations, "predominantly calcified" lesions at resection margins likely differ in terms of their behavior from "original" type Ⅴ lesions that occur centrally in the hepatic parenchyma in patients without prior resection.

Common statements

The cumulative occurrence of the different primary morphologies and their appended sub-criteria, respectively, as well as the occurrence of the different calcification patterns in association with the different primary morphologies, are shown in Figure 8.

The most frequently encountered CT-morphological pattern among the 228 patients was the diffuse infiltrating pattern type Ⅰ: 35.5% (n = 81), followed, in 20.6% (n = 47), by the primarily circumscribed tumor-like appearance (type Ⅱ), in 19.7% (n = 45) by the primarily cystoid type Ⅲ (type Ⅲa - intermediate, n = 27; type Ⅲb - widespread, n = 18), and in 17.1% (n = 39) by the small-cystoid/metastatic* morphology (type Ⅳ). Much less frequently observed was the mainly calcified appearance of type Ⅴ: 7% (n = 16), (Figure 8).

An analysis of the calcification pattern in relation to the primary morphology revealed the exclusive association of the central calcification with type Ⅳ primary morphology (Figure 8). Similarly, certain calcification patterns exhibited a clear predominance for other primary morphologies, which again underscores the delimitation of the individual primary morphological types from each other. These relationships in terms of calcification patterns extend into the primary morphological sub-criteria, demonstrating the clear subordination of the sub-criteria to their respective main primary morphological types.

DISCUSSION

AE presents with a wide variety of CT-morphological manifestations. The present paper, based on a large patient collective, proposes a new classification of hepatic AE lesions, which assigns them to five groups of primary morphologies (types Ⅰ-Ⅴ) and correlates these with a primary separate assignment of calcification patterns.

The CT morphological classification proposed in the present study seeks to facilitate the recognition and interpretation of lesions in hepatic AE and to aid in the frequently challenging differential diagnosis that includes neoplasms of the liver[17,18] such as cholangiocellular carcinoma (similar to type Ⅰ), biliary cystadenoma and cystadenocarcinoma (resemblance to type Ⅲ), or metastases of other tumors (similar to type Ⅱ or Ⅳ).

The most frequently encountered CT-morphological pattern among the 228 patients was the diffuse infiltrating pattern (type Ⅰ), followed by the primarily circumscribed tumor-like appearance (type Ⅱ), the primarily cystoid type (type Ⅲ) and by the small-cystoid/metastatic* morphology (type Ⅳ). Much less frequently observed was the mainly calcified appearance (type Ⅴ).

As a rule, the presence of multiple AE lesions within a single liver is associated with only one primary morphological pattern. In rare cases, multiple lesions in a given liver may be characterized by more than one primary morphology; these must be assessed separately over the course of the disease.

Based on the five primary morphologies, further descriptive sub-criteria were appended to types Ⅰ-Ⅲ. Thus, the presence of cystic components in types Ⅰ and Ⅱ and the presence of more solid portions at the edges in type Ⅲa and Ⅲb, lead to further characterization of the lesion.

Calcification patterns were first assessed independently of the primary morphology. This was shown to be useful as the extent and morphology of calcifications may change over the course of the disease or in response to therapy and thus give the respective primary morphology a quite different appearance.

An analysis of the calcification pattern in relation to the primary morphology revealed the exclusive association of the central calcification with type Ⅳ primary morphology. Similarly, certain calcification patterns exhibited a clear predominance for other primary morphologies, which again underscores the delimitation of the individual primary morphological types from each other. These relationships in terms of calcification patterns extend into the primary morphological sub-criteria, demonstrating the clear subordination of the sub-criteria to their respective main primary morphological types.

In the present classification scheme, the different calcification patterns can, in principle, be associated with any of the primary morphologies (with the exception of type Ⅴ, "mainly calcified"). Furthermore the special case of a central calcification can be observed, which is, when it occurs, exclusively linked to type Ⅳ disease.

Potential morphological changes occurring during the course of the disease or as a result of therapy,

especially as they relate to sub-criteria and calcification patterns, will be addressed in targeted studies.

In summary, the EMUC-CT classification allows for a very comprehensive description of hepatic AE lesions based on their CT-morphology. Assignment to one of five primary morphologies modified by an exact characterization of variable sub-criteria (insofar as sub-criteria are available for a given primary morphology) and in correlation to a respective calcification pattern should also assist in the interpretation of patients' clinical course and improve the comparability of CT findings in the context of scientific studies.

In order to describe the lesion completely according to the present classification, the criteria should be followed in order. Following is an example for the descriptions of individual types: Ⅰ. Diffuse infiltrating, without cystoid portion, with feathery calcifications; Ⅱ. Primarily circumscribed tumor-like, with cystoid portion, with focal calcifications; Ⅲa. Primarily cystoid - intermediate - with more solid portions at the edge, with calcifications primarily at the edge; Ⅳ. Small-cystoid/metastatic*, with a central calcification*; Ⅴ. Mainly calcified.

The knowledge of the primary morphologies of hepatic AE lesions according to these criteria may be of great assistance in the primary diagnostic work-up of this rare and morphologically variable disease entity. If changes in individual criteria are observed at follow-up monitoring, this can be clearly and reproducibly noted in the report of findings. Changes are more likely to relate to subcriteria of the primary morphologies and to calcification patterns; the primary morphological type often remains.

An exhaustive description of the findings of diagnostic imaging will naturally also enumerate the size and number of lesions, as well as their exact location within the liver (in addition to describing any extrahepatic manifestations). The precise characterization of the liver lesion based on the present classification will contribute to a much more comprehensive description of the disease manifestation. Unlike malignant diseases, the size of hepatic AE lesions often does not change significantly as a response to therapy; hence changings in size by themselves do not allow any unequivocal conclusions regarding the course of the disease. Changes in lesion morphology, however, could provide a number of additional criteria for assessing patients' clinical course. ^{18}F-FDG-PET, which is often used as an adjunct to CT, can yield some important data, though their value for assessing actual disease activity remains controversial[12-14,19,20]. Use of the morphological EMUC-CT classification could provide additional aspects regarding therapy planning as part of staging according to the PNM classification[16].

Unlike the WHO ultrasonographic classification of cystic echinococcosis (CE WHO-IWGE standardized classification)[1], the present CT classification of AE does not focus primarily on different stages of the disease: instead, it defines AE's very different morphological manifestations in the liver independent of the disease course. In the case of CE, a classification according to disease stages is supported by the fact that the form of individual lesions does not differ fundamentally between different individual hosts and, in comparison with one another, remains relatively constant within their stages (CE1-5). With AE, however, it would appear, as noted above, that there may be overlap between different morphological types (type Ⅰ/Ⅱ) and probably also transition in the disease course and response to therapy (type Ⅱ/Ⅲ), as well as alterations, for example, in the sense of a terminal phase (type Ⅴ) or recurrent lesions (type Ⅴ; type Ⅰ). It remains for further studies to determine whether and, if so, which individual primary morphology types are in fact to be interpreted as sequentially different disease stages or whether the alterations in lesions are due primarily to the modifiable criteria related to clinical course (subcriteria and calcification patterns).

A primary objective of the present classification is to facilitate the diagnosis of this rare disease entity in routine clinical practice. In addition, many provocative questions are raised as a by-product of this systematization:

Are the different morphologies the product of variable characteristics of the parasite or dependent on differences in host immunology? Does the parasite load at the time of initial infection play a role in the development of lesion morphology? How do the lesions develop over time, *e.g.,* with regard to their calcification pattern? In this regard, are transitions between different morphologies truly observable over the course of the disease and, if so, which? How are cystoid vs. solid components of different lesion types to be interpreted in the clinical course? Do the larger cystoid areas in type Ⅰ and possibly also in type Ⅱ lesions always represent necrotic zones or sometimes conglomerates of multiple small vesicular elements? Is there a correlation between different morphologies and (primary) behavior at ^{18}F-FDG-PET, or can conclusions be drawn regarding their activity based on PET imaging in cases of morphological changes in the lesions? Does the classification provide initial prognostic hints regarding patients' future disease course? Is there in this regard a correlation between different morphology types and the presence of extrahepatic disease or a tendency to intrahepatic infiltration of biliary or vascular structures (MRI is superior in answering this question)[17,21]? Can the classification, if, for example, correlations with specific immunohistochemical markers can be identified[22], serve as a building block for management decisions regarding surgical options with respect to resectability or for defining safe distances and resection margins for hepatic lesions?

While the small vascular elements within some lesions that are typical for AE are better visualized with MRI, CT offers a more nuanced view of other morphological properties and especially the calcifications[15,17]. Sound wave attenuation secondary to

the calcifications can, in turn, hinder ultrasonographic diagnostics. If corresponding parallel imaging data is available, a further goal could be a correlation of CT morphology as defined by the present classification with MRI findings or comparison with corresponding ultrasonographic lesion descriptions.

AE's rarity makes it difficult to assess inter-rater reliability. The lack of inter-rater reliability remains a limitation of the proposed classification. It was the objective of the present study to establish the crucial CT-morphological criteria for hepatic AE lesions and, for the first time, to systematically describe them.

In conclusions, the EMUC-CT classification presented in this paper is intended to facilitate the recognition and interpretation of hepatic lesions in AE based on CT-morphological criteria. It can also be used to more objectively interpret different clinical courses and enhance the comparability of CT findings in the context of scientific studies.

COMMENTS

Background
Human alveolar echinococcosis (AE) is the most lethal human helminthic infection and is one of the 17 neglected tropical diseases prioritized by the World Health Organization. Its incidence is low in endemic regions of Central and Western Europe (0.03-0.05/100000) and high in central Asia. Current studies suggest that the occurrence of alveolar echinococcosis is increasing worldwide and is spreading to previously unaffected regions. Morbidity and treatment costs of the disease are high.

Research frontiers
Despite the importance of computed tomography (CT), mostly combined with positron emission tomography, as an image modality in the work-up of hepatic AE, there is no CT-morphological classification of hepatic AE lesions.

Innovations and breakthroughs
Objective of the present study was to establish a CT-classification based on a large sample of patients with confirmed hepatic AE as a way of facilitating the diagnosis of the disease entity.

Applications
The CT-morphological classification proposed in the present study shall facilitate the diagnosis, interpretation, classification and comparison of CT-morphological findings in patients with alveolar echinococcosis of the liver, both in routine clinical practice and in the context of scientific studies.

Peer-review
The manuscript aims to provide a new CT-classification based on a large patient collective with confirmed hepatic AE. This draft provides a possibility for diagnostic tool of rare but sometimes fatal hepatic AE, and is valuable for sharing data in concern of determination of morphological status of hepatic AE, as well.

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ORIGINAL ARTICLE

Retrospective Study

Comprehensive treatments for hepatocellular carcinoma with tumor thrombus in major portal vein

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

AIM: To evaluate the efficacy of transcatheter arterial chemoembolisation (TACE) compared with surgical intervention and sorafenib for treatment of hepatocellular carcinoma (HCC) in patients with tumor thrombus extending to the main portal vein.

METHODS: From 2009 to 2013, a total of 418 HCC patients with tumor thrombus extending to the main portal vein were enrolled in this study and divided into four groups. These groups underwent different treatments as follows: TACE (n = 307), surgical intervention (n = 54), sorafenib (n = 15) and palliative

treatment (n = 42). Overall survival rates were determined by Kaplan-Meier method, and differences between the groups were identified through log-rank analysis. Cox's proportional hazard model was used to identify the risk factors for survival.

RESULTS: The mean survival periods for patients in the TACE, surgical intervention, sorafenib and palliative treatment groups were 10.39, 4.13, 5.54 and 2.82 mo, respectively. For the TACE group, the 3-, 6-, 12- and 24-mo survival rates were 94.1%, 85.9%, 51.5% and 0.0%, respectively. The corresponding rates were 60.3%, 22.2%, 0.0% and 0.0% for the surgical intervention group and 50.9%, 29.5%, 0.0% and 0.0% for the sorafenib group. Evidently, the results in the TACE group were significantly higher than those in the other groups ($P < 0.0001$). Furthermore, no significant difference among survival rates was observed between TACE with/without sorafenib (10.22 mo *vs* 10.52 mo, $P = 0.615$). No significant difference in survival rates was also found among the surgical intervention, sorafenib and palliative treatment groups ($P > 0.05$). These values significantly increased after TACE with/without sorafenib compared with other treatments ($P < 0.05$).

CONCLUSION: For HCC patients with tumor thrombus extending to the main portal vein, TACE can yield a higher survival rate than surgical intervention or sorafenib treatment.

Key words: Hepatocellular carcinoma; Portal vein; Tumor thrombus; Sorafenib; Transcatheter arterial chemoembolisation; Surgery

Core tip: This study evaluated the efficacy of transcatheter arterial chemoembolisation (TACE) compared with surgical intervention and sorafenib for treatment of hepatocellular carcinoma (HCC) in patients with tumor thrombus extending to the main portal vein. Results revealed that for HCC patients with tumor thrombus extending to the main portal vein, TACE can yield a higher survival rate than surgical intervention or sorafenib treatment.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide[1] and the third leading cause of cancer-related death[2]. Portal vein tumor thrombus (PVTT) was found invading the main trunk in 10%-15% of patients when they were diagnosed with HCC[3-5]. PVTT is usually correlated with a poor HCC prognosis. The mean survival period for HCC patients with PVTT was only 2.7-4.0 mo compared with 24.4 mo for HCC patients without PVTT[6,7]. Portal vein obstruction by tumor thrombus leads to portal vein hypertension, thereby resulting in heavy deterioration and impairment in liver function, intractable ascites, acute esophageal variceal bleeding and related death, particularly in patients with PVTT invading the main trunk of the portal vein. Furthermore, tumor cells usually spread out through the portal vein system and lead to invisible intrahepatic metastasis[8-11].

According to the guidelines of the European Association for the Study of the Liver[12], American Association for the Study of Liver Disease (AASLD)[13] and Classification Liver Cancer (BCLC) staging system, HCC with PVTT is considered entering an advanced stage, and PVTT is commonly regarded as an absolute or related contraindication for hepatic resection (HR) or transarterial chemoembolisation (TACE). Only sorafenib and palliative treatments are available for HCC treatment[1,11,14]. Interestingly, some reports showed that before the PVTT extended to the main trunk of the portal vein, the HR for HCC patients is feasible to achieve a survival benefit[15-17]. However, when PVTT extended to the main trunk of the portal vein, HR would not provide a survival benefit compared with palliative treatments. Instead, non-surgical treatments, such as TACE or TACE combined with sorafenib[18-20], would be better options. Nevertheless, some studies advocated that eradication of the primary tumor *via* hepatectomy and removal of PVTT through embolectomy[15] would still achieve a survival benefit despite that PVTT has been detected in the main trunk of the portal vein of HCC patients[21,22]. Thus, the proper therapy for HCC patients with PVTT existing in the main trunk of the portal vein remains debatable. The current retrospective study aimed to evaluate the efficacy and safety of different treatments, including TACE, HR, sorafenib and palliative treatments for treating HCC patients with PVTT invading the main trunk of the portal vein.

MATERIALS AND METHODS

This study was approved by the institutional review board of Guangxi Medical University and conducted in accordance with the Declaration of Helsinki and current ethical guidelines.

Patients

From January 2009 to December 2013, a total of 418 patients at the Hepatobiliary Surgery Department and Interventional Therapy Department of Guangxi Tumor Hospital, who were diagnosed with HCC combined with PVTT invading the main trunk of the portal vein and had satisfied the inclusion criteria below, were

recruited and retrospectively studied.

The inclusion criteria were: (1) All the candidates recruited were diagnosed with HCC associated with tumor thrombus involving the main trunk of the portal vein, which was defined by the presence of thrombus adjacent to the tumor and in the main trunk of the portal hepatic vein with undefined boundaries, as confirmed by two imaging modalities, namely, computed tomography (CT) and magnetic resonance imaging (MRI)[23], without distant metastasis and were evaluated with the Eastern Cooperative Oncology Group performance status (ECOG)[24] scores of 0-2 and moderate liver function (Child-Pugh A or B); (2) For candidates who underwent surgery, solitary tumor and/or multiple nodules can be eradicated *via* hepatectomy, and PVTT can be removed through embolectomy[15]; the remnant liver volume and liver function reserve were determined by volumetric computed tomography[25,26]. For patients without cirrhosis, approximately 30% of residual liver volume after surgery was considered sufficient, whereas for patients with chronic HBV and liver cirrhosis, the remnant liver volume should be > 50%. HCC and PVTT diagnoses were confirmed by histological examination of surgical samples; (3) For candidates receiving TACE, the inclusion criteria were similar to those in the HR group; these criteria were used when deciding whether to utilise TACE. Moreover, TACE was given to those patients with insufficient remnant liver volume and liver function reserve if they would undergo hepatic resection, with other unfavourable factors for surgery or without strong aspiration to receive HR. Moreover, sorafenib was given as an adjuvant therapy after TACE if possible; (4) For patients with unfavourable factors for HR or TACE, sorafenib was given; and (5) Routine palliative therapy was performed in patients who were not suitable for HR or TACE and did not use sorafenib.

TACE procedure

Contrast medium was injected into the arteries *via* a 4.1-French RC1 catheter, which was introduced into the abdominal aorta *via* the right superficial femoral artery by using the Seldinger technique[27]. Afterwards, the number, location, size and arterial branches supplying the tumors were identified. Iodised oil (10-20 mL), gel foam particles with doxorubicin (30-50 mg) and cisplatinum (50-100 mg) were injected into the arterial branches. Serum total bilirubin, albumin and prothrombin time were routinely monitored on the first, second and third day after TACE. After 1 month, CT follow-up was conducted to determine the effects of TACE. On the basis of liver function and tumor shrinkage, TACE was repeated at one-month intervals, and the TACE cycles were dependent on the tumor response to TACE and patient's liver function.

HR

Left, right, left partial, right partial and partial median hemihepatectomies were performed in 11, 10, 11, 11 and 11 patients, respectively. PVTT was removed in all patients through embolectomy[15]. The operative procedure for PVTT was decided on the basis of the location and extent of tumor thrombus: (1) when tumor thrombus involved the main trunk of the portal vein but not involved the branches of healthy side, surgery was performed to block the portal vein branch of the healthy side and longitudinally incised along the main trunk of the portal vein, the tumor thrombus was removed, and finally, the wall of the portal vein was closed *via* a continuous suture; and (2) when tumor thrombus had grown into the main trunk of the portal vein and branches of the healthy side, surgery was performed to block the portal vein branch of the retention sides to reduce bleeding and longitudinally cut open along the main trunk of the portal vein; the tumor thrombus was removed, and the wall of the portal vein was finally closed *via* a continuous suture. Ultrasound was generally used to detect whether tumor thrombus was completely removed.

Sorafenib administration (sorafenib monotherapy or sorafenib plus TACE)

Generally, about 400 mg of sorafenib (Bayer HealthCare AG, 200 mg/pill) was orally given twice daily. When grade 3 or 4 adverse events (such as skin, hematologic and gastrointestinal toxicities or organ dysfunction defined by the National Cancer Institute Common Terminology Criteria for Adverse Events[28]) occurred, the oral dose was reduced to 200 mg per day. If these adverse events continued after dose adjustment, sorafenib treatment was stopped until the symptoms were reduced or eliminated.

Follow-up and treatment of recurrence

After the initial therapy, serum alpha-fetoprotein (AFP) level and other laboratory tests were routinely monitored; ultrasonography, dynamic CT, MRI or angiography was performed at the end of the first month and then every 3 mo. When intrahepatic recurrence was suspended but not confirmed by imaging or serum AFP level, TACE was applied. When intrahepatic recurrence was confirmed after the initial HR, the second HR was performed on the basis of volumetric CT[25,26]. If HR was not feasible because of poor liver function, numerous intrahepatic metastases or other unfavourable factors, microwave coagulation, percutaneous ethanol injection, radiofrequency ablation or sorafenib therapy were applied instead of TACE. All patients were followed until December 30, 2013 or until death.

Statistical analysis

All the data were analysed using SPSS 21.0 statistical software. Normally and asymmetrically distributed data were determined as mean ± standard deviation (SD) and median (range) values, respectively. The

Table 1 Characteristics of hepatocellular carcinoma patient with portal vein tumor thrombus invading the main portal vein trunk and inferior vena cava *n* (%)

| | Group 1 (*n* = 307) | | | Group 2 (*n* = 54) | Group 3 (*n* = 15) | Group 4 (*n* = 42) | *P* value |
	TACE (*n* = 274)	TACE-sorafenib (*n* = 33)	*P* value				
Baseline characteristic							
Age, mean ± SD	48.62 ± 12.09	49.51 ± 11.23	0.764	47.40 ± 17.48	49.78 ± 21.26	51.49 ± 23.23	0.668
Sex (M)	233 (85.0)	26 (78.8)	0.350	46 (85.2)	12 (80.0)	36 (85.7)	0.960
Clinical characteristic							
Positive for HBsAg	224 (81.8)	29 (87.9)	0.505	46 (85.2)	14 (93.3)	36 (85.7)	0.665
Positive for anti-HCV	16 (5.8)	0 (0.0)	0.234	1 (1.9)	0 (0.0)	1 (2.3)	0.755
PLT (10^9/L)	198.25 ± 88.17	205.94 ± 102.04	0.146	246.37 ± 71.13	211.74 ± 101.21	297.10 ± 148.07	0.654
TBil (μmol/L)	19.1 (13.08-30.10)	16.70 (11.80-23.00)	0.347	21.1 (11.46-32.37)	17.23 (10.79-35.69)	25.1 (10.78-41.76)	0.065
ALB (g/L)	37.29 ± 5.14	37.74 ± 4.64	0.981	39.31 ± 7.81	36.73 ± 4.27	32.13 ± 8.23	0.851
ALT (U/L)	54.00 (35.00-79.00)	52.00 (35.00-99.00)	0.813	52.00 (32.00-77.00)	56.00 (31.00-72.00)	58.00 (31.00-81.00)	0.135
AST (U/L)	79.00 (49.00-144.00)	80.00 (50.00-157.50)	0.843	65.00 (28.00-101.00)	78.00 (45.00-127.00)	75.00 (21.00-167.00)	0.104
PT (s)	13.75 ± 1.52	13.84 ± 1.934	0.963	12.11 ± 1.41	13.49 ± 1.37	13.79 ± 1.54	0.060
AFP (mg/L)	873 (126-12100)	1210 (123-12100)	0.107	745 (310-12100)	691 (348-12100)	1207 (1001-12100)	0.793
Child-Pugh Score	5 (5-8)	5 (5-8)	0.914	5 (5-8)	6 (5-8)	6 (5-9)	0.255
Ascites	8 (2.9)	0 (0.0)	0.999	0 (0.0)	0 (0.0)	3 (7.1)	0.201
Pathological characteristic							
Tumor size (cm)	6.78 ± 2.96	7.14 ± 3.97	0.101	7.13 ± 2.73	6.97 ± 2.37	7.95 ± 3.34	0.437
Tumor number (≥ 3), *n*	178 (65.0)	22 (66.7)	0.846	17 (32.4)	9 (60.0)	27 (64.2)	0.982
Cirrhosis, *n*	223 (81.0)	27 (81.8)	0.952	46 (85.2)	11 (73.3)	36 (88.1)	0.697
Portal vein hypertension, *n*	76 (27.7)	8 (24.2)	0.671	12 (22.2)	3 (20.0)	14 (33.3)	0.609
Main portal vein trunk, *n*	211 (77.0)	21 (63.6)	0.091	44 (81.5)	6 (77.0)	17 (77.0)	< 0.001
Inferior vena cava, *n*	63 (23.0)	12 (36.4)		10 (18.5)	9 (77.0)	25 (77.0)	

PVTT: Portal vein tumor thrombus; SD: Standard deviation; HBsAg: Hepatitis B surface antigen; anti-HCV: Hepatitis C virus antibody; PLT: Platelet count; TBil: Total bilirubin; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein; Group 1: TACE; Group 2: Surgery + postoperative TACE; Group 3: Monotherapy of sorafenib; Group 4: Palliative therapy.

Table 2 Survival period and survival rate in different groups

	Mean overall survival (mo)	3-mo survival rate	6-mo survival rate	12-mo survival rate	24-mo survival rate
TACE administration	10.39	94.1%	85.9%	51.5%	0.0%
TACE subgroup	10.22	93.8%	86.7%	43.9%	0.0%
TACE-sorafenib subgroup	10.52	95.3%	83.3%	53.8%	0.0%
Liver resection + TACE	4.13	60.3%	22.2%	0.0%	0.0%
Targeted therapy of sorafenib	3.54	50.9%	29.5%	0.0%	0.0%
Palliate treatment	2.82	55.0%	0.0%	0.0%	0.0%

TACE: Transcatheter arterial chemoembolization.

baseline characteristics for patients with PVTT invading the main trunk and IVC were calculated using χ^2 tests. Survival time was defined as the period between the initial treatment and date of death or end of the study for survival patients. Survival curves were determined by Kaplan-Meier method, and differences between groups were identified using log-rank analysis. A *P* value < 0.05 was considered statistically significant.

RESULTS

Characteristics of patients

Table 1 summarises the clinical characteristics of the 418 HCC patients. Patients who used sorafenib or underwent palliative treatment were characterised with significantly higher levels of total bilirubin, higher frequencies of PHT and larger and multinodular HCC tumors (*P* < 0.001 for all). No statistical differences were found regarding the clinical or pathological variables, including sex, age, serum AFP level, hepatitis B surface antigen, Child-Pugh classification, ECOG score and location of PVTT among the four groups (*P* > 0.05).

Overall survival periods

The overall survival (OS) periods significantly increased in the TACE group compared with the other groups. For TACE administration, the OS rates at 3, 6, 12 and 24 mo were 94.1%, 85.9%, 51.5% and 0.0%, respectively. The mean survival period was 10.39 mo, which was significantly longer than that obtained from the three other groups (*P* < 0.05) (Figure 1, Table 2). We conducted subgroup analysis to explore

Group[1]	1	2	3	4
mean ± SD (mo)	10.39 ± 5.24	4.13 ± 1.78	3.54 ± 1.38	2.82 ± 1.40
1 (*P* value)	-	< 0.001	< 0.001	< 0.001
2 (*P* value)	< 0.001	-	0.356	0.398
3 (*P* value)	< 0.001	0.356	-	0.781
4 (*P* value)	< 0.001	0.398	0.781	-

Figure 1 Overall survival periods significantly increase in the transcatheter arterial chemoembolisation group compared with the other groups. [1]Group 1: TACE administration; Group 2: Surgery + postoperative TACE; Group 3: Monotherapy of sorafenib; Group 4: Palliative therapy.

the efficacy and safety of sorafenib as an adjuvant treatment for TACE. The 3-, 6-, 12- and 24-mo OS rates were 93.4%, 86.7%, 43.9% and 0.0% for TACE; correspondingly, these rates were 95.3%, 83.3%, 53.8% and 0.0% for TACE together with sorafenib. The mean survival periods were 10.22 and 10.52 mo for TACE alone and TACE together with sorafenib, respectively, which showed no significant difference (Figure 2, Table 2).

In the HR group, the 3-, 6-, 12- and 24-mo OS rates were 60.3%, 22.2%, 0.0% and 0.0%, respectively; correspondingly, these rates were 50.9%, 29.5%, 0.0% and 0.0%, and 55.0%, 0.0%, 0.0%, 0.0% in the sorafenib and palliative treatment groups, respectively. The mean survival periods were 4.13, 3.54 and 2.82 mo in the HR, sorafenib and palliative groups, respectively, in which the palliative group had the lowest mean survival period. Nevertheless, the difference of the mean survival period was not significant among these three groups (*P* > 0.05) (Figure 1, Table 2).

Compilations
In the HR group, 4 (7.4%) and 2 (3.7%) patients presented postoperative bile leakage and bleeding of esophageal venous plexus, respectively; in addition, 5 (9.3%) patients suffered from postoperative liver function deficiency, of whom 3 (5.6%) had developed refractory ascites and finally died of liver failure. A total of 7 (13.0%) patients suffered from pulmonary complication, and one of them was a 75-year-old

man who developed liver abscess and finally died because of serious infection. The PVTT of three patients was observed invading the main portal vein trunk preoperatively but found extending to the IVC during the operation for tumor thrombus; another PVTT was found in the right atrium. Although the tumor thrombus was successfully removed, the patient still died on the 31st day after surgery because of heart failure (1.5%). Table 3 shows a list of other complications. The frequency of complications (44.7%) and death in the hospital (9.3%) after HR were the highest.

Patients who underwent TACE usually had post-embolisation syndrome (nausea, vomiting, fever and pain). One patient also suffered from ectopic embolisation syndrome, and another suffered pulmonary complications and infection. However, no one suffered serious adverse events and hospital death (Table 3).

Most of the patients who used sorafenib suffered from grade 1 or 2 adverse events, and only six patients suffered grade 3 or 4 adverse events (Table 4). Nevertheless, adverse events disappeared after an oral dose reduction.

Analysis of prognostic factors for OS
Multivariate analysis of prognostic factors for OS showed that tumor size [hazard ratios (HR) = 3.31, 95%CI: 1.20-3.30, *P* = 0.008], tumor number (HR = 2.10, 95%CI: 1.22-3.63, *P* = 0.007), serum AFP level (HR = 1.84, 95%CI: 1.09-3.11, *P* = 0.023), Child-Pugh stage (HR = 1.99, 95%CI: 1.20-3.30, *P* = 0.008),

	TACE subgroup	TACE ± sorafenib subgroup	*P* value
Overall survival (mo ± SD)	10.22 ± 4.74	10.52 ± 4.27	0.615

Figure 2 Overall survival between subgroups of transarterial chemoembolisation administrations among all the hepatocellular carcinoma patients with portal vein tumor thrombus extending to the main portal vein trunk and inferior vena cava. TACE: Transarterial chemoembolization; HCC: Hepatocellular carcinoma.

Table 3 Complications and adverse events in different therapy groups _n_ (%)

Complication	Group 1 (*n* = 307)			Group 2 (*n* = 54)	Group 3 (*n* = 15)	*P* value
	TACE (*n* = 274)	TACE-sorafenib (*n* = 33)	*P* value			
Nausea, vomiting	49 (17.9)	19 (57.6)	< 0.001	12 (22.2)	12 (80.0)	< 0.001
Fever	62 (22.6)	8 (24.2)	0.835	11 (20.4)	0 (0.0)	< 0.001
Pain	119 (43.4)	7 (21.2)	0.047	24 (44.4)	0 (0.0)	< 0.001
Alopecia	3 (1.1)	11 (33.3)	< 0.001	3 (5.6)	6 (40.0)	< 0.001
Bleeding of tumor rupture	0 (0.0)	2 (6.0)	< 0.001	0 (0.0)	0 (0.0)	< 0.001
Liver failure	1 (0.4)	25 (75.8)	< 0.001	5 (9.3)	4 (26.7)	< 0.001
Bile leakage	0 (0.0)	0 (0.0)	0.999	4 (7.4)	0 (0.0)	< 0.001
Bleeding of esophageal venous plexus	0 (0.0)	0 (0.0)	0.999	2 (3.7)	0 (0.0)	< 0.001
Gastrointestinal hemorrhage	0 (0.0)	1 (3.0)	0.004	1 (1.9)	1 (6.7)	< 0.001
Heart failure	0 (0.0)	0 (0.0)	0.999	1 (1.9)	0 (0.0)	< 0.001
Infection	1 (0.4)	0 (0.0)	0.999	1 (1.9)	0 (0.0)	< 0.001
Ectopic embolism syndrome	1 (0.4)	0 (0.0)	0.999	0 (0.0)	0 (0.0)	< 0.001
Refractory ascites	0 (0.0)	0 (0.0)	0.999	3 (5.6)	0 (0.0)	< 0.001
Pulmonary complication	1 (0.4)	1 (3.0)	0.072	7 (13.0)	0 (0.0)	< 0.001
Therapy-related death	0 (0.0)	0 (0.0)	0.999	5[1] (9.3)	0 (0.0)	< 0.001

[1]Three patients died of postoperative liver failure, 1 patient was observed in right atrium and died of heart failure, and 1 patient died of badly infection. Group 1: TACE; Group 2: Surgery + postoperative TACE; Group 3: Monotherapy of sorafenib; Group 4: Palliative therapy.

cirrhosis (HR= 2.10, 95%CI: 1.02-4.32, *P* = 0.044), PHT (HR = 1.65, 95%CI: 1.02-2.65, *P* = 0.041), and PVTT location (HR = 5.41, 95%CI: 2.66-7.65, *P* < 0.001) were associated with worse OS (Table 5).

DISCUSSION

According to the guidelines of the European Association for Study of the Liver[12], AASLD[13] and Classification Liver Cancer (BCLC) staging system, PVTT was usually considered an absolute or relative contradiction for hepatic resection or TACE, and only sorafenib and palliative treatments are recommended[1,11,14]. However,

some studies[15-17] advocated that liver resection and removing tumor thrombus from the portal vein system[21,22], TACE[18-20] and TACE combined with sorafenib[29] still achieved survival benefit despite that the tumor thrombus had extended to the main trunk of the portal vein in HCC patients. Thus, the present study aimed to evaluate the efficacy and safety of different treatments for HCC patients with PVTT involving the main trunk of the portal vein.

TACE remains a safe and effective treatment strategy for HCC
TACE is usually considered a contradiction for HCC

Table 4 Adverse events related to sorafenib administration *n* (%)

Adverse event	Mono-therapy of sorafenib			TACE combined with sorafenib		
	All events	Grade 1-2 events	Grade 3-4 events	All events	Grade 1-2 events	Grade 3-4 events
Overall incidence	14 (93.3)	12 (80.0)	2 (13.3)	30 (90.9)	26 (78.8)	4 (12.1)
Alopecia	6 (40.0)	6 (40.0)	0 (0.0)	11 (33.3)	11 (33.3)	0 (0.0)
Anorexia	4 (26.7)	4 (26.7)	0 (0.0)	18 (54.5)	18 (54.5)	0 (0.0)
Diarrhea	13 (86.7)	12 (80.0)	1 (6.7)	27 (81.8)	26 (78.8)	1 (3.0)
Epistaxis	1 (6.7)	1 (6.7)	0 (0.0)	2 (6.0)	2 (6.0)	0 (0.0)
Fatigue	12 (80.0)	12 (80.0)	0 (0.0)	22 (66.7)	21 (63.6)	1 (3.0)
Gastrointestinal hemorrhage	1 (6.7)	1 (6.7)	0 (0.0)	1 (3.0)	1 (3.0)	0 (0.0)
Hand-foot skin reactions	11 (73.3)	10 (66.7)	0 (0.0)	12 (36.3)	11 (33.3)	1 (3.0)
Liver dysfunction	4 (26.7)	4 (26.7)	1 (6.7)	25 (75.8)	23 (69.7)	2 (6.0)
Nausea	12 (80.0)	12 (80.0)	0 (0.0)	19 (57.6)	19 (57.6)	0 (0.0)

TACE: Transcatheter arterial chemoembolization.

patients with portal vein obstruction by tumor thrombus because of the high risk of hepatic function insufficiency[30]. However, HCC growth mostly depends on 90% of the blood supply from the hepatic artery, whereas the normal liver parenchyma receives about 70% of its basic blood supply from the portal vein[31-33]. Based on this fact, TACE can provide a survival benefit to HCC patients by blocking the main nutrient vessels of tumor *via* an embolisation in the hepatic artery and subsequently allowing sustainable chemotherapeutic drugs to kill the HCC cells. This method is also effective in preventing compensatory circulation growth, reducing portal vein pressure and preventing intractable ascites and bleeding of esophageal varices[34-37]. Lee *et al*[38] reported that TACE is safe for the treatment of HCC with portal trunk obstruction when patients have sufficient collateral circulation around the portal trunk. Luo *et al*[18] showed that patients with major PVTT achieve better OS with TACE therapy than with conservative treatment. The 3-, 6-, 12- and 24-mo OS rates were 79.2%, 38.7%, 5.8% and 0% and 58.6%, 20%, 0% and 0%, respectively (*P* = 0.002). In our study, the survival rates (at 3, 6, 12 and 24 mo were 97.9%, 91.8%, 36.3%, 0.0% and 96.7%, 93.3%, 53.5%, 0.0%) and survival periods (10.22 mo *vs* 10.52 mo) increased mostly after TACE or TACE combined with sorafenib compared with other treatments.

TACE cannot completely block the nutrient transport to the tumor because of the small nutrient vessels from the portal vein; therefore, tumor necrosis is not completely achieved[18]. Generally, the cytotoxic effects of chemotherapy drugs follow log-cell kill kinetics, in which cells are killed proportionally. Therefore, tumor cells cannot be eliminated by one cycle of chemotherapy. With multiple treatment cycles, the possibility of killing residual tumor cells increases with enhanced prognosis[39]. Furthermore, among the patients with PVTT invading the main trunk of the portal vein, invisible intrahepatic metastasis is more likely to happen because the tumor cells were distributed *via* the portal vein system; in PVTT, these cells are located in a liver segment. Lipiodol can selectively accumulate in the invisible metastatic HCC when delivered intra-arterially and acts as a carrier for anticancer drugs. Hence, TACE can effectively block the nutrient vessels in the invisible metastatic HCC, thus allowing sustainable chemotherapeutic drugs to kill the microscopic HCC cells[35-37]. However, repeated TACE can damage the remnant liver parenchyma, particularly in cirrhotic patients, and result in liver function impairment or deterioration[40,41]. Thus, the number of TACE cycles should depend on the tumor response to TACE and patient's liver function.

Fortunately, patients who underwent TACE did not present serious adverse events or suffer from therapy-related death. Therefore, TACE should be considered a safe and effective treatment for HCC patients with PVTT extending to the main trunk and IVC.

Efficacy of sorafenib as an adjuvant treatment and single use of sorafenib

According to the BCLC group, the only recommended treatment for HCC patients with PVTT is sorafenib. Sorafenib is often used as an adjuvant therapy combined with TACE. As an oral small molecule tyrosine multikinase inhibitor of several intracellular proteins, sorafenib can intervene some factors regarding tumor progression, including the platelet derived growth factor receptor-β, Raf serine/threonine kinases and vascular endothelial growth factor receptor (VEGFR) receptors-1/2/3[42,43]. Sorafenib plays a critical role in tumor cell apoptosis and anti-angiogenesis of new born tumor, which further enhances the efficacy of TACE[44].

Basically, VEGF plays an important role in tumor recurrence and metastasis. Some reports[45,46] indicated that the VEGF level increases after TACE. Thus, anti-angiogenesis therapy *via* sorafenib is normally considered to contribute to preventing the development of new vessels supplying the tumor by suppressing the VEGFR level; consequently, the interaction between VEGF and VEGFR is decreased. Several studies[29,47,48] indicated that TACE combined with sorafenib would provide a better OS than single TACE. Several trials[49-51] also indicated that sorafenib used as a preoperative therapy would have benefit

Table 5 Factors affecting overall survival using Cox's proportional hazard model

Factor	Univariate analysis				Multivariate analysis		
	Patients, *n*	Mean OS (mo)	95%CI	*P* value	HR	95%CI	*P* value
Positive for HBsAg							
Yes	349	9.05	8.33-9.76	0.035	1.09	0.56-2.14	0.798
No	69	10.58	9.92-11.23				
Positive for anti-HCV							
Yes	19	6.00	2.49-9.51	0.026	1.45	0.44-3.88	0.454
No	399	9.42	8.90-9.94				
AFP (mg/L)							
> 400	134	7.99	6.94-9.05	< 0.001	1.84	1.09-3.11	0.023
⩽ 400	284	9.96	9.42-10.49				
Child-Pugh Stage							
Stage A	262	9.88	9.34-10.42	< 0.001	1.99	1.20-3.30	0.008
Stage B	156	8.28	7.21-9.37				
Tumor size (cm)							
> 5	301	8.48	7.79-9.18	< 0.001	3.31	1.57-6.98	0.002
⩽ 5	117	10.82	10.20-11.45				
Tumor number							
> 3	147	8.62	7.92-9.32	< 0.001	2.10	1.22-3.63	0.007
⩽ 3	271	10.55	9.89-11.20				
Cirrhosis							
Yes	343	8.85	8.23-9.46	0.007	2.10	1.02-4.32	0.044
No	75	10.82	10.06-11.59				
Portal hypertension							
Yes	113	7.00	6.03-7.98	< 0.001	1.65	1.02-2.65	0.041
No	345	10.14	9.62-10.65				
Tumor thrombus location							
Main portal vein trunk	299	10.40	9.89-10.91	< 0.001	4.51	2.66-7.65	< 0.001
Inferior vena cava	119	4.83	4.26-5.41				

PVTT: Portal vein tumor thrombus; HBsAg: Hepatitis B surface antigen; anti-HCV: Hepatitis C virus antibody; PLT: Platelet count; TBil: Total bilirubin; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein.

of shrinking the tumor size according to the response evaluation criteria in solid tumors and can downstage the HCC to undergo other therapies. However, the current study did not find that using sorafenib as an adjuvant therapy with TACE can significantly prolong the survival period compared with single TACE, probably because our study is basically focused on HCC patients with PVTT invading the main portal vein trunk or IVC, whereas the other studies emphasised on the patients without major PVTT or without PVTT. Similarly, a recent systematic review[50] evidenced that TACE combined with sorafenib would not prolong OS more than single TACE in unresectable HCC (HR = 0.81, 95%CI: 0.65-1.01, *P* = 0.061).

Nevertheless, single use of sorafenib would not provide a better survival benefit than palliative treatment. The survival rates at 3, 6, 12 and 24 mo were 50.9%, 29.5%, 0.0% and 0.0% in the single-use sorafenib group, respectively; correspondingly, these rates were 55.0%, 0.0%, 0.0% and 0.0% in the palliative group (*P* > 0.05). The survival period of the single-use sorafenib group (3.54 mo) was slightly longer than that of the palliative treatment group (2.82 mo). No obvious serious adverse events occurred in patients who used sorafenib. Nevertheless, for HCC patients associated with PVTT invading the main portal vein trunk, the single use of sorafenib as an adjuvant treatment can be recommended, but the efficacy

remains under discussion.

Efficacy and safety of HR

PVTT is generally considered an absolute or related contradiction for surgery[52]. However, some studies[16,53] advocated that HR can achieve survival benefit and enhance life quality. Hepatectomy along the portal tributary is effective in eradicating the main gross tumor, tumor's surgical margins and possible satellite nodules; an embolectomy is feasible to remove the tumor thrombus from the portal vein system, consequently reducing portal vein pressure and allowing the recovery of blood flow in the portal vein; this method helps improve liver function and prevent the intractable ascites, bleeding of esophageal varices and its related death. In addition, the method reduces the tumor burden and increases the efficacy of postoperative multimodality treatments, such as TACE, hepatic artery infusion, portal vein infusion and biotherapy. Ban *et al*[22] indicated that hepatectomy and embolectomy to treat HCC with tumor thrombus extending to the main trunk of the portal vein can provide a comparable survival benefit similar to that achieved by hepatectomy for PVTT located in the first branch of portal vein or above. In the present study, HR was therefore considered an effective treatment method in selected HCC patients with PVTT involving the major portal vein. However, we cannot determine

a survival benefit after HR compared with other treatments, which is probably because the patients involved in our study were characterised with high exposure to HBV, chronic liver cirrhosis and PTH. These variables can lead to heavier damage of perioperative liver function and worse hepatic impairment after HR. When tumor thrombus extended to the main trunk of the portal vein, the risk of portal vein hypertension and its related diseases was increased, and the liver function damage was heavier than that in HCC without the obstruction by tumor thrombus in the major portal vein. Furthermore, HCC cells spread out and were distributed through the portal vein system, thereby leading to an invisible intrahepatic metastasis, which is the main mechanism of postoperative sustainable damage of residual liver function and recurrence. Thus, HR was no longer an eradicative treatment for HCC patients with PVTT invading the main trunk of portal vein. All of these variables affected the long-term survival of the patients. Similar to our finding, Peng *et al*[54] reported that compared with TACE, HR provides survival benefits for patients with resectable HCC with PVTT invading the portal vein branches but not the main portal vein trunk. Shi *et al*[16] proposed that hepatectomy and thrombectomy are viable treatments until the PVTT infiltrates into the main trunk of the portal vein. Nevertheless, in our study, complications and mortality in hospital after HR were most frequently comparable with those after other therapies. Overall, HR should be considered with precaution for HCC patients with PVTT invading the main trunk of the portal vein because it usually cannot prolong the OS but may increase the risk of postoperative complications and liver failure. Hence, the efficacy of the studied method remains controversial.

Prognostic factors for survival

Our multivariable analysis revealed that larger and multiple nodular HCCs were related to poor prognosis. This finding is not consistent with other reports indicating that tumor size larger than 10 cm and multiple nodules are not conflicting with HR. Moreover, HBV, cirrhosis and portal vein hypertension were identified as poor prognostic factors. The patients in our study came from the Guangxi Zhuang Autonomous Region of China, where the population shows the highest HBV-related HCC incidence rate worldwide[55,56]. Cirrhosis usually developed from the liver, which was repeatedly impaired by HBV, and consequently developed to PHT. Furthermore, among the patients in our study, PTH not only developed from chronic HBV and cirrhosis, but also from the blockage by tumor thrombus in the main trunk of the portal vein. Thus, the perioperative liver function of patients in our study suffered heavier damage than patients from other areas without HBV and PVT in the major portal vein. On the basis of bad damage of perioperative liver function, large and multinodular HCC tumors generally need major hepatic resection (such as left/right hemihepatectomy), which leads to longer operation period and more loss of blood compared with partial hepatectomy. This finding explains the aggravation of the impairment of postoperative liver function and increase of risk in postoperative liver failure and death in the hospital. Such reason also helps explain why the postoperative survival was poor in the current study. Furthermore, our study did not find the difference of survival in those patients until PVTT extended to the inferior vein cava compared with those patients whose PVTT had invaded the inferior vein cava.

The limitations of the present study include its retrospective nature. Moreover, patients were treated at a single centre. Another limitation is the characteristics of the HCC patients, who came from some geographic areas with the highest incidence of HCC[57]. Therefore, the study result may be specifically suitable for Asian population and focusing mainly on HCC. Further randomised controlled trials with large sample size are needed.

This retrospective study suggests the following for treatment of HCC patients with tumor thrombus invading the main trunk of the portal vein and IVC: (1) TACE provided the most significant survival benefit among other therapies without inducing serious adverse events. Thus, TACE should be recommended as a safe and effective therapy; (2) sorafenib as an adjuvant treatment and combined with TACE slightly prolonged the OS than single TACE. However, the single use of sorafenib did not obviously prolong the survival compared with palliative treatment. Therefore, sorafenib remains a good option as an adjuvant therapy, but the efficacy of its single use remains to be evaluated; and (3) although hepatic resection released the portal vein hypertension and its related disease, this method did not provide survival benefit but rather induced multiple complications and increased the risk of postoperative liver failure and related death. Thus, liver resection should be carefully selected, and the efficacy of this method remains controversial.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related death. Portal vein tumor thrombus (PVTT) was found invading the main trunk in 10%-15% patients with HCC. HCC patients with PVTT are generally considered to have lost the optimal opportunity to undergo transarterial chemoembolisation (TACE) and surgical intervention, and only sorafenib and palliative treatments are available. Studying the efficacy of TACE compared with surgical intervention and sorafenib for HCC with PVTT provides clinical significance for further application of this strategy to treat HCC.

Research frontiers

This study compared and evaluated the efficacy of four kinds of interventions for HCC patients with PVTT extending to the main portal vein. Results indicated

that only TACE with/without sorafenib provided a comparable survival benefit, whereas surgical intervention or sorafenib did not lead to better survival than palliative treatments.

Innovations and breakthroughs

This study revealed that for HCC patients with tumor thrombus extending to the main portal vein, TACE can yield a higher survival rate than surgical intervention or sorafenib.

Applications

This study evaluated the efficacy of TACE compared with surgical intervention and sorafenib treatment for HCC with PVTT. The results offered novel treatment choices for clinical surgeons to treat HCC patients with PVTT extending to the main portal vein.

Terminology

TACE is used for some patients with liver cancer that cannot be treated surgically or *via* radiofrequency ablation. TACE is also a minimally invasive technique to treat liver tumors, particularly HCC.

Peer-review

This study investigated the efficacy of TACE compared with surgical intervention and sorafenib for HCC patients with tumor thrombus extending to the main portal vein. The results are significant and applicable to clinical practices and studies.

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ORIGINAL ARTICLE

Clinical Trials Study

Near-infrared fluorescence sentinel lymph node detection in gastric cancer: A pilot study

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Institutional review board statement: This study was approved and reviewed by the Local Medical Ethics Committee of the Leiden University Medical Center (LUMC).

Clinical trial registration statement: The trial was approved by the Medical Ethics Committee of the Leiden University Medical Center and was performed in accordance with the ethical standards of the Helsinki Declaration of 1975. This trial is registered with the Netherlands Trial Register as NTR4280.

Informed consent statement: All involved persons provided informed consent prior to study inclusion.

Conflict-of-interest statement: Tummers QRJG, Boogerd LSF, de Steur WO, Verbeek FPR, Boonstra MC, Handgraaf HJM, van de Velde CJH, Hartgrink HH and Vahrmeijer AL have no conflicts of interest or financial ties to disclose; Frangioni JV FLARE™ technology is owned by Beth Israel Deaconess Medical Center, a teaching hospital of Harvard Medical School;

Dr. Frangioni has started three for-profit companies, Curadel, Curadel ResVet Imaging, and Curadel Surgical Innovations, which has optioned FLARE™ technology for potential licensing from Beth Israel Deaconess Medical Center.

Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate feasibility and accuracy of near-infrared fluorescence imaging using indocyanine green: nanocolloid for sentinel lymph node (SLN) detection in gastric cancer.

METHODS: A prospective, single-institution, phase

I feasibility trial was conducted. Patients suffering from gastric cancer and planned for gastrectomy were included. During surgery, a subserosal injection of 1.6 mL ICG:Nanocoll was administered around the tumor. NIR fluorescence imaging of the abdominal cavity was performed using the Mini-FLARE™ NIR fluorescence imaging system. Lymphatic pathways and SLNs were visualized. Of every detected SLN, the corresponding lymph node station, signal-to-background ratio and histopathological diagnosis was determined. Patients underwent standard-of-care gastrectomy. Detected SLNs outside the standard dissection planes were also resected and evaluated.

RESULTS: Twenty-six patients were enrolled. Four patients were excluded because distant metastases were found during surgery or due to technical failure of the injection. In 21 of the remaining 22 patients, at least 1 SLN was detected by NIR Fluorescence imaging (mean 3.1 SLNs; range 1-6). In 8 of the 21 patients, tumor-positive LNs were found. Overall accuracy of the technique was 90% (70%-99%; 95%CI), which decreased by higher pT-stage (100%, 100%, 100%, 90%, 0% for respectively Tx, T1, T2, T3, T4 tumors). All NIR-negative SLNs were completely effaced by tumor. Mean fluorescence signal-to-background ratio of SLNs was 4.4 (range 1.4-19.8). In 8 of the 21 patients, SLNs outside the standard resection plane were identified, that contained malignant cells in 2 patients.

CONCLUSION: This study shows successful use of ICG:Nanocoll as lymphatic tracer for SLN detection in gastric cancer. Moreover, tumor-containing LNs outside the standard dissection planes were identified.

Key words: Gastric cancer; Sentinel lymph node; Near-infrared fluorescence imaging; Image-guided surgery; Indocyanine green

Core tip: Sentinel lymph node (SLN) detection using indoyanine green adsorbed to nanocolloid (ICG: Nanocoll) was investigated in 26 patients with gastric cancer. Adsorption of ICG to nanocolloid results in better retention in SLNs and staining of less 2nd tier nodes. After subserosal injection, fluorescent SLN detection using the Mini-FLARE™ system was performed. A mean number of 3.1 SLNs per patient were found and overall accuracy was 90%. In 8 patients, SLNs outside the standard resection planes were identified, that contained malignant cells in 2 patients. To conclude, NIR fluorescence imaging using ICG:Nanocoll as lymphatic tracer identified SLNs in- and outside standard dissection planes.

Tummers QRJG, Boogerd LSF, de Steur WO, Verbeek FPR, Boonstra MC, Handgraaf HJM, Frangioni JV, van de Velde CJH, Hartgrink HH, Vahrmeijer AL. Near-infrared fluorescence

INTRODUCTION

Gastric cancer is still one of the most frequent causes of cancer deaths worldwide with an incidence rate varying between countries[1,2]. The highest estimated mortality rates are in Eastern Asia (24 per 100000 in men, 9.8 per 100000 in women), the lowest in North America (2.8 and 1.5, respectively)[3].

Surgical resection of the tumor is the only curative treatment option. Depending on the size, infiltration depth, and location of the tumor, surgery can be performed endoscopically, or by partial or total gastrectomy. In addition to resection of the affected part of the stomach, a lymph node (LN) dissection is typically performed. This can either be done by extensive lymphadenectomy or by a sentinel lymph node (SLN) procedure, depending on T status and size of the tumor. Nodal involvement in gastric cancer occurs in only 2%-18% when the depth of cancer invasion is limited to the mucosal or submucosal layer (T1), and in about 50% when tumors invade the subserosal layer (T2)[4]. In patients with tumor-negative lymph nodes, a SLN procedure could avoid the risk of morbidity and mortality of an unnecessary lymphadenectomy. Additionally, in patients who are undergoing a partial or total gastrectomy combined with lymphadenectomy, identification of potentially involved LNs outside the standard plane of resection is possible by detecting the SLN. In this way, also in tumors with higher T stages, one can find the true first tumor draining LN(s), and not leaving them in situ. As the lymphatic drainage route of gastric cancer is generally multidirectional and complicated[5], intraoperative assistance in identification of potentially involved lymph nodes could improve gastric cancer treatment.

SLN detection in gastric cancer was first described by Kitagawa et al[6] Since then, multiple studies were performed. A prospective multicenter trial in 433 patients with T1 or T2 stadium tumors showed an accuracy rate of 99% for identification of metastasis in SLNs with the use of a dual tracer consisting of radiolabeled tin colloid and blue dye[7].

Near-infrared (NIR) fluorescence imaging is an innovative technique to visualize tumors, vital structures, lymphatic channels, and LNs[8]. Soltesz et al[9] in a preclinical setting and Kusano et al[10] in a clinical setting were the first to report the SLN procedure in gastric cancer using NIR fluorescence imaging. Since then, multiple studies confirmed the feasibility of this technique for both open and laparoscopic surgery[11-15]. All clinical studies reported to date utilized indocyanine green (ICG) as the lymphatic tracer. However, the use

of ICG resulted in detection of more fluorescent lymph nodes per patient than expected due to migration through the SLN to second tier nodes. Consequently, resection and pathological assessment of multiple nodes was still needed. Adsorption of ICG to a nanocolloid (ICG:Nanocoll) increases its hydrodynamic diameter, which may result in better retention of the lymphatic tracer in the SLN, and thereby staining less second-tier nodes. This results in intraoperative identification of true SLNs, and avoids analyzing non-SLNs during pathological assessment for tumor-status of the SLN. This principle was already successfully described for breast cancer[16] and skin melanoma[17].

The aim of this study was to investigate feasibility of ICG adsorbed to nanocolloid as a lymphatic tracer for the intraoperative detection of the SLN in gastric cancer patients with different pT stages, and to determine the prognostic utility of the detected SLN.

MATERIALS AND METHODS

Tracer preparation
ICG:Nanocoll was prepared by diluting 25 mg ICG (Pulsion Medical Systems, Munich, Germany) in 5 mL water and diluting 0.5 mg Nanocoll (GE Healthcare, Eindhoven, the Netherlands) in 3 mL saline. Portions of these solutions were mixed to obtain 1.6 mL ICG:Nanocoll containing 0.05 mg ICG and 0.1 mg Nanocoll. Preparation was performed in the operating room, following preparation instructions of the institutional pharmacist.

Clinical trial
The trial was approved by the Medical Ethics Committee of the Leiden University Medical Center and was performed in accordance with the ethical standards of the Helsinki Declaration of 1975. Registration within the Netherlands Trial Register was performed (NTR4280).

Twenty-six patients with different T stages of gastric cancer, planned for a partial or total gastrectomy, were included between February 2013 and March 2015. Patients underwent standard-of-care preoperative imaging using a computed tomography (CT) scan. No standard endoscopic ultrasound or staging laparoscopy was performed. All procedures were performed by surgeons with broad experience in gastric cancer surgery.

After opening of the abdominal cavity the tumor was exposed without causing damage to lymphatic vessels around the tumor as much as possible. When no metastasized disease was found, 1.6 mL ICG:Nanocoll was administered subserosally in 4 quadrants around the tumor. Directly after injection NIR fluorescence images of lymphatic pathways were acquired using the Mini-FLARE™ NIR fluorescence imaging system[18]. Fluorescence imaging was performed on multiple time points during surgery. A SLN was defined as fluorescent

hotspot that appeared after injection of the tracer. When multiple fluorescent hotspots appeared in the same LN basin all fluorescent LNs were defined as SLNs. The anatomical location of the fluorescent hotspots was determined using the lymph node stations as defined by the Japanese Research Society for the Study of Gastric Cancer[19]. Patients underwent a standard-of-care partial or total gastrectomy with modified D2 resection, consisting of resection of the peri-gastric LNs and LN station 7, 8 and 9. After resection, the specimen was analyzed *ex vivo* using the FLARE™ NIR fluorescence imaging system at the Pathology Department. The marked fluorescent hotspots were resected from the specimen, transected, fixed in formalin and embedded in paraffin for routine hematoxylin and eosin staining, and analyzed for tumor status.

The *in vivo* signal-to-background ratio (SBR) of the SLN was calculated by dividing the fluorescence intensity of the SLN by the fluorescence intensity of the directly surrounding fatty tissue. Accuracy rate was defined by the number of patients in which tumor-negative SLNs were found when no tumor-positive lymph nodes were found in the entire specimen and the number of patients in which tumor-positive SLNs were found when tumor-positive lymph nodes were found in the whole specimen divided by the total number of patients. Accuracy rate was expressed as percentage with a 95%CI. A false-negative patient was defined as a patient in whom tumor-negative SLNs were found, while tumor-positive LNs were found in the resection specimen.

Confidence intervals for the binomial proportions were calculated using exact binomial confidence intervals. Numerical data were summarized with median (range).

Intraoperative near-infrared fluorescence imaging
Intraoperative imaging procedures were performed using the Mini-Fluorescence-Assisted Resection and Exploration (Mini-FLARE™) image-guided surgery system, as described earlier[18]. Briefly, the system consists of 2 wavelength isolated light sources: a "white" light source, generating 26600 lx of 400 to 650 nm light, and a "near-infrared" light source, generating 1.08 mW/ca^{me} of about 760 nm light. Color video and NIR fluorescence images are simultaneously acquired and displayed in real time using custom optics and software that separate the color video and NIR fluorescence images. A pseudo-colored (lime green) merged image of the color video and NIR fluorescence images is also displayed. The imaging head is attached to a flexible gooseneck arm, which permits positioning of the imaging head at extreme angles virtually anywhere over the surgical field. For intraoperative use, the imaging head and imaging system pole stand are wrapped in a sterile shield and drape (Medical Technique Inc., Tucson, AZ).

Color	NIF fluorescence	Color NIR merge

Figure 1 Identification of sentinel lymph nodes using near-infrared fluorescence imaging. A: Identification of sentinel lymph node (SLN) (arrow) 15 min after injection of ICG:Nanocoll using near-infrared (NIR) fluorescence imaging. The injection site around the tumor is indicated by a dashed arrow; B: Patient with tumor-positive lymph node (indicated by arrow). Injection sites and fluorescent SLN are clearly detected. Lymphatic vessels are also visible between the injection site and SLN. The SLN is marked using sutures.

Table 1 Patient and tumor characteristics

Characteristic	Median	Range
Age (yr)	64	30-82
Tumor size (mm)	35	10-90
	N (n = 26)	
Gender		
M	19	73%
F	7	27%
Tumor location		
Cardia	8	31%
Corpus	6	23%
Antrum	12	46%
Tumor pT stage		
pTx	2	8%
pT1	5	19%
pT2	5	19%
pT3	10	39%
pT4	4	15%
Type of resection		
Total gastrectomy	11	42%
Partial gastrectomy	14	54%
No resection	1	4%
Preoperative CTx	23	88%

RESULTS

Patient characteristics

Twenty-six patients with gastric cancer undergoing partial or total gastrectomy were included in this study (Table 1). Median age was 64 years (range 30-82) and 19 patients were male. T-stadium of tumors was pTx, pT1, pT2, pT3, and pT4 in respectively 2, 5, 5, 10, and 4 patients. Median tumor size was 31 mm (range 10-90 mm). Tumors were located in the cardia in 8, corpus in 6 and antrum in 12 patients. Eleven patients

underwent a total gastrectomy, 14 patients underwent a partial gastrectomy and in 1 patient no resection was performed due to metastasized disease. Twenty-three patients received neoadjuvant chemotherapy consisting of Epirubicine, Oxaliplatin and Capecitabine or Epirubicine, Cisplatine and Capecitabine.

Sentinel lymph node detection

Three patients (No.3, No.12, and No.16) did not receive an injection of ICG:Nanocoll because metastatic disease was found during surgery. In 1 patient (No.8), ICG:Nanocoll was injected through the wall of the stomach. After this technical failure, this patient was excluded for further analysis.

Table 2 shows the characteristics of the intraoperatively detected SLNs in each patient. In 21 of the remaining 22 patients (95%, 77%-100%, 95%CI interval), at least 1 SLN was found during surgery (mean of 3.1 SLNs per patient; range 1-6). SLNs were identified as bright fluorescent spots in the surrounding tissue of the stomach. Figure 1A shows a bright fluorescent spot, which was found histologically to be a tumor-negative lymph node. Figure 1B shows an example of a tumor-positive lymph node and visualization of lymphatic vessels running from the injection site to the lymph node. The mean SBR of the SLNs was 4.4 (range 1.4-19.8). In total, 533 LNs were identified in the resection specimens by the pathologist, resulting in a mean number of 24 resected LNs (range 11-44) per patient.

In 19 out of 21 patients, an accurate SLN was found. The overall accuracy of the SLN procedure was 90% (95%CI: 70%-99%) and a higher pT-

Table 2 Characteristics of detected sentinel lymph nodes

Patient No.	T stage	Tumor location	Pre-operative CTx	Number of detected SLNs	Location of detected SLNs by LN St	Mean SBR of SLNs	Tumor status in SLNs	SLN Identification accurate?	SLN outside Standard dissection plane?
1	1	M	No	3	3	5.4	Neg	Yes	No
2	4	M	Yes	3	3; 3 and 6	1.9	Pos	No	No
3	4	D	Yes	No tracer injected because of metastasized disease						
4	1	D	Yes	3	4; 6 and 6	4.3	Neg	Yes	No
5	2	U	Yes	4	3; 3; 4 and 4	3.9	Neg	Yes	No
6	3	M	Yes	3	4; 4 and 7	5.3	Neg	Yes	No
7	1	M	No	4	3; 3; 6 and 6	3.1	Neg	Yes	No
8	2	U	Yes	Technical failure of tracer administration						
9	3	U	Yes	2	1 and 1	8.7	Neg	Yes	No
10	3	D	No	3	6; 6 and 12	4.7	Pos	No	Yes
11	3	D	Yes	4	5; 6; 6 and 12	4.9	Pos	Yes	Yes
12	4	U	Yes	No tracer injected because of metastasized disease						
13	2	U	Yes	3	1; 7 and 9	3.5	Neg	Yes	No
14	2	D	Yes	4	6 and 12	5.1	Pos	Yes	Yes
15	2	M	Yes	0	NA	NA	NA	NA	No
16	4	M	Yes	No tracer injected because of metastasized disease						
17	3	U	Yes	2	8 and 12	2.6	Neg	Yes	Yes
18	3	D	Yes	2	5 and 6	11.4	Neg	Yes	No
19	x	D	Yes	2	3 and 3	3.6	Neg	Yes	No
20	x	D	Yes	2	3 and 14	3.6	Neg	Yes	Yes
21	3	M	Yes	5	3; 3; outside standard planes of 9 (3x)	2.8	Pos	Yes	Yes
22	3	D	Yes	4	3; 6; 6 and 14	3.1	Pos	Yes	Yes
23	3	U	Yes	1	1	6.2	Pos	Yes	No
24	3	D	Yes	6	3; 3; 6; 6; 6 and 11	4.0	Pos	Yes	Yes
25	1	D	Yes	4	3; 3; 4 and 6	4.6	Neg	Yes	No
26	1	D	Yes	4	5; 6; 6 and 6	5.6	Neg	Yes	No

CTx: Chemotherapy; D: Antrum of stomach; LN: Lymph node; M: Corpus of stomach; NA: Not Applicable; SBR: Signal-to-background ratio; SLN: Sentinel lymph node; St: Lymph node station according to Japanese Research Society for the Study of Gastric Cancer; U: Cardia of stomach.

stadium was associated with a lower accuracy rate. Accuracy rates for pTx, pT1, pT2, pT3, and pT4 were respectively 100%, 100%, 100%, 90%, and 0%.

Histological analysis of the SLNs showed lymph node metastases in 8 out of 21 patients. In 6 patients, the SLNs that were identified using NIR fluorescence imaging were tumor-positive (true positive). In the other 2 patients, tumor-positive lymph nodes were not identified using NIR fluorescence imaging (false-negative). One false-negative patient (No.2) had a T4 tumor. The tumor positive lymph nodes (3 out of 33 LNs) in this patient were found in the peripancreatic fatty tissue and in lymph node station 3, where a SLN was also detected. The second false-negative patient (No.10) had a T3 tumor. Four out of 11 LNs that contained tumor cells were not detected by NIR fluorescence imaging. Of particular importance, all 7 tumor-positive LNs that were not detected by NIR fluorescence imaging were completely effaced by tumor tissue and no lymphatic tissue could be identified.

In 8 patients, SLNs outside the standard resection plane were identified. In 4 patients these were located in the hepatoduodenal ligament (LN station 12), in 2 patients near the border of the pancreas (LN station 14), in 1 patient outside the standard plane near LN station 9 and in 1 patient in LN station 11. In 2 patients, the extra-detected lymph nodes outside the standard plane of resection contained tumor cells (No.21 and No.22) (Figure 2).

No adverse events regarding the use of ICG:Nanocoll or NIR fluorescence imaging were encountered.

DISCUSSION

The current feasibility study demonstrates that the SLN detection in gastric cancer, using ICG adsorbed to nanocolloid as the lymphatic tracer, is feasible and safe. In 21 out of 22 patients at least 1 SLN was identified, and in 19 out of 21 patients an accurate SLN was found.

In fewer than 50 percent of patients with a T1 or T2 tumor, lymph nodes show tumor involvement. In these patients, the SLN procedure has the potential to avoid an unnecessary lymphadenectomy, and its associated potential morbidity and mortality. Kitagawa *et al*[7] reported an accuracy of nodal evaluation for metastasis of 99 percent, underlining the clinical applicability of the technique in this selected patient group. The SLN procedure in gastric cancer was validated for previously untreated cT1-T2 tumors with a diameter of less than 4 mm. However, in the Western world patients often present with a higher T stadium,

Color	NIF fluorescence	Color NIR merge

Figure 2 Identification of tumor-positive sentinel lymph nodes outside the standard dissection planes. Identification of tumor-positive sentinel lymph node (arrow) outside the standard resection specimen in patient No.21. LNs are identified after gastrectomy and located outside the standard dissection plane near LN station 9.

and are often pretreated with chemotherapy. In these patients who need an extensive lymphadenectomy, the described technique could assist in identifying potentially involved lymph nodes located outside the standard plane of resection. Moreover, morbidity and mortality rates increase when a more extended lymph node dissection is performed[20]. A more targeted and personalized treatment, including identification and dissection of truly or potentially involved lymph nodes, could result in improved gastric cancer treatment. Therefore, in both early gastric cancer and in resectable cases of advanced gastric cancer, accurate identification of true SLNs is of great importance.

Many different lymphatic tracers have been reported for SLN identification. The largest prospective multicenter trial until now used a combination of blue dye and radiolabeled tin colloids[7]. However, both tracers have disadvantages. Blue dye could alter the surgical field by dark staining, and only permits identification of superficially located lymph nodes. Moreover, in previous studies comparing radiolabeled colloids, blue dye, and NIR fluorescence for SLN detection in breast cancer patients, only 84%-88% of the identified SLNs stained blue compared to 100% that were NIR fluorescent[16,21]. For the SLN procedure in skin melanoma blue dye staining was successful in only 73%[22]. Radiolabeled colloids only permit acoustic guidance during SLN identification, but no visual guidance. Besides, radioactive isotopes are scarce in many areas of the world. NIR fluorescence imaging could overcome these limitations as it only needs an imaging system and fluorescent tracer, and allows real-time optical identification of lymph nodes in up to about 6 mm of tissue, for example in visceral dense fat tissue[8].

Since Kusano *et al*[10] reported the first SLN procedure in gastric cancer using NIR fluorescence imaging, multiple studies confirmed the feasibility of this technique for both open and laparoscopic surgery[11-15]. All reported studies to date used ICG alone as lymphatic tracer, which resulted in detection of many fluorescent lymph nodes per patient. For example, Tajima *et al*[14] reported a mean number of 7.2 ± 7 SLN per patient and Fujita *et al*[11] a mean number of 9.3 ± 6.4 SLN per patient when using ICG

as lymphatic tracer. This was possibly due to migration through the SLN to second tier nodes, and resection and pathological assessment of multiple nodes was still needed.

By combining ICG with nanocolloid, its hydrodynamic diameter increases from ⩽ 1 nm (ICG) to 20-80 nm (ICG:Nanocoll). It has been shown that the hydrodynamic diameter of a lymphatic tracer has a major impact on the lymphatic migration and accumulation in lymph nodes. Molecules with a hydrodynamic diameter less than approximately 10 nm (for example ICG) have the potential to migrate through the SLN to second tier nodes, while larger molecules with a hydrodynamic diameter of < 100 nm (ICG:Nanocoll) are retained in the SLN[23]. In the current study, a mean number of 3.1 SLN per patient was found. This lower number of detected SLNs is in accordance with our hypothesis that better retention in SLNs is obtained when a lymphatic tracer with a higher hydrodynamic diameter is used. This highly improves the clinical applicability of SLN detection using NIR fluorescence imaging in gastric cancer.

Although the number of patients was limited in the current study, an excellent accuracy rate was obtained in lower pT stages, in which the clinical value of a SLN procedure is becoming more and more accepted. These data are consistent with previous studies where the SLN procedure was performed for T1 and T2 tumors. However, even for advanced gastric cancer, identifying the first draining lymph nodes can be of added value. In the current study, LNs from 8 patients were identified outside the standard resection margin using NIR fluorescence imaging; they would otherwise not have been resected. In 2 patients, the extra-detected LNs outside the standard plane of resection contained tumor cells. These LNs were only resected because NIR fluorescence imaging identified them. Larger studies are needed to determine the additional value of the described technique in advanced gastric cancer.

In 2 patients, tumor-positive LNs were not identified using NIR fluorescence imaging. One explanation for the false positivity of these LNs might be the fact that these tumors were relatively large, respectively 60 and 45mm diameter, and consequently, adequate injection of the

tracer in four quadrants around the tumor might be hampered. However, other fluorescent LNs that that did contain tumor cells, were found in tumors with a median size of 52mm (range 27-90). Moreover, all 7 of these LNs were completely effaced by tumor tissue. Such LNs lose function, lymph doesn't flow in or out, and no lymphatic tissue is present to trap the fluorescent tracer. Subsequently, these tumor-positive LNs can't be identified, a principle that counts for the SLN procedure in all solid cancers. In one of these patients however, the identified SLN was found in the same LN basin as one of the tumor-involved LNs. This underlines the theory that whenever SLNs are visualized, the entire lymph node basin should be resected instead of only the SLN by lymph node picking, because it is shown that most of the metastatic non-SLNs are positioned in the same basin as the detected SLNs[24,25].

A well-known difficulty in SLN mapping in gastric cancer is the presence of skip metastasis: involvement of extra-perigastric lymph nodes without the detection of perigastric lymph node metastasis. The incidence of skip metastasis among patients with gastric cancer and metastasis is reported to be as high as 11%[26]. The described technique could assist in identifying these potentially involved extra-perigastric lymph nodes.

One of the limitations of this study is the administration technique, which is performed during surgery in the subserosal layer of the gastric wall. Opening the abdominal cavity, and exposing the affected part of the stomach could damage lymphatic vessels. This potentially hampers lymphatic flow to SLNs and was overcome as much as possible by avoiding dissections near the primary tumor. Besides, injecting the tracer in the submucosal layer seems more appropriate in case of tumor invasion limited to the mucosa or submucosa. However, it is shown that subserosal injection leads to drainage of the tracer to the same lymph nodes as submucosal injection, because of communication through vertical connections of lymphatic vessels in the gastric wall[27]. These limitations could be overcome by submucosal endoscopic administration of the lymphatic tracer before surgery. One of the additional advantages of this administration technique is that it allows visual tumor demarcation during surgery through the stomach wall, which assists in intraoperative tumor identification, and determination of resection margins. Especially in patients who experience good response on neoadjuvant chemotherapy this could be of added value.

Another limitation of the current feasibility study is that no true SLN procedure was performed, but instead intraoperative detection of SLNs. However, all fluorescent hotspots that were detected during surgery were directly marked using sutures. After the surgical procedure, they were mapped from the specimen for pathological assessment. By doing this, the same LNs were analyzed as if they would have been resected directly during surgery, and feasibility of ICG:Nanocoll as lymphatic tracer could still be showed.

Finally, pathological assessment of the LNs consisted of standard-of-care transection and hematoxylin and eosin staining. Multiple transections and additional keratin staining could possibly have resulted in the detection of tumor-tissue in the SLNs of the 2 false-negative patients, and thereby increasing accuracy rate. However, small tumor deposits in the detected SLNs were unlikely to be present as the tumor-positive LNs in the specimen were completely effaced by tumor.

In conclusion, this is the first study using ICG combined with nanocolloid as lymphatic tracer for the detection of the SLN in gastric cancer patients by NIR fluorescence imaging. In T1 and T2 gastric tumors, an excellent accuracy was observed. Moreover, this technique allowed identification of tumor-positive lymph nodes outside the standard dissection planes.

ACKNOWLEDGMENTS

COMMENTS

Background

Treatment of gastric cancer consists of surgical resection of the tumor and identification and resection of potentially involved lymph nodes. The latter can either be done by extensive lymphadenectomy or by a sentinel lymph node (SLN) procedure. Identification of these lymph nodes can be challenging, and assistance in identification of potentially involved lymph nodes could improve gastric cancer treatment. Near-infrared (NIR) fluorescence imaging is an innovative technique to visualize lymph nodes and lymphatic channels, and could therefore improve identification of SLNs.

Research frontiers

NIR fluorescence imaging is gaining more and more scientific and clinical attention worldwide, and physicians are exploring the possibilities and capabilities of clinically available contrast agents like indocyanine green (ICG). Optimizing optical and accuracy properties of lymphatic tracers, as performed in the current study, can have major impact on its clinical relevance.

Innovations and breakthroughs

Several studies to date are reported on the use of ICG as fluorescent tracer for identification of SLNs. However, the use of ICG alone resulted in detection of more fluorescent lymph nodes per patient than expected due to migration through the SLN to second tier nodes. Consequently, resection and pathological assessment of multiple nodes was still needed. Adsorption of ICG to a nanocolloid (ICG:Nanocoll) increases its hydrodynamic diameter, which may result in better retention of the lymphatic tracer in the SLN, and thereby staining less second-tier nodes. To the best of our knowledge, this is the first study reported on the use of ICG:Nanocoll as lymphatic tracer in gastric cancer.

Applications

Gastric cancer is one of the most frequent causes of cancer deaths worldwide, and optimizing its treatment can have major impact on patient outcome. The described agents for NIR fluorescence imaging (ICG and Nanocoll) are commercially available. Therefore this technique can be easily applied to the treatment of gastric cancer.

Terminology

NIR fluorescence imaging is an optical imaging technique using light in the NIR spectrum (700-900 nm). Indocyanine green is a cyanine dye with fluorescence properties in the NIR spectrum.

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ORIGINAL ARTICLE

Observational Study

Dual-input two-compartment pharmacokinetic model of dynamic contrast-enhanced magnetic resonance imaging in hepatocellular carcinoma

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Shaoxing People's Hospital (Shaoxing Hospital of Zhejiang University).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

AIM: To investigate the feasibility of a dual-input two-compartment tracer kinetic model for evaluating tumorous microvascular properties in advanced hepatocellular carcinoma (HCC).

METHODS: From January 2014 to April 2015, we prospectively measured and analyzed pharmacokinetic parameters [transfer constant (K_{trans}), plasma flow

(F_P), permeability surface area product (PS), efflux rate constant (k_{ep}), extravascular extracellular space volume ratio (v_e), blood plasma volume ratio (v_p), and hepatic perfusion index (HPI)] using dual-input two-compartment tracer kinetic models [a dual-input extended Tofts model and a dual-input 2-compartment exchange model (2CXM)] in 28 consecutive HCC patients. A well-known consensus that HCC is a hypervascular tumor supplied by the hepatic artery and the portal vein was used as a reference standard. A paired Student's *t*-test and a nonparametric paired Wilcoxon rank sum test were used to compare the equivalent pharmacokinetic parameters derived from the two models, and Pearson correlation analysis was also applied to observe the correlations among all equivalent parameters. The tumor size and pharmacokinetic parameters were tested by Pearson correlation analysis, while correlations among stage, tumor size and all pharmacokinetic parameters were assessed by Spearman correlation analysis.

RESULTS: The F_P value was greater than the PS value (F_P = 1.07 mL/mL per minute, PS = 0.19 mL/mL per minute) in the dual-input 2CXM; HPI was 0.66 and 0.63 in the dual-input extended Tofts model and the dual-input 2CXM, respectively. There were no significant differences in the k_{ep}, v_p, or HPI between the dual-input extended Tofts model and the dual-input 2CXM (*P* = 0.524, 0.569, and 0.622, respectively). All equivalent pharmacokinetic parameters, except for v_e, were correlated in the two dual-input two-compartment pharmacokinetic models; both F_P and PS in the dual-input 2CXM were correlated with K_{trans} derived from the dual-input extended Tofts model (*P* = 0.002, *r* = 0.566; *P* = 0.002, *r* = 0.570); k_{ep}, v_p, and HPI between the two kinetic models were positively correlated (*P* = 0.001, *r* = 0.594; *P* = 0.0001, *r* = 0.686; *P* = 0.04, *r* = 0.391, respectively). In the dual input extended Tofts model, v_e was significantly less than that in the dual input 2CXM (*P* = 0.004), and no significant correlation was seen between the two tracer kinetic models (*P* = 0.156, *r* = 0.276). Neither tumor size nor tumor stage was significantly correlated with any of the pharmacokinetic parameters obtained from the two models (*P* > 0.05).

CONCLUSION: A dual-input two-compartment pharmacokinetic model (a dual-input extended Tofts model and a dual-input 2CXM) can be used in assessing the microvascular physiopathological properties before the treatment of advanced HCC. The dual-input extended Tofts model may be more stable in measuring the v_e; however, the dual-input 2CXM may be more detailed and accurate in measuring microvascular permeability.

Key words: Hepatocellular carcinoma; Dynamic contrast-enhanced magnetic resonance imaging; Pharmacokinetics

Core tip: Dynamic contrast-enhanced magnetic resonance imaging provides a more comprehensive assessment of microvascular parameters in tumors; however, selection of a pharmacokinetic model that takes into account actual physiopathological status is an essential component of evaluating tumor microvascular permeability and perfusion. Here, we confirm that a dual-input two-compartment tracer kinetic model is suitable for evaluating microvascular properties in advanced hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common primary malignant tumors and the second leading cause of cancer-related deaths worldwide[1]. Some patients present with advanced disease at the time of diagnosis and are treated with molecular-targeted treatment, transarterial chemoembolization (TACE), and radiofrequency ablation against HCC[2]. Assessing the therapeutic efficacy of these therapy modalities is closely related to the tumorous microvasculature properties that are linked to the angiogenic potential of the tumor[3]. A tracer kinetic model of T1-weighted dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has significant potential to obtain information about the tumor microvascular properties by estimating the pharmacokinetic parameters of the microvascular perfusion and permeability[4]. Some studies have shown the value of assessing microvascular properties in monitoring the effects of interventional therapy or antiangiogenic drug treatment of HCC, as well as metastases in the liver, by using single-input single-compartment or two-compartment tracer kinetic models to evaluate tumor pharmacokinetic parameters[3,5-8].

According to the dynamic distribution of the contrast agent, a well-mixed space of contrast agent is defined as a compartment where the contrast agent concentration is spatially uniform[9,10]. The tracer compartment model is divided into a single-compartment model such as the Tofts model, which assumes that the contrast agent is confined to only one compartment (*i.e.*, vascular space), and two-compartment models such as the extended Tofts model and the exchange model, in which the contrast

agent transits vascular space to the interstitial space[9]. The tissue under investigation and its underlying microvascular physiology, as well as the temporal resolution and spatial resolution of scanning MRI, are important factors that led us to select a tracer kinetic model. The parenchyma in most tumors consists of two compartments [tumorous intravascular space and extravascular extracellular space (EES)]; thus, a two-compartmental kinetic model may provide a better reflection of the microcirculation of tumor[11]. Moreover, HCC is supplied by both the portal vein and the hepatic artery in different proportions at various stages[12]. Therefore, we hypothesized that a dual-input two-compartment model may accurately evaluate the pharmacokinetic parameters in advanced HCC.

To date, microvascular property assessment by dual-input two-compartment tracer kinetic model with commonly used extracellular gadoliniun contrast agent has not been reported in HCC in a clinical practice setting. Hence, the aim of this study was to prospectively explore whether a dual-input two-compartment tracer kinetic model could evaluate the tumorous microvascular properties in advanced HCC by analyzing perfusion and permeability parameters derived from a dual-input extended Tofts model and a dual-input 2CXM.

MATERIALS AND METHODS

Patient population
This study was approved by the local Institutional Review Board, and informed consent was obtained from all patients. From January 2014 to April 2015, 42 patients with HCC were recruited, all of whom did not receive any antineoplastic treatment before their MRI scan. The inclusion and exclusion criteria are listed in Table 1. We staged Barcelxona-Clínic Liver Cancer classification (BCLC) for all enrolled HCC patients according to the criteria of the 2012 European Association for the Study of the Liver (EASL)[13]. Because microvascular physiologic parameters in tumors are functional biomarkers that could not be measured from the pathological sample *in vitro*, we looked to a well-known consensus as a reference standard that HCC is a hypervascular tumor supplied by both the portal vein and the hepatic artery in different proportions.

MRI technique
An MRI scan of the whole liver was performed using a 12-channel phased array body coil on the 3.0-T MRI system (Magnetom Verio, Siemens, Erlangen, Germany) with Syngo 2009B software. The scan protocol consisted of transverse T2-weighted turbo spin-echo images (TR/TE, 1370/81; slice thickness, 6 mm; interslice gap, 1.2 mm; matrix size, 207 × 320; received bandwidth, 220 kHz) and diffusion weighted

Table 1 Inclusion and exclusion criteria of the enrolled patients

Eligibility criteria	Exclusion criteria
Histopathology confirmed	MRI examination contraindication
Non-invasive diagnosis criteria [(EASL) 2012]¹ Cirrhotic patients Hypervascular in the arterial phase Washout in the portal venous or delayed phase	Significant renal insufficiency
Non-antineoplaston therapy	Severe motion artifacts on MRI images
The largest diameter of lesion ⩾ 2 cm	Hepatic vein/portal cancer embolus
Age of patients ⩾ 18 yr	Inferior vena cava embolus Inability of informed written consent

¹European Association for the Study of the Liver suggested non-invasive diagnosis criteria on 4-phase multidetector CT scan[13]. EASL: European Association for the Study of the Liver; MRI: Magnetic resonance imaging.

echoplanar images (TR/TE, 7400/73; slice thickness, 6 mm; interslice gap, 1.2 mm; b value = 0 s/mm², 600 s/mm²; matrix, 99 × 146; received bandwidth, 1802 kHz). Multi-flip angle T1 Mapping and DCE T1-weighted three-dimensional volume interpolated excitation (VIBE) fat-suppression sequence with breath-free (TR/TE, 3.5/1.17 msec; slice thickness, 5 mm; interslice gap, 1 mm; matrix, 288 × 164; field of view, 350 × 284 mm; scan slices were 30 in unenhanced T1WI and enhanced TIWI; flip angle were 5°, 10°, 15° in unenhanced TIWI, flip angle was 10° in enhanced TIWI; temporal resolution, 6 s) were also obtained. DCE-MR imaging data were acquired after a 5-s delay subsequent to the injection of contrast medium through a 20-guage peripheral intravenous line in the medial cubital vein (0.1 mmol/kg body weight of Gadodiamide contrast medium; Omniscan, GE Medical Systems, Amersham, Ireland) at 3.5 mL/s, followed by a saline chase of 20 mL at a rate of 2 mL/s. DCE-MR imaging included 35 acquiring phases and lasted for 227.5 s.

Model design
In this study, a dual-input two-compartment tracer kinetic model was utilized to analyze the perfusion and permeability of tumorous vascularity in HCC. This type of model accounts for the hepatic artery and portal vein input to the HCC and assumes contrast agent in two compartments (tumorous intravascular space and EES). We applied the hepatic perfusion index (HPI) to observe the contribution of arterial flow to the tumor. The tissue that the blood vessels containing contrast agent supply can be measured by Vascular Input Function (VIF) in both the hepatic artery and the portal vein. The definitions of all symbols are listed in Table 2.

The tracer in intravascular space is able to diffuse to the EES through the capillary walls, and K$_{trans}$ denotes

Table 2 Summary of parameter terms used in the dual-input extended Tofts model and 2-compartment exchange model

Symbol	Definition	Unit
$C_{tiss}(t)$	Concentration of tracer in the tissue	mmol/L
$CA(t)$	Concentration of tracer in whole blood in a major feeding artery	mmol/L
$C_v(t)$	Concentration of tracer in the portal vein	mmol/L
Fa	Arterial fraction of the tissue perfusion	%
HLV	Hematocrit in major (large) vessels	none
HPI	Hepatic perfusion index	none
F_P	Flow rate of the blood plasma through the intravascular plasma space	mL/mL per minute
v_P	Ratio of blood plasma volume to tissue volume	%
v_e	Ratio of EES volume to tissue volume	%
k_{ep}	Efflux rate constant	min^{-1}
K_{trans}	Transfer constant	min^{-1}
PS	Endothelial permeability surface area product	mL/mL per minute
⊗	Convolution operator	None

EES: Extravascular extracellular space.

the tracer transfer constant between blood and EES, which combined the plasma flow (F_P) and the capillary permeability-surface area product (PS). The efflux rate constant (k_{ep}) is the ratio of the transfer constant from EES to blood plasma. v_P and v_e are the volume fraction in the vascular space and EES, respectively[14].

An extended Tofts model evaluates K_{trans}, v_e, v_P, and k_{ep}[14]. This model assumes that a neglect of plasma mean transit time results in a situation where the concentration of contrast agent within the plasma compartment is equal to the concentration in the supplying artery[15]; therefore, the concentration of contrast agent in the tissue, $C_{tiss}(t)$ in this model, can be written as follows:

$$C_{tiss}(t) = v_P CA(t)/(1-HLV) + K_{trans} CA(t)/(1-HLV) \otimes \exp(-k_{ep}t) \quad \text{(Eq. 1A)}$$

The equation (Eq. 1A) is the formulation of a single-input extended Tofts model; thus, we insert a dual inlet equation (Eq. 2) to produce the equation of the dual-input extended Tofts model (Eq. 1B).

$$CA(t) \rightarrow faCA(t) + (1-fa)C_v(t) \quad \text{(Eq. 2)}$$

$$C_{tiss}(t) = v_P[faCA(t) + (1-fa)C_v(t)]/(1 - HLV) + K_{trans}[faCA(t) + (1-fa)C_v(t)]/(1 - HLV) \otimes \exp(-k_{ep}t) \quad \text{(Eq. 1B)}$$

The 2CXM is the most common exchange model and can separately evaluate F_P and PS, in addition to V_P, V_e, and K_{ep}[10,14]. As for a single-input 2CXM, the concentration of the contrast agent in the tissue, $C_{tiss}(t)$, can be written as follows:

$$C_{tiss}(t) = F_P CA(t)/(1 - HLV) \otimes A.\exp(-\alpha t) + (1-A).\exp(-\beta t) \quad \text{(Eq. 3A)}$$

We also insert a dual-inlet equation (Eq. 2) into the equation (Eq. 3A) to obtain the final equation of the dual-input 2CXM as follows:

$$C_{tiss}(t) = F_P[faCA(t) + (1 - fa)C_v(t)]/(1 - HLV \otimes A.\exp(-\alpha t) + (1 - A).\exp(-\beta t) \quad \text{(Eq. 3B)}$$

Figure 1 Concentration-time curve of the portal vein was an inferior fit (arrow) compared to that of the abdominal aorta without non-rigid registration (A); however, the concentration-time curve of the portal vein was a better fit after non-rigid registration (B).

where A, α, and β can be obtained from the model parameters v_P, v_e, and PS, respectively:

$$A = PS/v_P; \quad \alpha = PS/v_e; \quad \beta = F_P/v_P$$

Data postprocessing and analysis

Because there were more than 2 lesions of HCC in the liver in some patients, we measured the one with the largest diameter and also measured the mass volume. Each data set was measured by the same radiologist (who has 17 years of abdominal diagnosis experience), who kept the approach consistent at each time point of the procedure. All data were postprocessed using Omni Kinetics software (GE Healthcare, China). After data loading, we registered all acquired data using 3D non-rigid registration to relieve the motion artifact caused by breath and heart beating (Figure 1); then, we extracted an average signal-time course of the tumor and converted the signal-time curve to a concentration-time curve. The signal intensity of all pixels in the image was converted to contrast agent concentration using the precontrast T1 value of 1600

Figure 2 ROI 1 was placed on the abdominal aorta near the entrance of the celiac trunk, replacing the hepatic artery, and ROI 2 was placed on the portal vein as a dual input model to fit the vascular input function; the concentration-time curve maps of the ROI 1 and ROI 2 are shown to the right.
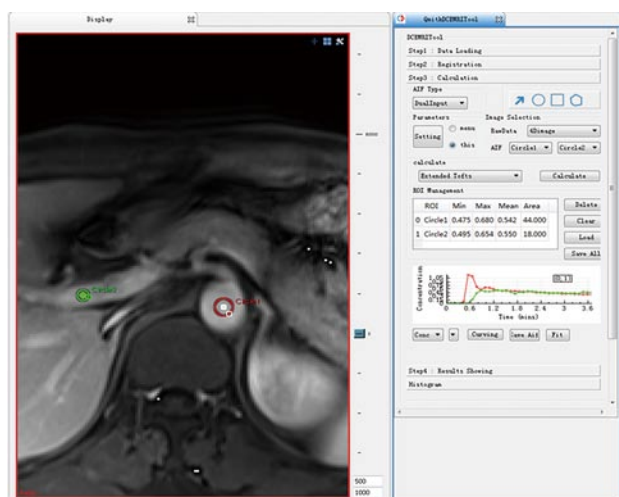
ms for blood and 1580 ms for tissue, and an assumed hematocrit of 0.42 was used to convert from whole blood concentration to plasma concentration for all patients. Before calculating the pharmacokinetic parameters, we drew the ROI on both the portal vein and the hepatic artery, which was replaced by the abdominal aorta near the entrance of celiac trunk as a dual-input model to fit the VIF (Figure 2). The size and location of the ROI drawn on the lesion in the same patient are consentaneous in both models. We drew the ROI (by hand) on the parenchyma of the HCC at its largest diameter on the T1WI images in each patient, avoiding necrosis, hemorrhage, steatosis, and peripheral blood vessels, and then, we used the dual-input extended Tofts model and the dual-input 2CXM, respectively, to fit the data and calculate tumorous perfusion and permeability parameters: K_{trans} in the dual-input extended Tofts model, F_p and PS in the dual-input 2CXM, and v_p, v_e, k_{ep}, and HPI in both models (Figure 3). All measurements were performed three times, and the mean value is presented.

Statistical analysis

Statistical analyses were performed with the SPSS statistical software package (version 19.0; SPSS Inc, Chicago, IL, United States). Tumor size and pharmacokinetic parameters were assessed by Pearson correlation analysis. Correlation between stage, tumor size and all pharmacokinetic parameters was assessed by Spearman correlation analysis. We compared all equivalent parameters obtained from the dual-input extended Tofts model and dual-input 2CXM using a paired Student's *t*-test or a nonparametric paired Wilcoxon rank sum test for non-normal distribution data to confirm the consistency of these two models.

Pearson correlation analysis was also performed to analyze the correlation between all parameters. A *P*-value < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Hai-yang Xing from Shaoxing University.

RESULTS

In this prospective study, 42 HCC patients based on pathological diagnosis and non-invasive criteria underwent T1WI DCE-MRI. However, 14 patients were excluded because of portal or hepatic vein/inferior vena cava embolus (6 patients), severe motion artifacts (4 patients), MRI examination failure because of claustrophobia (1 patient), or different contrast agent injection rates (3 patients). In total, 28 patients were enrolled. Patients' demographic characteristics, tumor volume and the tumor stage are shown in Table 3. A significant correlation was found between tumor size and stage (*P* = 0.013, *r* = 0.463). Neither tumor size nor tumor stage significantly correlated with any of the pharmacokinetic parameters obtained in any of the models.

DCE-MRI microvascular physiopathological parameters obtained with the dual-input two-compartment tracer kinetic model are shown in Table 4. There were no significant differences in the k_{ep}, v_p, or HPI between the dual-input extended Tofts model and the dual-input 2CXM (*P* = 0.524, *P* = 0.569, *P* = 0.622, respectively). Except for v_e, all equivalent pharmacokinetic parameters derived from the two tracer kinetic models are correlated: both F_p and PS in the dual-input 2CXM are correlated with K_{trans} in the dual-input extended Tofts model (*P* =0.002, *r* = 0.566; *P* =0.002, *r* = 0.570, respectively; Figure 4); k_{ep}, v_p, and HPI were positively correlated (*P* = 0.001, *r* = 0.594; *P* = 0.0001, *r* = 0.686; *P* = 0.04, *r* = 0.391, respectively; Figures 5-7). v_e was significantly less in the dual-input extended Tofts model than in the dual-input 2CXM (*P* = 0.004), and there was no significant correlation between the two proposed tracer kinetics models (*P* = 0.156, *r* = 0.276; Table 4, Figure 8). The value of F_p was larger than that of PS in the dual-input 2CXM (F_p = 1.07 mL/mL per minute, PS = 0.19 mL/mL per minute); K_{trans} derived from the dual-input extended Tofts model was 0.29 min^{-1}; HPI was 0.66 and 0.63 in the dual-input extended Tofts model and the dual-input 2CXM, respectively (Table 4).

DISCUSSION

In recent decades, non-surgical therapies, such as antiangiogenic targeted drugs, radiofrequency ablation, and TACE, play an important role in the HCC therapy regime, and the therapeutic efficacy evaluation of these therapy methods has become an important issue. Response Evaluation Criteria In Solid Tumors

Figure 3 Lesion of the hepatocellular carcinoma in a 68-year-old man on dynamic contrast-enhanced T1WI (A); pharmacokinetic parameter map (K_{trans}, k_{ep}, v_e, v_p, and hepatic perfusion index) derived from the dual-input extended Tofts model (B-F), and the pharmacokinetic parameter maps (F_p, PS, k_{ep}, v_e, v_p, and hepatic perfusion index) derived from the dual-input 2-compartment exchange model (G-L).

(RECIST) or modified RECIST (mRECIST) are common criteria in evaluating a therapeutic effect. Specifically, mRECIST takes into account the contrast enhancement in the arterial phase to evaluate the viable tumor component[16,17]. These modified evaluation criteria imply that the assessment of tumor vascular properties not only contributes to HCC diagnosis but also is helpful in evaluating the therapeutic efficacy of treatments for this tumor. Recently, research has suggested that DCE-MRI allows the quantitative measurement of

pharmacokinetic parameters related to perfusion and permeability and provides a more comprehensive assessment of a tumor's physiologic properties[7]. However, finding a suitable tracer kinetic model that takes into account the actual physiopathological status to evaluate the tumor microvascular parameters under the condition of sufficient MRI temporal resolution and spatial resolution is critical.

Several studies have assessed the microvascular properties of HCC using single-input, single-compart-

Table 3 Patients' demographic characteristics, tumor volume, and tumor stage				
Age (yr)	Gender	Diagnosis criteria	Size (cm³)	Stage (BCLC)[1]
64.857 ± 10.384	Female 5	Confirmed by histology 8	409.588	A2 (2)
	Male 23	Diagnosed by EASL 20		A3 (3)
				A4 (1)
				B (11)
				C (11)

[1]Barcelona-Clínic Liver Cancer classification[13]. EASL: European Association for the Study of the Liver.

ment or two-compartment tracer kinetic models in monitoring the therapeutic response to interventional therapy or antiangiogenic drug treatment[7,8,18]. However, according to Thng *et al*[11] and Van *et al*[12], the parenchyma in HCC consists of the vascular space and the interstitial space and is supplied by both the portal vein and the hepatic artery in different proportions. We assume that the dual-input two-compartment tracer kinetic model may be suitable for evaluating the microvascular properties of HCC under the conditions of a high field strength MR machine. The purpose of this prospective study was to investigate the feasibility of a dual-input extended Tofts model and a dual-input 2CXM for evaluating microvasculature properties in advanced HCC.

The liver is a hypervascular organ supplied by the portal vein (75%) and the hepatic artery (25%)[19]. However, neovascularization in HCC is predominantly supplied by the hepatic artery, but it is supplemented by the portal vein[20]. HPI describes the relative contribution of arterial *vs* portovenous flow to the total liver perfusion, which is a semi-quantitative descriptor of liver vascularity[11]. In this study, we applied this perfusion parameter to observe the contribution of arterial flow to the HCC in order to verify the assumption that a dual-input model is appropriate in evaluating the pharmacokinetic parameters in most cases of HCC. Our study shows that HPI was 0.66 and 0.63 in the dual-input extended Tofts model and dual-input 2CXM, respectively. This finding is consistent with the assumption that a dual input is a realistic blood supply model in most cases of HCC and also shows that hepatic artery blood flow accounts for the majority but not all of total blood flow to advanced HCC.

K_{trans} is an important pharmacokinetic parameter to assess vascular permeability and therapeutic effects after non-operation treatment in HCC. Previous studies have suggested that a larger drop of K_{trans} is correlated with favorable clinical outcomes after sunitinib or Floxuridine therapy[7,8]. Depending on the balance between PS and F_p in the tissue of interest, K_{trans} has three physiologic interpretations: in a high PS status (PS >> F_p), K_{trans} is approximately equal to F_p; conversely, in high F_p situations (F_p >> PS), K_{trans}

is approximately equal to PS; and under mixed flow and permeability limited conditions, K_{trans} is the product (EF_p) of the initial extraction fraction (E) and F_p[21]. Tofts *et al*[21] and Bergamino *et al*[22] noted that the extended Tofts model provides accurate permeability values for only tissues that are weakly vascularized or highly perfused with a relatively high F_p. Our study results show that F_p is greater than PS in the dual-input 2CXM, and the value of K_{trans} in the dual-input extended Tofts model is close to that of PS. Moreover, both F_p and PS are correlated with K_{trans}. These findings are consistent with the second interpretation of the relationship among K_{trans}, F_p, and PS proposed by Tofts *et al*[21] and demonstrate that the dual-input two-compartment tracer kinetic model conforms to the physiologic properties in cases of HCC (high perfusion with relative high F_p) and could be applied to evaluate the perfusion and permeability of pre-therapeutic HCC.

However, the most important role of quantitative DCE-MRI is the assessment of treatment efficacy in advanced HCC after non-surgical methods. These treatments may not produce a change in K_{trans} because F_p and PS may change in opposite directions. On the other hand, F_p and PS may be changed in varying proportions. In these types of situations, it is important to understand which part of the vasculature is affected by the treatment. The dual-input 2CXM is able to provide a separate evaluation of PS and F_p, while the extended Tofts model only provides an assessment of K_{trans}, which reflects a combination of these two parameters; therefore, the dual-input 2CXM may have an advantage over the dual-input extended Tofts model.

k_{ep} is the reflux ratio of the transfer constant between EES and blood plasma. The amounts of k_{ep} in the two models are much larger than the K_{trans} or PS in our study, which also shows no significant difference but certain correlation between the two models with respect to this parameter. As a hypervascular tumor, the contrast agent in the tumor vasculature leaks into EES, and, with the hemodynamics progress, the contrast agent concentration in the EES increases but the tracer in the tumor vasculature decreases because the tracer is not only leaking into EES but also being eliminated from the plasma, which may cause a relative larger k_{ep}. Like K_{trans}, k_{ep} is an important predictable biomarker, and research has shown that a decrease in k_{ep} is correlated with favorable clinical outcomes after antiangiogenic drug treatment[8].

Theoretically, hypervascular tumors are usually composed of a larger vascular space (v_p) relative to the interstitial space (v_e) and show a pattern of rapid arterial enhancement followed by washout, whereas a hypovascular tumor usually consists of a larger v_e relative to the v_p and shows progressive enhancement[10]. However, our study shows that median v_e is far greater than v_p in both the extended Tofts model and the 2CXM model. This result seems

Table 4 Comparison of tumor dynamic contrast-enhanced-magnetic resonance imaging pharmacokinetic parameters from 28 scanned patients using two models [Median (IQR)]

	k_{trans} (min⁻¹)	PS (mL/mL · min⁻¹)	F_p (mL/mL · min⁻¹)	k_{ep} (min⁻¹)	v_e	v_p	HPI
Dual-Input Extended Tofts model	0.29 ± 0.38	-	-	1.35 ± 1.42	0.51 ± 1.16	0.12 ± 0.21	0.66 ± 0.24
Dual-Input Exchange model	-	0.19 ± 0.36	1.07 ± 1.73	0.95 ± 1.60	1.22 ± 1.19	0.14 ± 0.17	0.63 ± 0.29
Z/t value	NA	NA	NA	-0.638	-2.869	-0.568	0.499[1]
P value	NA	NA	NA	0.524	0.004	0.569	0.622[1]

[1]Paired *t*-test. NA: Not available; HPI: Hepatic perfusion index.

Figure 4 Scatterplots showing the correlation between K_{trans} (min⁻¹) obtained with the extended Tofts model and F_p (A) and PS (B) obtained with the 2-compartment exchange model.
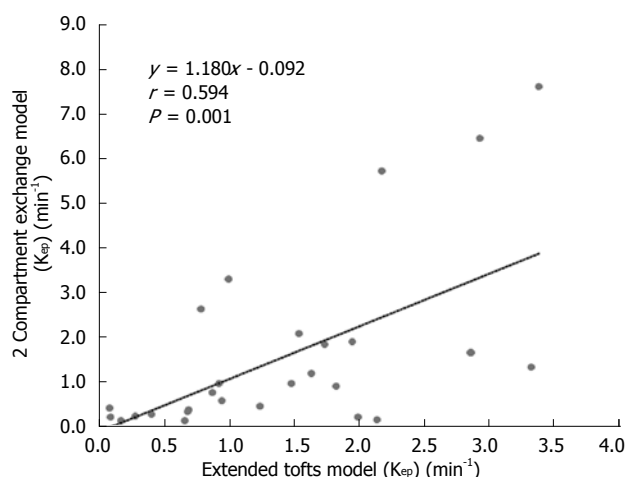
contradictory. The possible reasons for this result are that high blood flow in tumor vasculature results in great hydrodynamic pressure, the contrast agent enters into EES, and the available amount of extracellular space for this tracer to leak into increases; meanwhile, HCC, as a malignant tumor, usually contains a relatively larger EES. An additional reason may be the technology field. One animal DCE-MRI study assessing rat HCC shows that v_e is significantly larger than v_p in the extended Tofts model (also called the extended Kety model). The authors believe that k_{ep} and the sum of v_p and v_e are relatively invariant, and the underestimation of v_p leads to overestimations of v_e which are most severe in the Tofts (Kety) model because v_p is not considered in that model[23]. However, more studies are needed to elucidate the relationship between v_e and v_p.

In this study, v_e is significantly larger in the 2CXM than in the extended Tofts model, with no correlation between the two models. We believed that the relatively lower temporal resolution applied in the 2CXM may have caused this outcome. The 2CXM is more complicated and requires a higher temporal resolution than the extended Tofts model[22]. We acquired data for the 2CXM and the extended Tofts model using the same temporal resolution (6 second), which may have caused the v_e derived from the 2CXM to be overestimated.

There is no significant correlation between tumor size and stage and the microvascular perfusion and permeability in HCC. We believe the main reason for this result is the component we selected to measure the microvascular properties in HCC because we avoid the necrosis, hemorrhage, and adipose degeneration in the tumors, and these pathologic changes are usually relative to the tumor size and stage.

Some limitations of our study should be noted. First, no standard reference was used for any of the parameters. The perfusion and permeability parameters are functional biomarkers, and these parameters could not be measured from the pathological sample *in vitro*. We unavoidably used a well-known consensus as a reference standard that advanced HCC is supplied by the hepatic artery and portal vein with relatively high perfusion. We did not obtain histopathologic confirmation in the 18 HCC patients diagnosed by EASL criteria, as we did not know the grade of the tumor and the degree of cirrhosis around the mass. HCC grade may have influenced the parameters of perfusion and permeability. To date, most research related to HCC has not mentioned the relationship between the grade and the microvascular properties, and we believe it is worthy of future study. Second, the sample size is relatively small, and the relationship between v_e and v_p must be further validated in larger prospective studies.

Figure 5 Scatterplot showing the significant correlation of Kep estimated with the extended Tofts model and the 2-compartment exchange model.

Figure 7 Scatterplot showing the correlation of hepatic perfusion index estimated with the extended Tofts model and the 2-compartment exchange model.

Figure 6 Scatterplot showing that v_P values are correlated between the extended Tofts model and the 2-compartment exchange model.

Figure 8 Scatterplot showing that v_e is not correlated in the extended Tofts model and the 2-compartment exchange model.

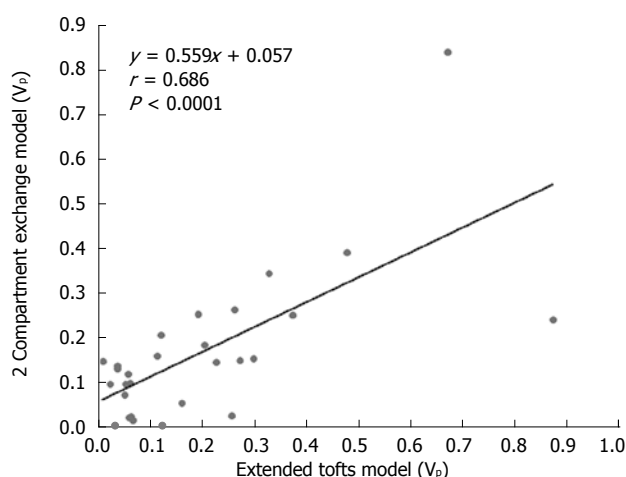In addition, the temporal resolution of the acquired image data in this study is 6 s. This resolution may not be high enough for the 2CXM model to measure the tumor interstitial parameter v_e. Finally, patients were instructed to freely breathe during imaging acquisition; this operation method and the patients' heart beat may together result in movement artifacts. We used non-rigid registration as much as possible to diminish the influence of imaging quality caused by movement artifacts.

In conclusion, all of the results derived from this prospective study indicate that the dual-input two-compartment tracer kinetic model could reflect the microvascular properties before the treatment of advanced HCC. Additionally, the dual-input extended Tofts model is more stable in measuring the v_e due to the suitable temporal resolution. Considering the therapeutic efficacy assessment of HCC treated by the antiangiogenic targeted drug, TACE, and radiofrequency ablation, the dual-input 2CXM may be more detailed and accurate than the dual-input extended Tofts model because the dual-input 2CXM can separately evaluate F_P and PS. This function is especially important if F_P and PS may change in opposite directions because K_{trans} cannot reflect this change in the extended Tofts model. However, this assumption needs to be adequately verified by comparing microvasculature parameters derived from the two tracer kinetic models in advanced HCC after antiangiogenic-targeted drug treatment or TACE and is also one of the major purposes of our next study.

ACKNOWLEDGMENTS

COMMENTS

Background

The parenchyma in hepatocellular carcinoma (HCC) consists of the vascular and interstitial space and is supplied by both the portal vein and the hepatic artery in different proportions; however, to date, the assessment of microvascular properties using a dual-input two-compartment tracer kinetic model with commonly used extracellular gadoliniun contrast agent has not been reported in HCC in a clinical practice setting.

Research frontiers

Some studies have shown the value of assessing microvascular properties in monitoring the effects of interventional therapy or antiangiogenic drug treatment of HCC, as well as metastases in the liver, using single-input single compartment, single-input two-compartment or dual-input single-compartment tracer kinetic models to evaluate a tumor's pharmacokinetic parameters. This study was designed to confirm that a dual-input two-compartment tracer kinetic model could be applied in advanced HCC.

Innovations and breakthroughs

This is the first study to explore and confirm that the dual-input two-compartment tracer kinetic model is suitable to evaluate microvascular physiologic properties in advanced HCC.

Applications

A dual-input two-compartment tracer kinetic model should be applied in assessing tumor microvascular pharmacokinetic parameters in advanced HCC.

Terminology

Dynamic contrast-enhanced-magnetic resonance imaging (MRI) is one of the functional imaging methods of MRI and is used to evaluate microvascular perfusion and permeability in lesions by observing pharmacokinetic parameters.

Peer-review

Although this manuscript is quite "technical", it is interesting to read (also from the clinical point of view). Potential shortcomings (*e.g.*, the rather small sample size) of the study are duly mentioned by the authors at the end of the "discussion".

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ORIGINAL ARTICLE

Observational Study

Prevalence of and risk factors for non-alcoholic fatty liver disease in a Chinese population: An 8-year follow-up study

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Abstract

AIM: To investigate the prevalence of and risk factors for non-alcoholic fatty liver disease (NAFLD) in a Chinese population.

METHODS: A total of 1948 adults from China was followed for 8 years. A cross-sectional study was performed to investigate the prevalence of NAFLD at baseline, and then the participants were followed for 8 years to investigate risk factors for the development of NAFLD.

RESULTS: A total of 1948 participants were enrolled at baseline, of whom 691 were diagnosed with NAFLD. During the 8-year follow-up, 337 baseline NAFLD-free participants developed NAFLD. They had a greater

increase in body mass index (BMI), serum uric acid, fasting plasma glucose, very low-density lipoprotein cholesterol and a considerable decrease in high-density lipoprotein cholesterol. 123 participants who had NAFLD at baseline lost NAFLD during the 8-year follow-up period. They had a greater decrease in BMI, fasting plasma glucose, triglycerides, total cholesterol, low-density lipo-protein cholesterol, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase.

CONCLUSION: NAFLD is prevalent in Chinese popu-lation with a rapidly increasing tendency. It can be reversed when patients lose their weight, control their hyperlipidemia and hyperglycemia, and reduce the liver enzyme levels.

Key words: Non-alcoholic fatty liver disease; Follow-up; Prevalence; Risk factors

Core tip: This study followed a large sample (*n* = 1928) for a long term (time = 8 years) to observe the development of non-alcoholic fatty liver disease due to the lifestyle and nutrition changes in a Chinese population.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) refers to hepatic steatosis accounting for over 5% of the total weight of the liver, which is not caused by excessive consumption of alcohol (women ≤ 70 g/wk, men ≤ 140 g/wk)[1,2]. NAFLD is seen worldwide and it is considered the most common chronic liver disease in Western countries, with a prevalence ranging from 17% and 46% in the general population[3,4]. It has also become prevalent in China with the rapid economic development[5-8]. Although NAFLD is not a severe disease, it is among the causes of fatty liver and one of the leading etiologies of chronic liver diseases[9]. NFLD includes a wide clinical and histological spectrum, such as simple steatosis, steatohepatitis, fibrosis, and cirrhosis[10]. Simple steatosis is considered a benign condition, while non-alcoholic steatohepatitis progresses to advanced fibrosis or cirrhosis in nearly 30% of cases, and may lead to end-stage liver disease and hepatocellular carcinoma[9,11-14]. It has

been projected that NAFLD will be the most rapidly growing indication for liver transplantation in the next decades[15]. In addition, NAFLD is a well-known risk factor for cardiovascular disease, type 2 diabetes and metabolic syndrome[16-18].

This report consists of two studies of the same population of adult urban subjects. A total of 1948 adults from Zhejiang, China was followed for 8 years. A cross-sectional study was performed to investigate the prevalence and correlative factors of NAFLD and the risk factors for the development of NAFLD after a prospective 8-year follow-up was performed.

MATERIALS AND METHODS

Study population

This study was performed among adults who underwent routine health examinations at the First Affiliated Hospital, College of Medicine, Zhejiang University between 2006 and 2014. Subjects were excluded if they fulfilled one of the following criteria in 8 years: (1) excess alcohol consumption (men > 140 g/wk, women > 70 g/d); (2) presence of markers of hepatitis B virus infection (hepatitis B surface antigen) and hepatitis C virus infection (anti-hepatitis C virus antibodies); (3) a history of autoimmune hepatitis (*e.g.*, autoimmune hepatitis, primary biliary cirrhosis) or other chronic liver disease with clear causes; and (4) absence of uncontrolled biliary diseases (*e.g.*, bile ductal stone, stenosis, biliary dilatation). A total of 1948 adults above 20 years old were included in the final analysis. The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University.

Baseline examinations

Body weight and standing height were measured to calculate body mass index (BMI). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded with an automated sphygmomanometer as the subjects sitting calmly. Approximately 10 mL venous blood samples were collected from all subjects following a 12 h overnight-fast. Hemoglobin (Hb), platelet (PLT), white blood cell (WBC), serum uric acid (SUA), fasting plasma glucose (FPG), triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, very low-density lipoprotein (VLDL) cholesterol, high-density lipoprotein (HDL) cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (γ-GT) were measured with an automatic biochemistry analyzer (Beckman Coulter Inc., CA) using standard methods.

NAFLD was diagnosed based on the ultrasonic criteria suggested by the Chinese Medical Association[2]. The criteria are described as the following items: (1) diffuse enhancement of near field echo in the hepatic

Table 1 Baseline characteristics of the subjects with and without non-alcoholic fatty liver disease

Variable	NAFLD	Non-NAFLD	t value	P value
Age (yr)	43.38 (0.42)	40.14 (0.35)	5.944	< 0.001
Gender, male/ female (n)	598/93	685/572	203.663	< 0.001
BMI (kg/m^2)	25.69 (0.09)	21.83 (0.07)	33.044	< 0.001
SBP (mmHg)	128.62 (0.6)	117.49 (0.43)	15.253	< 0.001
DBP (mmHg)	79.73 (0.36)	72.71 (0.27)	15.608	< 0.001
Hb (g/L)	151.3 (0.46)	141.62 (0.41)	15.679	< 0.001
PLT (× 10^9/L)	209.44 (2.12)	206.1 (1.5)	1.299	0.194
WBC (× 10^9/L)	6.48 (0.06)	5.91 (0.04)	8.294	< 0.001
SUA (μmol/L)	367.92 (3.45)	291.89 (2.32)	18.742	< 0.001
FPG (mmol/L)	5.01 (0.05)	4.59 (0.02)	8.019	< 0.001
TG (mmol/L)	2.47 (0.07)	1.3 (0.03)	15.096	< 0.001
TC (mmol/L)	4.94 (0.03)	4.6 (0.02)	8.128	< 0.001
LDL (mmol/L)	2.35 (0.03)	2.16 (0.03)	4.487	< 0.001
VLDL (mmol/L)	1.41 (0.03)	1.01 (0.02)	10.996	< 0.001
HLDL (mmol/L)	1.16 (0.01)	1.44 (0.01)	-20.396	< 0.001
ALT (U/L)	36.24 (0.9)	19.75 (0.41)	16.649	< 0.001
AST (U/L)	27.21 (0.43)	22.1 (0.25)	10.200	< 0.001
γ-GT (U/L)	49.97 (1.51)	25.56 (0.99)	13.512	< 0.001

NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Hb: Hemoglobin; PLT: Platelet; WBC: White blood cell; SUA: Serum uric acid; FPG: Fasting plasma glucose; TG: Triglycerides; TC: Total cholesterol; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; HDL: High-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase.
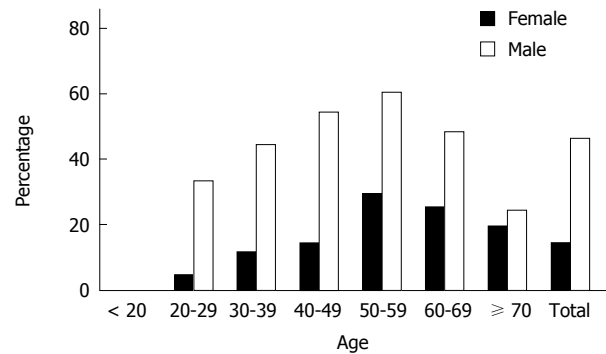
region (stronger than in the kidney and spleen region) and gradual attenuation of the far field echo; (2) unclear display of intra-hepatic lacuna structure; (3) mild to moderate hepatomegaly with a round and blunt border; and (4) color Doppler ultrasonography shows a reduction of the blood flow signal in the liver or it is even hard to display, but the distribution of blood flow is normal. NAFLD was diagnosed if item 1 and any one or more of items 2-4 are matched. Hepatic ultrasonic examination was performed and conducted by a trained ultrasonographist in a blind manner.

Follow-up examination
The population was followed for 8 years, and the end-point examination was repeated in 2014. During this period, patients who took any kind of prescription medicine are excluded. Serological tests were measured with the same automatic analyzer using the same methods. Training course was carried out to make sure that the ultrasonic criteria of NAFLD remain the same and hepatic ultrasonic examination was still performed in a blind manner.

Statistical analysis
All statistical analyses were conducted with the SPSS software package version 13.0 for Windows (SPSS, Inc., Chicago, IL). Baseline analyses were done using descriptive statistics expressed as mean ± SD, and differences between continuous variables were assessed using Student's *t*-test or the Mann-Whitney *U*-test, depending on the normality of the data. Cate-

Figure 1 Age and sex distribution of overall non-alcoholic fatty liver disease prevalence. The overall non-alcoholic fatty liver disease prevalence is presented by the distribution according to age and sex.

gorical variables were compared using Pearson's chi-squared (χ^2) test or Fisher's exact test. Stepwise Binary logistic regression (Forward: Wald; Entry: 0.05, Removal: 0.10) was used to analyse the risk factors associated with the presence of NAFLD. A *P* value < 0.05 (two-tailed) was considered statistically significant.

RESULTS

Prevalence and clinical characteristics of NAFLD
A total of 1948 adult subjects were eventually enrolled in this study, which consisted of 1283 males (65.86%) and 665 females (34.14%). Among 1948 subjects, 691 were diagnosed with NAFLD with the prevalence of 35.47% at baseline (Table 1). Compared with those without NAFLD, the subjects with NAFLD were significantly older, male dominated, and had significantly higher BMI, SBP and DBP, levels of Hb, WBC count, SUA, FPG, TG, TC, LDL cholesterol, VLDL cholesterol, HDL cholesterol, ALT, AST and γ-GT. Meanwhile, NAFLD subjects had significantly lower high-density lipoprotein cholesterol levels. NAFLD also had relatively higher level of PLT count with no significance. Furthermore, the overall prevalence of NAFLD was significantly higher in males than in females (46.61% *vs* 13.98%, *P* < 0.001). The gender difference in NAFLD prevalence was noted in groups younger than 60 years but absent in those older than 60 years (Table 2, Figure 1). In addition, the overall prevalence of NAFLD increased with age (in trend analysis, *P* < 0.001) and reached a peak in the group of 50-60 years (51.74%). This trend was not only in the overall prevalence but also in both males and female's prevalence (Table 2 and Figure 1).

Risk factors associated with the presence of NAFLD
Stepwise binary logistic regression model was used to explore the independent risk factors associated with the presence of NAFLD. All the 18 variables in Table 1 were recruited into the original equation, and 10 variables remained in the final equation after removing 8 variables (Table 3). Our results suggest that those

Table 2 Age and sex distribution of overall non-alcoholic fatty liver disease prevalence							
Age (yr)	Total	NAFLD	Overall	Males	Females	χ^2 value	*P* value
< 20	3	0	0.00%	0.00%	0.00%	-	-
20-29	342	69	20.18%	28.07%	4.39%	26.471	< 0.001
30-39	559	183	32.74%	44.14%	10.94%	63.114	< 0.001
40-49	608	245	40.30%	55.01%	14.16%	97.223	< 0.001
50-59	259	134	51.74%	60.00%	28.99%	19.499	< 0.001
60-69	119	47	39.50%	48.00%	25.00%	6.139	0.013
≥ 70	58	13	22.41%	25.00%	19.23%	0.275	0.600
Total	1948	691	35.47%	46.61%	13.98%	203.663	< 0.001

NAFLD: Non-alcoholic fatty liver disease.

Table 3 Logistic regression analysis of risk factors associated with the prevalence of non-alcoholic fatty liver disease							
Variables	B	S.E.	Wals	Sig.	OR	95%CI of OR	
Age	0.025	0.007	14.767	< 0.001	1.025	1.012	1.038
BMI	0.450	0.036	158.411	< 0.001	1.569	1.462	1.682
PLT	0.004	0.001	6.898	0.009	1.004	1.001	1.006
SUA	0.006	0.001	28.372	< 0.001	1.006	1.004	1.008
FPG	0.334	0.081	16.884	< 0.001	1.397	1.191	1.638
TG	0.280	0.089	9.919	0.002	1.323	1.112	1.575
VLDL	0.336	0.118	8.148	0.004	1.400	1.111	1.764
HDL	-1.053	0.298	12.469	< 0.001	0.349	0.194	0.626
ALT	0.049	0.008	37.985	< 0.001	1.050	1.034	1.067
AST	-0.064	0.014	20.903	< 0.001	0.938	0.912	0.964

BMI: Body mass index; PLT: Platelet; SUA: Serum uric acid; FPG: Fasting plasma glucose; TG: Triglycerides; VLDL: Very low-density lipoprotein; HDL: High-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 4 Comparison of variables between baseline and end point in subjects who developed non-alcoholic fatty liver disease			
Variables	Difference	*t* value	*P* value
BMI (kg/m^2)	-1.454	-17.198	0.000
SBP (mmHg)	-4.875	-6.105	0.000
DBP (mmHg)	-2.225	-4.021	0.000
Hb (g/L)	-3.537	-6.972	0.000
PLT (× 10^9/L)	-7.352	-3.276	0.001
WBC (× 10^9/L)	-0.283	-3.728	0.000
SUA (μmol/L)	-29.304	-8.814	0.000
FPG (mmol/L)	-0.463	-10.909	0.000
TG (mmol/L)	-0.224	-2.630	0.009
TC (mmol/L)	-0.268	-6.823	0.000
LDL (mmol/L)	0.054	1.368	0.172
VLDL (mmol/L)	-0.295	-11.043	0.000
HDL (mmol/L)	0.126	10.200	0.000
ALT (U/L)	-3.099	-2.672	0.008
AST (U/L)	0.006	0.009	0.993
γ-GT (U/L)	-10.43	-4.912	0.000

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Hb: Hemoglobin; PLT: Platelet; WBC: White blood cell; SUA: Serum uric acid; FPG: Fasting plasma glucose; TG: Triglycerides; TC: Total cholesterol; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; HDL: High-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase.

Table 5 Comparison of variables between baseline and end point in subjects who seceded from non-alcoholic fatty liver disease			
Variables	Difference	*t* value	*P* value
BMI (kg/m^2)	0.801	4.759	0.000
SBP (mmHg)	-2.285	-1.509	0.134
DBP (mmHg)	-0.279	-0.274	0.784
Hb (g/L)	-0.434	-0.496	0.621
PLT (× 10^9/L)	0.754	0.203	0.839
WBC (× 10^9/L)	-0.040	-0.274	0.785
SUA (μmol/L)	12.800	1.820	0.072
FPG (mmol/L)	-0.204	-2.329	0.022
TG (mmol/L)	0.643	6.159	0.000
TC (mmol/L)	0.209	2.721	0.007
LDL (mmol/L)	0.357	4.532	0.000
VLDL (mmol/L)	0.073	1.544	0.125
HDL (mmol/L)	-0.005	-0.194	0.846
ALT (U/L)	8.950	5.917	0.000
AST (U/L)	5.084	5.997	0.000
γ-GT (U/L)	9.557	4.612	0.000

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Hb: Hemoglobin; PLT: Platelet; WBC: White blood cell; SUA: Serum uric acid; FPG: Fasting plasma glucose; TG: Triglycerides; TC: Total cholesterol; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; HDL: High-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase.

10 variables were significantly associated with the presence of NAFLD including age, BMI, PLT count, SUA, FPG, TG, VLDL cholesterol, HDL cholesterol, ALT and AST.

Development of NAFLD after 8-year follow-up

After 8-year follow-up, 337 (17.30%) subjects free of NAFLD at baseline developed NAFLD and 123 (6.31%) subjects diagnosed with NAFLD at baseline had not

NAFLD any more (Tables 4 and 5). These changes made the prevalence of NAFLD rise by 10.99% to 46.46%. Paired-samples *t*-test was employed to analyse the changes from baseline to the end point in these two groups, respectively. Subjects who developed NAFLD had a significant change in almost all the parameters during the follow-up. There was an especially greater increase in BMI, SUA, FPG, VLDL cholesterol and a considerable decrease in HDL cholesterol, as their absolute *t*-values were greater. It is worth noting that these 5 variables were all significantly associated with the presence of NAFLD. Subjects who seceded from NAFLD had a greater decrease in BMI, FPG, TG, TC, LDL cholesterol, ALT, AST, and γ-GT.

DISCUSSION

The prevalence of NAFLD in the Western countries was estimated to be 20% to 30%[19], and varied between 17% and 46% depending on the different population included in the study[4]. With the improvement of economy and westernization of lifestyle in China, the prevalence of NAFLD in the Chinese population had a rapidly increasing tendency, especially in the urban population. Our study showed that the prevalence of NAFLD was 35.47 % in 2006 and went up to 46.46% in 2014 in the same population after follow-up for 8 years. The results also indicated that the prevalence of NAFLD increased with age. The peak of age showed in 50-60 year old subjects. It is important to note that elder people had significantly more known risk factors for NAFLD, such as obesity, diabetes mellitus and hyperlipidaemia[4]. On the other hand, dysfunction of preadipocytes in the elder people impairs the capacity of fat tissue to store lipids, and leads to fat redistribution from subcutaneous to intraabdominal visceral depots including the liver[20,21]. Our results also suggest that there was a significant difference in the prevalence of NAFLD between men and women before the age of 60. This difference might be a consequence of the protective role of estrogens in females. Difference in sex hormone levels would probably correlate to the differences in the amount and distribution of body fat between the sexes[22,23]. As men usually store fat in the abdomen, women store more fat in the subcutaneous tissue. What's more, logistic regression analyses revealed that gender was not an independent risk factor associated with the prevalence of NAFLD. Different values of serological markers in different genders might directly affect the prevalence of NAFLD.

Our results showed that age, BMI, PLT count, SUA, FPG, TG, VLDL cholesterol, HDL cholesterol, ALT and AST were 10 risk factors independently associated with the prevalence of NAFLD. In the follow-up study, we found not only progressive subjects but also rehabilitative cases. It is the most interesting finding in our study that NAFLD was not always progressing.

BMI, SUA, FPG, VLDL cholesterol and HDL cholesterol play the greatest role in the development of NAFLD. They are risk factors for both the prevalence and development of NAFLD. Subjects with NAFLD can be reversed if they lose their weight, control their hyperlipidemia and hyperglycemia, and reduce the liver enzyme levels.

High BMI is without doubt a major risk factor for NAFLD. In this study, the prevalence of NAFLD was 19.94% in non-obese subjects (BMI $\leqslant$ 25 kg/m^2) and reached 75.05% in obese subjects (BMI > 25 kg/m^2). In patients with morbid obesity (BMI > 40 kg/m^2) who undergo bariatric surgery, the prevalence of NAFLD may even be in excess of 90%[9]. Previous studies showed that modest weight loss is associated with amelioration of hepatic steatosis and other histological improvements[24,25]. Several recent studies suggested the importance of body weight control, not only in the obese but also in non-obese subjects, for reducing the risk of or preventing NAFLD[26,27].

As universally acknowledged, the proportion of NAFLD is also higher in patients with type 2 diabetes or metabolic syndrome[4]. FPG, TG, VLDL cholesterol and HDL cholesterol were all markers relevant to glucose and lipid metabolism. In this study, we suggest that not only type 2 diabetes and metabolic syndrome but also moderate elevation in parameters mentioned above were responsible for a high prevalence of NAFLD.

Our results demonstrate a close relationship between high SUA and NAFLD. Hyperuricemia is a common finding in recent studies and SUA is independently associated with histological findings of NAFLD regardless of insulin resistance and metabolic syndrome status[28]. As the underlying mechanism is not well studied, further studies are needed to characterize the role of SUA in the development of NAFLD.

In this study, liver enzymes were associated with the prevalence and development of NAFLD. In many previous studies, elevations in the liver enzymes were non-invasive indicators of NAFLD[29,30]. These enzymes indirectly reflect histological changes of livers and severity of NAFLD. It is not surprising that all three enzymes[1-3] significantly decreased in subjects who seceded from NAFLD during the follow-up.

From both clinical experience and research data, the more alcohol people intake, the higher blood TG levels. Patients with NAFLD often have elevated TG. The biopsy also proved that their steatosis correlates directly with alcohol intake. Women may be affected at even lower levels of intake (*e.g.,* half dose). In another word, the alcohol sensitivity of women is different from that of men. Women are more likely to develop NAFLD than men even with lower dose of alcohol intake. That is why we put a lower bar for women (70 g in women *vs* 140 g in men) in this study according to the Guidelines for the diagnosis and treatment of nonalcoholic fatty liver diseases[2].

There are several limitations in this study. NAFLD was diagnosed by ultrasonography, which is not sen-

sitive for mild NAFLD and cannot determine the severity of NAFLD. However, ultrasonography as a non-invasive method is widely used in population-based studies with high diagnostic value for detecting NAFLD[31]. Although excluding any medical intervention, during the 8-year follow-up, dietary habits are not fully followed due to the difficult standardization. The role of diet change in NAFLD development and resolution should be also further studied. In addition, admission bias cannot be eliminated because most subjects who participated in health examinations were from population with stable income and high education in urban area.

Our results provide the prevalence of NAFLD and the risk factors for its prevalence and development in a Chinese population. Our findings may make clear the high prevalence in China.

In conclusion, our results showed that NAFLD is prevalent in the Chinese population with a rapidly increasing tendency. NAFLD can be reversed when patients lose their weight, control their hyperlipidemia and hyperglycemia, and reduce the liver enzyme levels.

COMMENTS

Background
This study focused on the long-term outcome of non-alcoholic fatty liver disease (NAFLD) in China. The prevalence of NAFLD in China has approximately doubled in the past decade with the increasing pandemic of obesity. Epidemiological data and long-term outcomes of NAFLD in Chinese populations remain unknown and need to be updated, which can be used as a predictor for metabolic disorders and a basis for public health interventions.

Research frontiers
Not all individuals with NAFLD develop hepatic steatosis. There also appears to be racial-ethnic variations. So NAFLD racial-ethnic study becomes a hotpot currently. Our study focused on a Chinese population and observed the long-term outcome to provide the initial clues of risk factors for further mechanism study.

Innovations and breakthroughs
The breakthrough of the current study is the finding of natural prognosis of NAFLD by a self-comparison after 8-year follow-up. Among a total 1948 participants, 337 baseline NAFLD-free participants developed NAFLD and 123 participants who had NAFLD at baseline lost NAFLD. Analysis of their clinical characters and laboratory results indicates their metabolic fate without antilipemic drug interventions.

Applications
Currently, there is no approved therapy for NAFLD/non-alcoholic steatohepatitis (NASH). Treatment strategies may be grouped into those which address weight loss, reduce lipids, are antioxidants, or target the liver. Our research findings provide the target and biomarkers for the therapies.

Terminology
NAFLD is a common hepatic disease. Pathologically, it can present as simple steatosis, NASH, and eventually progress to cirrhosis, an end-stage liver disease. The NAFLD is the most common cause of chronic liver disease in the Western world, and it has been increasing in China in the past years. Notably, 1%-5% of patients with simple steatosis can eventually develop actual cirrhosis and even to hepatocellular carcinoma.

Peer-review
Three reviewers have reviewed the manuscript. They all recognized the

significance of the study. The only concern is the different criteria for alcohol consumption between males and females. It comes from the sensitivity threshold between different genders. The definition refers to the NAFLD guideline.

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ORIGINAL ARTICLE

Prospective Study

Operative link on gastritis assessment stage is an appropriate predictor of early gastric cancer

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Abstract

AIM: To assess the predictive value of Operative Link on Gastritis Assessment (OLGA) and Operative Link on Gastric Intestinal Metaplasia Assessment (OLGIM) stages in gastric cancer.

METHODS: A prospective study was conducted with 71 patients with early gastric cancer (EGC) and 156 patients with non-EGC. All patients underwent endoscopic examination and systematic biopsy. Outcome measures were assessed and compared, including the Japanese endoscopic gastric atrophy (EGA) classification method and the modified OLGA method as well as the modified OLGIM method. *Helicobacter pylori* (*H. pylori*) status was determined for all study participants. Stepwise logistic regression modeling was performed to analyze correlations between EGC and the EGA, OLGA and OLGIM methods.

RESULTS: For patients with EGC and patients with non-EGC, the proportions of moderate-to-severe EGA cases were 64.8% and 44.9%, respectively (*P* = 0.005), the proportions of OLGA stages III-IV cases were 52.1% and 22.4%, respectively (*P* < 0.001), and the proportions of OLGIM stages III-IV cases were

42.3% and 19.9%, respectively ($P < 0.001$). OLGA stage and OLGIM stage were significantly related to EGA classification; specifically, logistic regression modeling showed significant correlations between EGC and moderate-to-severe EGA (OR = 1.95, 95% CI: 1.06-3.58, $P = 0.031$) and OLGA stages Ⅲ-Ⅳ (OR = 3.14, 95%CI: 1.71-5.81, $P < 0.001$), but no significant correlation between EGC and OLGIM stages Ⅲ-Ⅳ ($P = 0.781$). *H. pylori* infection rate was significantly higher in patients with moderate-to-severe EGA (75.0% *vs* 54.1%, $P = 0.001$) or OLGA/OLGIM stages Ⅲ-Ⅳ (OLGA: 83.6% *vs* 55.8%, $P < 0.001$; OLGIM: 83.6% *vs* 57.8%, $P < 0.001$).

CONCLUSION: OLGA classification is optimal for EGC screening. A surveillance program including OLGA stage and *H. pylori* infection status may facilitate early detection of gastric cancer.

Key words: Early gastric cancer; Operative Link on Gastritis Assessment/Operative Link on Gastric Intestinal Metaplasia Assessment stage; Endoscopic gastric atrophy classification; Screening; Endoscopy

Core tip: Japanese endoscopic gastric atrophy classification, Operative Link on Gastritis Assessment (OLGA), and Operative Link on Gastric Intestinal Metaplasia Assessment (OLGIM) have been proven separately as effective methods to evaluate severity of gastric atrophy and intestinal metaplasia. However, these methods have not been compared for prognosticating neoplastic development. This study compared the correlations of these three methods with early gastric cancer (EGC) and found that OLGA classification is optimal for EGC screening. A surveillance program based on OLGA stage and *Helicobacter pylori* infection status may represent a practical approach for detecting gastric cancer at an early stage.

INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide[1]. The prognosis of GC is meaningfully associated with tumor stage, as highlighted by the 5-year overall survival rate of patients with early gastric cancer (EG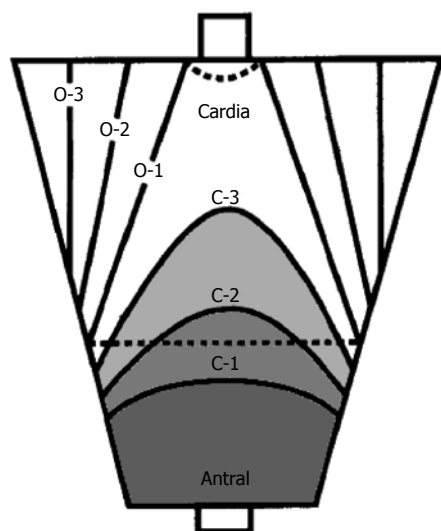C) exceeding 90%[2,3]. The presence of atrophic gastritis (defined as loss of appropriate gland function), intestinal metaplasia (defined as replacement of gastric epithelium by intestinal-type epithelium, IM) and *Helicobacter pylori* (*H. pylori*) infection are well-known risk factors of GC. As atrophic gastritis and IM progress to GC in only a small proportion of patients[4], identifying the characteristics of the background mucosa in EGC may help clinicians to select a subgroup of patients who may benefit from a surveillance program.

In recent years, the Operative Link on Gastritis Assessment (OLGA), which is based on the histopathology findings of biopsy specimens, was proposed as an effective method to rank gastritis into stages with corresponding carcinoma risks[5,6]. It has been reported that a high-risk stage (defined as stage Ⅲ or Ⅳ of the OLGA classification) is strongly correlated with a high risk of GC[7,8]. However, in consideration of the low interobserver agreement of OLGA classification, the Operative Link on Intestinal Metaplasia Assessment (OLGIM) was developed as an alternative, and was subsequently recommended as an effective method to predict GC risk due to its higher interobserver agreement and strong association with the OLGA stage[9,10].

The endoscopic gastric atrophy (EGA) assessment that uses Kimura-Takemoto classification was first applied in a study of Japanese subjects to evaluate the extent of endoscopic atrophic border (EAB) and the severity of gastric atrophy[11]. A subsequent study showed that moderate-to-severe grade of EGA was closely associated with an increased risk of GC[12]. EGA is regarded as an assessment of endoscopic gastric atrophy, in contrast to the OLGA and OLGIM methods which are identified as the assessments of histologic atrophy and IM. While all three methods have proven effective in assessing gastric atrophy and predicting the development of GC, their use remains limited and has not extended worldwide. The OLGA and OLGIM classifications are applied primarily in Europe and America; on the other hand, the EGA assessment is applied primarily in Japan and Vietnam. None of these three evaluation methods has been widely applied in China and other Asian countries, despite the fact that they harbor a high prevalence of GC.

Determining the optimal assessment method for predicting EGC makes sense for both patient care and allocation of medical resources. To the best of our knowledge, no research study to date has reported a comparative analysis of the associations between the three evaluation methods and EGC; as such, the relationship between EGA assessment and the OLGA/OLGIM stages remains unclear. We designed this prospective study to evaluate the characteristics of the background mucosa of EGC using three criterions, ultimately to investigate whether the EGA, OLGA or OLGIM method has the highest correlation with EGC so that the optimal means of assessment can be used

Figure 1 Endoscopic atrophic pattern described by Kimura-Takemoto[11]. The spread of atrophic gastritis is divided into closed-type gastritis (C-type) and open-type gastritis (O-type). C-1 represents highly localized antral gastritis, and C-2 and C-3 represent increasing extension through the lesser curvature and greater curvature, respectively. O-type indicates gastritis reaching the cardia, with O-1 reaching the lesser curvature, O-2 reaching half of the stomach, and O-3 having extensive atrophic gastritis, affecting almost the entire stomach.

in development of an appropriate surveillance program for detecting EGC in China.

MATERIALS AND METHODS

Patients and classification

The study was conducted prospectively at Shanghai Ren Ji Hospital from May 2013 to July 2015. Consecutive patients, ranging in age from 40 to 80 years, with diagnosis of functional dyspepsia or suspicion of EGC and who underwent esophagogastroduodenoscopy were recruited to the study. Patients were excluded from study participation based upon diagnosis of advanced GC, order or receipt of post-subtotal gastrectomy, or presence of any conditions that may interfere with clinical examination or treatment, such as acute upper gastrointestinal bleeding and severe systemic diseases (*e.g.*, a severe cardiac condition, serious infection, or renal failure). Patients who lacked histology data were also excluded.

The study protocol was approved by the local ethics committee, and all patients provided written informed consent. Patients were selected and classified into two groups. Patients with a pathological diagnosis of EGC or high-grade neoplasia (HGN) (categories 4-5 according to the revised Vienna classification)[13] were defined as the EGC group. Patients with a pathological diagnosis of non-gastritis, gastritis or low-grade neoplasia (LGN) (revised Vienna category 1-3) were defined as the non-EGC group.

Endoscopic procedure

All patients were examined by a single experien-

ced endoscopist, using a conventional endoscope (GIF-H260; Olympus Medical Systems, Tokyo, Japan), a magnifying endoscope (GIF-H260 Z; Olympus Medical Systems), and an electric endoscopic system (EVIS 260 Spectrum; Olympus Medical Systems). All patients were originally diagnosed using the Kimura-Takemoto EGA assessment[11] (Figure 1). The extent of atrophic gastritis was categorized according to the following two primary patterns: closed-type gastritis (C-type) and open-type gastritis (O-type). For the C-type, C1 sub-categorization represented highly localized antral gastritis, C2 sub-categorization represented increasing extension through the lesser curvature, and C3 sub-categorization represented increasing extension through the greater curvature. For the O-type, in which the gastritis reached the cardia, O1 sub-categorization indicated reaching the lesser curvature, O2 sub-categorization indicated reaching half of the stomach, and O3 sub-categorization indicated extensive atrophic gastritis that affected almost the entire stomach. According to the patient's EGA classification, the endoscopic atrophic pattern was divided into the following three degrees: mild (C1-C2), moderate (C3-O1) and severe (O2-O3). Then, biopsy samples (*n*) were obtained for histology from the following standardized sites: antrum (*n* = 3, including 1 for exclusive use in the rapid urease test (RUT) (Pronto Dry; Medical Instruments Corporation, Solothurn, Switzerland) and corpus (*n* = 2, including 1 from the lesser curvature and 1 from the greater curvature). If a suspicious lesion was found, 2-3 extra biopsy samples were obtained from the lesion.

Treatment

Patients with suspected EGC or with a diagnosis of intraepithelial neoplasia by pathology were examined by magnifying endoscopy with narrow-band imaging (ME-NBI). The treatment for each patient was determined according to results from conventional endoscopy (CE) and ME-NBI, as well as biopsy pathologic diagnoses. When the biopsy pathology turned out to be gastritis or LGN (revised Vienna categories 1-3), the patients received follow-up. For those diagnosed with HGN and GC (revised Vienna categories 4-5) by biopsy pathology, endoscopic submucosal dissection (ESD) or surgery were chosen according to the indications of endoscopic resection (ER)[13].

Histopathology

The retrieved tissues were fixed in formalin (10%) and embedded in paraffin. All biopsy specimens were examined by a single experienced pathologist, blinded to the endoscopic diagnosis, using the World Health Organization classification of tumors (digestive system)[14] and the revised Vienna classification[13]. Gastric adenocarcinomas were sub-divided into D-type (well or moderately differentiated adenocarcinoma or

Atrophy score		Corpus			
		No atrophy (score 0)	Mild atrophy (score 1)	Moderate atrophy (score 2)	Severe atrophy (score 3)
Antrum	No atrophy (score 0)	STAGE 0	STAGE I	STAGE II	STAGE II
	Mild atrophy (score 1)	STAGE I	STAGE I	STAGE II	STAGE III
	Moderate atrophy (score 2)	STAGE II	STAGE II	STAGE III	STAGE IV
	Severe atrophy (score 3)	STAGE III	STAGE III	STAGE IV	STAGE IV

Figure 2 Modified operative link for gastritis assessment staging frame (with exclusion of the biopsies from incisura angularis)[16].

Table 1 Clinicopathological characteristics and endoscopic gastric atrophy classification

	EGC group	Non-EGC group	P value
Total patients, *n* (%)	71 (100)	156 (100)	
Sex, *n* (%)			> 0.050
Male	51 (71.8)	98 (62.8)	
Female	20 (28.2)	58 (37.2)	
Age (yr)	64.0 ± 9.1	62.2 ± 7.4	> 0.050
H. pylori infection rate	70.4%	61.5%	> 0.050
EGA classification			< 0.001
C0	1	6	
C1	3	18	
C2	21	62	
C3	7	29	
O1	23	30	
O2	12	11	
O3	4	0	
Atrophic rate	98.6%	96.2%	> 0.050
OLGA stage			< 0.001
0	1	6	
I	16	65	
II	17	49	
III	27	27	
IV	10	9	
OLGIM stage			< 0.001
0	6	45	
I	17	47	
II	18	33	
III	20	23	
IV	10	8	
IM rate	91.5%	71.2%	0.001
IM subtype			0.019
Complete IM	13	41	
Incomplete IM	52	70	

EGA: Endoscopic gastric atrophy; EGC: Early gastric cancer; *H. pylori*: *Helicobacter pylori;* IM: Intestinal metaplasia; OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment.

papillary adenocarcinoma) and UD-type (mucinous cell carcinoma, signet-ring cell carcinoma, or poorly differentiated adenocarcinoma). If both characteristics were present, the lesion was regarded as UD-type. Atrophic gastritis and IM[6] were scored using a visual analog scale based on the updated Sydney system[15], in which 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. Presence of atrophic gastritis and stage of IM were determined according to the modified OLGA stage system and the modified OLGIM stage system,

without biopsy from the incisura angularis[16] (Figure 2).

H. pylori evaluation

For patients who underwent esophagogastro-duodenoscopy, biopsy samples were obtained from the antrum (*n* = 3, 1 for RUT and 2 for *H. pylori* detection) and from the corpus (*n* = 2, both for *H. pylori* detection). For *H. pylori* detection, sections were stained with modified Giemsa and histologically evaluated. In addition, peripheral blood was collected to determine *H. pylori* IgG antibody titers by enzyme-linked immunosorbent assay (ELISA) (*H. pylori*-EIA-Well; Radim, Rome, Italy). Any two positive findings among the tests of four biopsy sites, RUT, and anti-*H. pylori* IgG were considered as having a positive *H. pylori* status. Patients with only one positive result were considered as having an inconclusive *H. pylori* status. Only those patients with all tests having negative results were considered as negative (non-infected) *H. pylori* status.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 statistical software (SPSS Inc., Chicago, IL, United States). Continuous parameters are expressed as mean ± SD, and discrete parameters are expressed as numbers and percentages. Differences between the two groups were evaluated by Pearson's χ^2 test, Mann-Whitney Wilcoxon test and Student's *t*-test, as appropriate. A logistic regression model (stepwise forward procedure) was used for correlation analysis between EGA, OLGA, OLGIM and EGC. All *P*-values reported are two-sided, and a *P*-value < 0.05 was considered statistically significant.

RESULTS

Clinicopathological characteristics

Overall, 227 patients were enrolled in the study. The clinicopathological characteristics of these patients, in the EGC group (*n* = 71) and the non-EGC group (*n* = 156), are summarized in Table 1. The EGC group had a total of 75 EGC lesions (mean diameter, 21.9 ± 9.3 mm), and 66 of the patients in this group underwent ESD treatment while 5 underwent surgery. Of the 75

Table 2 Endoscopic gastric atrophy classification and OLGA/OLGIM stage in early gastric cancer and non- early gastric cancer patients

	EGC group	Non-EGC group	*P* value	OR (95%CI)
EGA classification[1]			0.005	2.26 (1.27-4.04)
None-to-mild	25	86		
Moderate-to-severe	46	70		
OLGA stage			< 0.001	3.76 (2.07-6.85)
0-Ⅱ	34	121		
Ⅲ-Ⅳ	37	35		
OLGIM stage			< 0.001	2.95 (1.60-5.45)
0-Ⅱ	41	125		
Ⅲ-Ⅳ	30	31		

[1]None-to-mild EGA, C0-C2 types of EGA classification; Moderate-to-severe EGA, C3-O3 types of EGA classification. CI: Confidence interval; EGA: Endoscopic gastric atrophy; EGC: Early gastric cancer; OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment; OR: Odds ratio.

Table 3 Relation between early gastric cancer classification and OLGA/OLGIM stage *n* (%)

	EGA classification		*P* value
	None-to-mild	Moderate-to-severe	
OLGA stage			0.001
0-Ⅱ	87 (56.1)	68 (43.9)	
Ⅲ-Ⅳ	24 (33.3)	48 (66.7)	
OLGIM stage			< 0.001
0-Ⅱ	94 (56.6)	72 (43.4)	
Ⅲ-Ⅳ	17 (27.9)	44 (72.1)	

EGA: Endoscopic gastric atrophy; OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment.

EGC lesions, 72 (96.0%) were differentiated-type and 3 (4.0%) were undifferentiated-type. As for the tumor size, 14 (18.7%) were ≤ 1 cm, 33 (44%) were 1-2 cm, 15 (20%) were 2-3 cm and 13 (17.3%) were > 3 cm. Moreover, 70 (93.3%) of the tumors were intramucosal and 5 (6.7%) were submucosal.

The mean patient age, sex, *H. pylori* infection rate, and atrophic rate were not significantly different between the EGC and non-EGC groups. On the other hand, the EGA classification, OLGA stage, OLGIM stage, IM rate and IM type were significantly different between the two groups.

EGA classification and OLGA/OLGIM stages
As shown in Table 2, the proportions of moderate-to-severe EGA cases in the EGC group and the non-EGC group were 64.8% (46/71) and 44.9% (70/156), respectively. The proportions of OLGA gastritis stages Ⅲ-Ⅳ cases in the EGC group and the non-EGC group were 52.1% (37/71) and 22.4% (35/156), respectively. The proportions of OLGIM stages Ⅲ-Ⅳ cases in the EGC group and the non-EGC group were 42.3% (30/71) and 19.9% (31/156), respectively.

Relation between EGA classification and OLGA/OLGIM stage is summarized in Table 3. OLGA stage and OLGIM stage were significantly related to EGA classification. Table 4 shows the relation between OLGA stage and OLGIM stage. For 128 (56.4%) of the total 227 cases, low-risk stages (0 + Ⅰ + Ⅱ) and high-risk stages (Ⅲ + Ⅳ) were consistent when either the OLGA or OLGIM criteria were used. Ninety-nine (43.6%) of the total 227 cases were staged inconsistently, including 80 patients (35.2%) who were down-staged by OLGIM criteria compared with OLGA criteria, with 20 (8.8%) patients who were considered as low-risk when the OLGIM criteria were used but as high-risk when the OLGA criteria were used, and 19 patients who were down-staged by OLGA criteria compared with OLGIM criteria. As for correlations between EGA, OLGA, OLGIM and EGC, logistic regression modeling showed that moderate-to-severe EGA and OLGA stages Ⅲ-Ⅳ were significantly associated with EGC (Table 5).

H. pylori infection
The EGC group had a slightly higher *H. pylori* infection rate than the non-EGC group (70.4% *vs* 61.5%), but the difference was not significant (*P* = 0.195) (Table 1). The *H. pylori* infection rate in moderate-to-severe EGA patients was significantly higher than that in the none-to-mild EGA patients (75.0% *vs* 54.1%, *P* = 0.001). In addition, the *H. pylori* infection rate in OLGA/OLGIM stages Ⅲ-Ⅳ patients was significantly higher than that in the OLGA/OLGIM stages 0-Ⅱ patients (OLGA: 83.6% *vs* 55.8%, *P* < 0.001; OLGIM: 83.6% *vs* 57.8%, *P* < 0.001). However, the *H. pylori* infection rate in the patients with complete IM was not different from that in the patients with incomplete IM (68.5% *vs* 68.0%, *P* = 0.949) (Table 6).

DISCUSSION
China has a high prevalence of GC, reflecting its huge population, distinctive dietary (high-salt) structure and high *H. pylori* infection rate. Recognizing risk factors of EGC and establishing an appropriate surveillance system for patients with a high risk of GC will help to lengthen the survival time of patients and reduce waste of social resources. In the current study, we found that moderate-to-severe EGA and high-risk (Ⅳ) OLGA/OLGIM stages had a remarkable correlation with EGC, and these results are consistent with the published literature[7-10,12,17]. Rugge *et al*[10] stated that most HGN or invasive gastric neoplasia cases were consistently connected with high-risk OLGA/OLGIM stages (97.6% for OLGA stages, and 92.7% for OLGIM stages); however, in our study, 47.9% (34/71) and 57.7% (41/71) of patients with EGC were staged as low-risk (0-Ⅱ) according to the modified OLGA/OLGIM methods. In addition to the differences of pathological diagnosis and race of our study population, another

Table 4 Relation between OLGA/OLGIM stage and IM stage

		OLGIM				
		Stage 0	Stage I	Stage II	Stage III	Stage IV
OLGA	Stage 0	7	0	0	0	0
	Stage I	29	41	8	3	0
	Stage II	14	15	32	5	0
	Stage III	1	7	10	33	3
	Stage IV	0	1	1	2	15

OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment.

Table 5 Logistic regression analysis of three risk factors for early gastric cancer

	OR (95%CI)	*P* value
Moderate-to-severe EGA	1.95 (1.06-3.58)	0.031
OLGA stages III-IV	3.14 (1.71-5.81)	< 0.001
OLGIM stages III-IV	-	0.781

CI: Confidence interval; EGA: Endoscopic gastric atrophy; EGC: Early gastric cancer; OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment; OR: Odds ratio.

Table 6 Comparison of *H. pylori* infection rates between different groups

	Group	*H. pylori* infection rate	*P* value
EGA classification	None-mild degree	54.1%	0.001
	Moderate-severe degree	75.0%	
OLGA stage	0-II	55.8%	< 0.001
	III-IV	83.6%	
OLGIM stage	0-II	57.8%	< 0.001
	III-IV	83.6%	
IM subtype	Complete	68.5%	0.949
	Incomplete	68.0%	

EGA: Endoscopic gastric atrophy; EGC: Early gastric cancer; IM: Intestinal metaplasia; OLGA: Operative Link for Gastritis Assessment; OLGIM: Operative Link on Intestinal Metaplasia Assessment.

the existence of IM and the incomplete IM subtype to be significantly correlated with EGC, and these findings are consistent with those from other studies[20,21].

Quach *et al*[22] studied the relation between EGA classification and OLGA stage using 280 patients with functional dyspepsia. The results indicated a significant association between moderate-to-severe EGA and high-risk OLGA stage and extensive IM; our findings in the current study confirmed this conclusion. Moreover, the present study investigated the relation between OLGA stage and OLGIM stage. Approximately one-third of the cases were down-staged by OLGIM criteria, as compared with OLGA criteria, and less than one-tenth of the cases were considered as low-risk using the OLGIM criteria and as high-risk according to OLGA. Because a down-stage existed using OLGIM criteria, as compared with OLGA criteria[10], and more than one-half of the patients with EGC in our study were staged as 0-II by OLGIM, it may be prudent to consider that low OLGIM stages are simply considered equal to low risk for EGC.

The three assessments used to evaluate gastric atrophy and IM were all risk factors of EGC; nevertheless, they have their own characteristics. EGA focuses on the recognition of the endoscopic atrophic border and its range in the stomach. As the endoscopic gastric atrophy classification, EGA could be assessed in real-time as patients are undergoing endoscopy. Furthermore, EGA is intuitive and can be evaluated without taking biopsy specimens, which reduces the risk of gastric bleeding as well as saves costs associated with performance of the biopsy procedure. However, EGA is subjective and may result in designation of a different stage by different endoscopists, regardless of whether they are experienced or not. One recent report examined interobserver and intraobserver agreement for EGA[23]. The result showed that although intraobserver agreement for gastric mucosa atrophy was good to excellent (kappa value: 0.585-0.871), the interobserver agreement was only moderate for experienced endoscopists (kappa value: 0.29-0.474). The low interobserver agreement may give rise to low reproducibility of endoscopic findings, and may influence the detection of EGC to some extent. On the contrary, histologic atrophy and IM assessments based on OLGA/OLGIM system are more objective, and they are designated by pathologists who are blinded to

important difference was our strategy of obtaining and using only four gastric biopsy specimens for staging by the modified OLGA/OLGIM methods. Despite the fact that five standard biopsy specimens have been recommended by the updated Sydney system (two from the antrum, two from the corpus, and one from the incisura)[15], whether biopsy samples from the incisura angularis may provide extra clinical information useful towards determining the extent of premalignant conditions remains an unresolved controversy[18] (Figure 3). Current guidelines suggested at least four biopsies (two from the antrum, and two from the corpus) for adequate staging[19]. Marcos-Pinto *et al*[16] applied a modified OLGA/OLGIM staging system, with exclusion of biopsy of the incisura, and showed a downgrade of stages in comparison with standard OLGA stages. We took only four gastric biopsy specimens, which might have resulted in downgrade of high-risk OLGA/OLGIM stages. Our study also showed

IM score		Corpus			
		No IM (score 0)	Mild IM (score 1)	Moderate IM (score 2)	Severe IM (score 3)
Antrum	No IM (score 0)	STAGE 0	STAGE I	STAGE II	STAGE II
	Mild IM (score 1)	STAGE I	STAGE I	STAGE II	STAGE III
	Moderate IM (score 2)	STAGE II	STAGE II	STAGE III	STAGE IV
	Severe IM (score 3)	STAGE III	STAGE III	STAGE IV	STAGE IV

Figure 3 Modified operative link on intestinal metaplasia assessment staging frame (with exclusion of the biopsies from incisura angularis)[16]. IM: Intestinal metaplasia.

the patients' clinical information and whose material for assessment is subject to less interference than that of endoscopists. The interobserver agreement of OLGA/OLGIM by expert pathologists was reportedly higher than that for EGA[9,24]. However, OLGA/OLGIM staging depends on the biopsy specimens taken by endoscopists, which may be down-staged in cases when severe lesions were missed. That might be why, in the present study, the percentage of OLGA/OLGIM stage III-IV cases was lower in EGC than in moderate-to-severe EGA. We analyzed the correlation between EGC and endoscopic, histologic gastritis. The odds ratios of high-risk EGA, OLGA and OLGIM were 2.26, 3.76 and 2.95, respectively. In view of the tight relation of the three methods, stepwise logistic regression modeling was performed to determine which classification performs better in suggesting the occurrence of EGC. It showed that moderate-to-severe EGA and OLGA stages III-IV were prominently related to EGC (P = 0.031 for EGA and P < 0.001 for OLGA), with the odds ratios of high-risk EGA and OLGA being 1.95 and 3.14, respectively. Thus, OLGA stages III-IV appeared to be more relevant to the occurrence of EGC. In addition, since *H. pylori* infection is considered a high-risk factor for GC[25,26] and has been demonstrated as significantly related to high-risk OLGA/OLGIM stages[27] and to moderate-to-severe EGA (the present study), we emphasized the importance of *H. pylori* infection in the detection of EGC. Considering the advantages and disadvantages of the three methods, we suggest that OLGA classification combined with *H. pylori* detection be put into routine use in a surveillance program for EGC.

Up to now, the suitable surveillance intervals for patients under precancerous conditions remain controversial. According to the recent guidelines[19], endoscopic surveillance is recommended for patients with extensive atrophic gastritis or IM, who should obtain follow-up every 3 years. In contrast, some researchers from Japan have suggested that patients with extensive atrophic gastritis or IM obtain follow-up every 1 year, those with moderate atrophic gastritis every 2 years, and those with none-to-mild every 3 years[28,29]. Based on the findings from the present

study, although moderate-to-severe EGA and high-risk OLGA/OLGIM stages were all high-risk factors of EGC, the OLGA classification may be more appropriate for EGC screening. We suggest that patients aged more than 40 years undergo upper gastrointestinal endoscopy for GC screening, with OLGA stage being detected in the meanwhile. The surveillance intervals for patients with OLGA stages III-IV need to be shortened, even when there is no obvious lesion, and endoscopists should be sufficiently cautious and take more biopsy specimens if necessary in order to avoid missed diagnosis of EGC. Prospective studies are needed to investigate the appropriate surveillance intervals for patients with OLGA stages III-IV.

This study had several limitations. First, all the endoscopic assessments were performed by a single highly experienced endoscopist, and all the histopathological diagnoses were made by a single experienced pathologist, which may lead to deviations of data analysis. Second, this was a single-center study; therefore, we cannot exclude the possibility of selection bias. However, to the best of our knowledge, this is the first study to identify that OLGA stage is more appropriate for predicting EGC than OLGIM stage and EGA classification, which can further help in establishment of a thorough surveillance program for EGC.

In conclusion, our study showed that moderate-to-severe EGA and high-risk OLGA/OLGIM stages are all high-risk factors of EGC. The three assessments had tight relation with each other, and *H. pylori* infection was significantly associated with high-risk stage of both endoscopic and histologic atrophy and IM. However, we suggest OLGA classification as the optimal method for EGC screening. A surveillance program including OLGA stage and *H. pylori* infection is expected to be a practical approach that will help to achieve greater detection of gastric cancers at an early stage.

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COMMENTS

Background

Japanese endoscopic gastric atrophy (EGA) classification, Operative Link on Gastritis Assessment (OLGA), and Operative Link on Gastric Intestinal Metaplasia Assessment (OLGIM) have been proven separately as effective methods to evaluate severity of gastric atrophy and intestinal metaplasia. However, these methods have not been compared for prognosticating neoplastic development. The present study was designed to compare these three methods, so as to select the optimal method for early gastric cancer (EGC) screening.

Research frontiers

There is increasing focus on the correlations between gastric atrophy and intestinal metaplasia with GC. EGA classification is considered as endoscopic gastric atrophy assessment, while OLGA/OLGIM is considered as histologic gastric atrophy/intestinal metaplasia assessments. Recent investigations have shown that moderate-to-severe EGA and high-risk OLGA/OLGIM stages are high-risk factors of EGC.

Innovations and breakthroughs

The present study analyzes the advantages as well as disadvantages of endoscopic and histologic gastric atrophy or intestinal metaplasia (EGA classification, OLGA/OLGIM stages), and compares the correlation between EGC and these three methods. The findings show that OLGA classification is optimal for EGC screening. It is suggested that the OLGA classification be adopted to help detect more gastric cancers at an early stage.

Applications

This study provides additional evidence supporting the importance of OLGA stage in predicting the development of EGC, which may lead to development of an appropriate surveillance program for EGC screening.

Terminology

OLGA and OLGIM are gastritis staging systems that primarily rank the risk of GC according to the extent and severity of gastric atrophy and intestinal metaplasia. EGA assessment, first defined by Kimura-Takemoto and mostly used in Japan, is divided into six types according to the extent of gastric atrophy detected under endoscopic observation.

Peer-review

The authors evaluated the characteristics of background mucosa in patients with EGC by using different classifications (EGA, OLGA and OLGIM) and compared the correlations between these three methods and EGC. They concluded that all three methods are risk factors of EGC and OLGA classification is optimal for EGC screening.

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META-ANALYSIS

Does aspirin or non-aspirin non-steroidal anti-inflammatory drug use prevent colorectal cancer in inflammatory bowel disease?

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Abstract

AIM: To determine whether aspirin or non-aspirin non-steroidal anti-inflammatory drugs (NA-NSAIDs) prevent colorectal cancer (CRC) in patients with inflammatory bowel disease (IBD).

METHODS: We performed a systematic review and meta-analysis. We searched for articles reporting the risk of CRC in patients with IBD related to aspirin or NA-NSAID use. Pooled odds ratios (OR) and 95%CIs were determined using a random-effects model. Publication bias was assessed using Funnel plots and Egger's test. Heterogeneity was assessed using Cochran's Q and the I^2 statistic.

RESULTS: Eight studies involving 14917 patients and 3 studies involving 1282 patients provided data on the risk of CRC in patients with IBD taking NA-NSAIDs and aspirin respectively. The pooled OR of developing CRC after exposure to NA-NSAIDs in patients with IBD was 0.80 (95%CI: 0.39-1.21) and after exposure to aspirin it was 0.66 (95%CI: 0.06-1.39). There was significant heterogeneity ($I^2 > 50\%$) between the studies. There was no change in the effect estimates on subgroup analyses of the population studied or whether adjustment or matching was performed.

CONCLUSION: There is a lack of high quality evidence on this important clinical topic. From the available evidence NA-NSAID or aspirin use does not appear to be chemopreventative for CRC in patients with IBD.

Core tip: Colorectal cancer (CRC) remains a serious complication of inflammatory bowel disease (IBD) and chemoprevention is an attractive alternative to prophylactic surgery or intensive surveillance programs. Aspirin and non-steroidal anti-inflammatory drugs have chemopreventative activity against "sporadic" CRC. We have synthesized the available data for the prevention of IBD associated CRC and found no potential protective effect for either medication. There is a lack of available data on the potential effects of these medications in preventing CRC in patients with IBD and there is a need for high quality, focused studies on this topic.

INTRODUCTION

One of the most serious complications of inflammatory bowel disease (IBD) is the development of colorectal cancer (CRC). Worldwide, the risk of developing CRC ranges between 5-40/100000 people depending on location, with a marked increase in Western populations[1,2], this is increased to 300/100000 in patients with longstanding ulcerative colitis (UC)[3]. The cumulative probability of developing CRC in patients with UC increases from 2%, 8% and 18% after 10, 20 and 30 years of disease respectively[3]. In patients with CD, a meta-analysis of population-based studies has demonstrated an increased risk of CRC, with a standardized incidence ratio of 1.9 (95%CI: 1.4-2.5)[4]. Whilst the risk of CRC in CD appears to be stable, a recent study from Denmark[5] has reported a decrease in CRC incidence in patients with UC over the past 30 years. It is currently unclear why there may be a reduction in incidence, but a plausible hypothesis is that increased use of IBD medications may reduce inflammation-driven colorectal carcinogenesis.

Identified risk factors for developing CRC in patients with IBD include primary sclerosing cholangitis[6], degree of inflammation[7-9], duration of disease, extent of colonic involvement[10], as well as family history of colorectal cancer[10,11]. International society guidelines advocate regular surveillance colonoscopy examinations to identify malignant and pre-malignant lesions[12]. These are resource intensive and not

without risk. As such primary prevention of CRC in these patients is an attractive alternative. Several treatment modalities have been proposed as potential chemopreventative agents and studied mainly *via* retrospective case-control and cohort studies[13]. These include 5-aminosalicylic acid preparations[14-16], ursodeoxycholic acid (in patients with concomitant PSC)[17,18], thiopurine analogues[19], aspirin and non-aspirin non-steroidal anti-inflammatory drugs (NA-NSAIDs), and statins[20].

There are plausible biological mechanisms for how NA-NSAIDs, and aspirin, may prevent CRC development in patients with IBD. In epidemiological, laboratory and clinical studies aspirin has consistently been shown to reduce the incidence of several tumors, including "sporadic" CRC not related to a defined genetic predisposition or IBD[21]. The exact anti-neoplastic mechanism(s) of aspirin and NA-NSAIDs is not yet clear but several cell signaling pathways have been implicated as targets for COX-dependent and COX-independent mechanisms of action[22,23]. Aspirin use also appears to prevent CRC metastasis, as well as the risk of primary CRC[24].

As well as the potential benefits for chemoprevention there are concerns about negative effects of aspirin and the NA-NSAIDs on the lower gastrointestinal tract. Adverse effects of NA-NSAIDs on the colon include a NSAID colonopathy with diaphragm-like stricturing and mucosal inflammation and ulceration, complicated diverticular disease including bleeding[25], and microscopic colitis[26,27]. A possible association between the use of NSAIDs including aspirin and the onset or relapse of IBD has been repeatedly suggested. However, lack of controlled prospective trials make it difficult to draw definite conclusions[28,29].

Aspirin use is associated with several side effects. The main concern is the risk of upper gastrointestinal bleeding and hemorrhagic stroke. Most studies using aspirin have not shown increased death rates from gastrointestinal bleeding suggesting that any bleeds related to aspirin are small and relatively insignificant[30].

There are currently conflicting data on the putative role of NA-NSAIDs and aspirin in the prevention of CRC in IBD. We therefore performed a systematic review and meta-analysis in order to identify if there is evidence that aspirin and NA-NSAIDs have chemopreventative activity against CRC in patients with IBD.

MATERIALS AND METHODS

We followed a pre-specified and peer-reviewed protocol; the PRISMA statement, a 27 item checklist deemed essential for reporting systematic reviews and meta-analyses of randomized controlled trials and observational studies[31].

Search strategy
We searched multiple electronic databases including

MEDLINE (1965 to July 2015), EMBASE (1974 to July 2015), ISI Web of Science (1945-July 2015) and the Cochrane Register of Controlled Trials. The MeSH search terms included were Inflammatory Bowel Disease AND CRC AND Aspirin OR NSAIDs. Free text terms and variations were used. No limits or language restrictions were applied. We performed a recursive search of the bibliographies of relevant review articles and of the included studies. Articles were assessed by two independent reviews (Burr NE and Subramanian V) to assess eligibility for inclusion. Any disagreements were resolved by consensus decision.

Study selection

Studies were eligible for inclusion if they reported on risk of developing CRC in patients with IBD on either NA-NSAIDs or aspirin compared to a control population. Studies published only in abstract form were not included. Two reviewers (Burr NE and Subramanian V) independently screened titles and abstracts identified by the preliminary searches to identity potentially eligible studies. Both reviewers independently assessed the full text articles of potentially relevant studies for inclusion in the pooled analysis. Data from included studies were independently extracted by two investigators (Burr NE and Subramanian V). Information was collected on characteristics of the study (population studied, country of origin, study design, definition of drug exposure) and medication use including NA-NSAIDs, aspirin and development of CRC. Agreement between the reviewers was greater than 95% and differences between the datasets were resolved by consensus decision.

Statistical analysis

The odds ratio (OR) with 95% confidence interval (CI) of developing CRC in patients with IBD on aspirin or NA-NSAIDs compared with controls was extracted from the study. When insufficient (no information on odds ratio or drug exposure) data had been published, we contacted the study authors. As randomization and blinding is not possible in observational studies and baseline differences between the groups can confound the results we used the authors' ORs with adjustment for potential confounding factors wherever available. The pooled OR estimate was calculated from an inverse-variance-weighted average of the individual studies[32]. A DerSimonian-Laird random effects model was used a priori[33]. As a further sensitivity analysis a fixed effects model was used for comparison. Stata version 12 (StataCorp, College Station, Texas, United States), was used for all of the data analysis.

We used the Cochran's Q statistic to test hete-rogeneity among pooled estimates[34]. Statistical heterogeneity was also measured by the I^2 statistic, which quantifies the proportion of inconsistency in individual studies that cannot be explained by chance[35]. Values of I^2 equal to 25%, 50%, and 75% represent low, moderate, and high heterogeneity,

respectively. To test for publication bias, we used a test for asymmetry of the funnel plot proposed by Egger *et al*[36]. This test detects funnel plot asymmetry by determining whether the intercept deviates significantly from zero in a regression of the normalized effect estimate (estimate divided by the standard error) against precision (reciprocal of the standard error of the estimate) weighted by the reciprocal of the variance of the estimate.

The quality of the primary studies assessing the risk of bias was evaluated using the Newcastle-Ottawa Scale for non-randomized studies (NOS)[37]. Studies score for a maximum of 4 for selection, 2 for comparability and 3 for outcomes (cohort) or exposures (case-control). We regarded scores of 0-3 as low, 4-6 as medium and 7-9 as high methodological quality.

We performed pre-planned subgroup analyses to assess the following factors on the trial outcome and on the heterogeneity of the analyses: (1) matching or adjustment for confounders (any or none); and (2) the population studied (population-based or other).

RESULTS

Our searches retrieved 9 potentially relevant articles, of which 3[14,38,39] provided data on aspirin exposure and risk of CRC and 8[14,39-45] on NA-NSAID exposure and risk of CRC in patients with IBD. Figure 1 outlines the fate of the selected articles. The studies were either retrospective case-control, nested case-control or cohort studies by design and 5[14,40-42,44] included population-based analysis. Table 1 lists all the included studies and their characteristics. We contacted authors of papers for missing data but did not obtain any extra information. The quality assessment of the studies using the Newcastle Ottawa scale is also detailed in Table 1. Only 3 studies[39,41,42] provided multivariate analysis of data for risk of developing CRC in IBD patients exposed to aspirin or NA-NSAIDs.

Cumulative risk of developing CRC in IBD patients exposed to NA-NSAIDs

Eight studies, including 14917 patients with IBD provided data on the risk of developing CRC after exposure to NA-NSAIDs. Using a random effects model, the pooled adjusted OR of developing CRC after exposure to NA-NSAIDs in patients with IBD was 0.80 (95%CI: 0.39-1.21) (Figure 2). The heterogeneity between the studies was high (Cochran's Q = 38.15, P = 0.00 and I^2 = 81.6%).
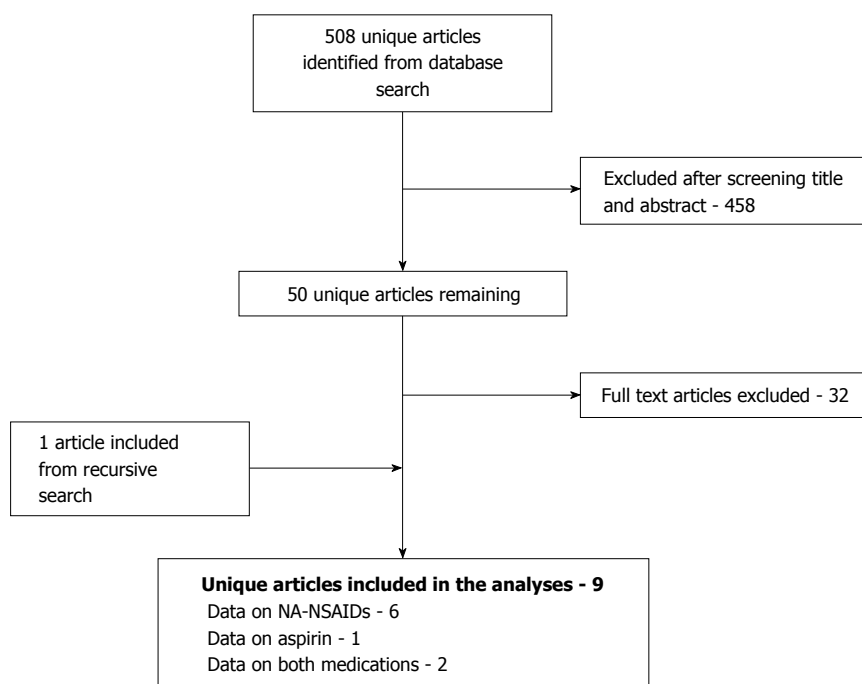
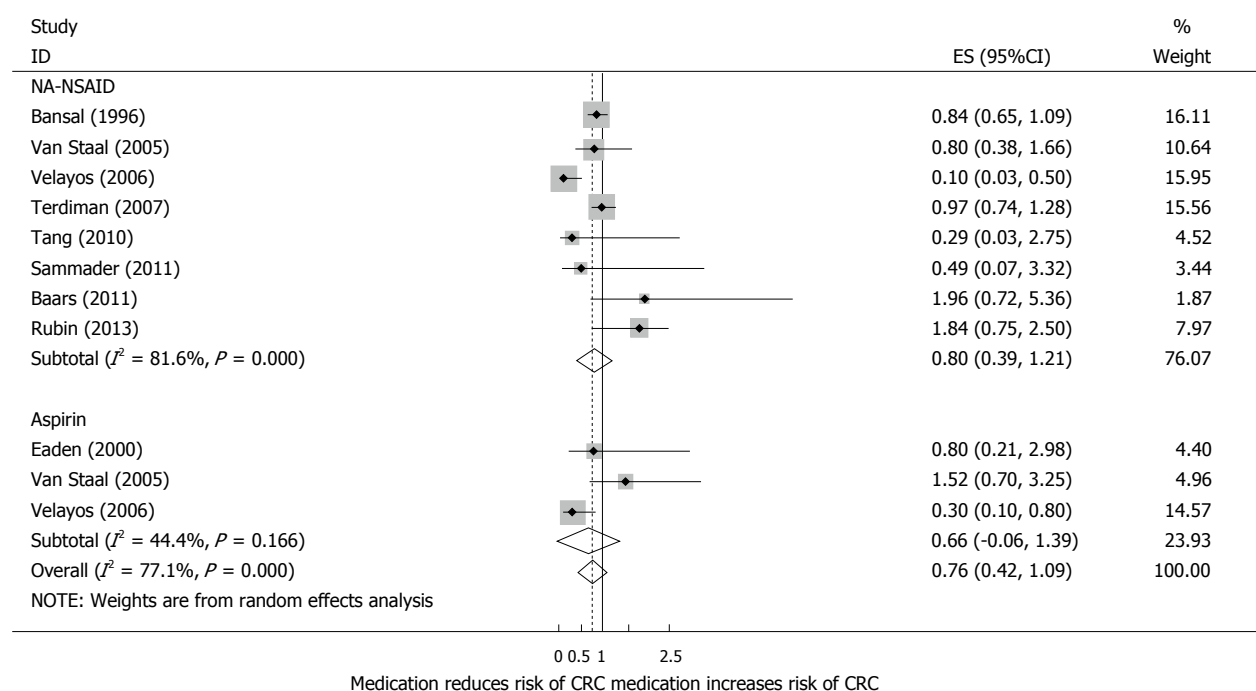### Sensitivity analysis and publication bias

Pre-planned subgroup analyses showed that there was no difference in the overall effect estimate when comparing the population studied or whe-ther adjustment or matching for confounders was performed (Table 2). There was no heterogeneity among population-based studies (Cochran's Q = 2.39,

Table 1 Characteristics of studies included in the analysis

Author	Design	Population	Definition of IBD	Drug exposure	Exclusion criteria	No. of patients	OR (95%CI)[1]	Adjustment/ matching	NOS quality assessment
Bansal *et al*[42], 1996	Case-control	US veterans affairs	Clinical database	NSAID associated diagnosis	Not specified	11446	0.84 (0.65-1.09)	Adjusted for age, sex and ethnicity	6
Eaden *et al*[38], 2000	Case-control	UK Hospital	Clinical, pathological and radiological records	Prescribed 5-10 years before diagnosis	Colorectal surgery, IBD diagnosed at time of cancer diagnosis	206	0.80 (0.21-2.98) Aspirin	Non-adjusted	4
Van Staal *et al*[14], 2005	Nested case-control	UK general practice	Clinical records	Prescribed in the 6 mo prior to diagnosis	Colorectal surgery, previous history of CRC	700	1.52 (0.7-3.25) (Aspirin) 0.80 (0.38-1.66) (NA-NSAID's)	Non-adjusted	5
Velayos *et al*[39], 2006	Case-control	US Hospital	Clinical, pathological and endoscopic records	2 records of use in notes	Previous CRC, IBD diagnosed at same time as CRC, incomplete data	376	0.3 (0.1-0.8) (Aspirin) 0.1 (0.03-0.5) (NA-NSAID)	Matched on gender, duration of disease and extent of disease	8
Terdiman *et al*[40], 2007	Case-control	US insurance claims	Clinical records	Prescribed in the year before diagnosis	Colorectal surgery	1536	0.97 (0.74-1.28)	Non-adjusted	5
Tang *et al*[43], 2010	Retrospective cohort	US Hospital	Clinical database	Ever used	No colonic involvement of IBD	48	0.29 (0.03-2.75)	Non-adjusted	5
Samadder *et al*[41], 2011	Case-control	N. Israel community	Patient questionnaires	Weekly for > 3 yr	Previous history of CRC	60	0.49 (0.07-3.32)	Matched for age, gender and ethnicity	6
Baars *et al*[44], 2011	Case-control	Netherlands nationwide pathology	Pathology reports	Ever used	IBD diagnosed at the same time as CRC	551	1.96 (0.72-5.36)	Non-adjusted	6
Rubin *et al*[45], 2013	Case-control	US Hospital	Pathology reports	Not specified	Incomplete records	200	1.84 (0.75-2.5)	Non-adjusted	5

[1]OR for colorectal cancer (CRC) chemoprotective effect of non-aspirin non-steroidal (NA-NSAID) use in patients in inflammatory bowel disease (IBD) unless otherwise stated. For cohort studies was used only for the Tang *et al*[43] study. The scale for case-control studies was used for the other studies. NOS: Newcastle-Ottawa Score.

Figure 1 Flow chart showing the results from the search strategy. NA-NSAIDs: Non-aspirin non-steroidal anti-inflammatory drugs.

Figure 2 Forest plot of odds ratios and 95%CI for effect of non-aspirin non-steroids anti-inflammatory drugs or aspirin on colorectal cancer development in patients with inflammatory bowel disease. Random effects model.
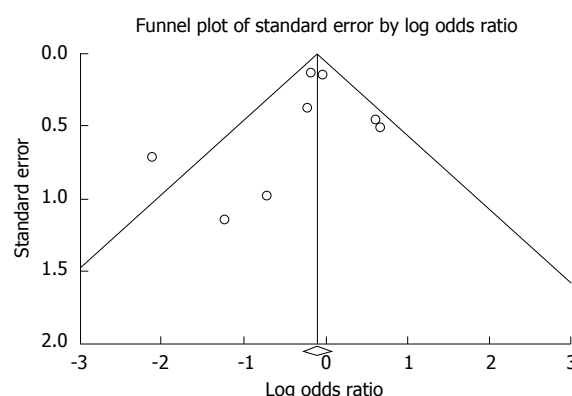
Table 2 Subgroup analyses for studies reporting on risk of colorectal cancer in patients with inflammatory bowel disease taking non-aspirin non-steroidal anti-inflammatory drugs

	No. of studies	Pooled OR (95%CI)
Matched y/n		
Matched/adjusted	3	0.47 (0.18-1.13)
None	5	1.04 (0.65-1.43)
Study location		
Hospital	6	0.92 (0.78-2.62)
Population	2	0.88 (0.72-1.04)

Figure 3 Funnel plot for publication bias for studies looking at the odds ratio of developing colorectal cancer in patients with inflammatory bowel disease on non-aspirin non-steroids anti-inflammatory drugs.

$P = 0.79$ and $I^2 = 0\%$) but high heterogeneity between hospital-based studies (Cochran's $Q = 14.17$, $P < 0.005$ and $I^2 = 92\%$)). There was some funnel plot asymmetry compatible with publication bias (Figure 3). However Egger's regression asymmetry test was non-significant ($P = 0.56$). The regression asymmetry test is probably underpowered as there are only 8 studies included in this meta-analysis[46].

Cumulative risk of developing CRC in IBD patients exposed to aspirin

Three studies, including 1282 patients with IBD, provided data on risks of developing CRC after exposure to aspirin. The pooled adjusted OR of developing CRC after exposure to aspirin in patients with IBD was 0.66 (95%CI: 0.06-1.39) (Figure 2). A random effects model was chosen a priori. The heterogeneity between the studies was high (Cochran's $Q = 0.166$. and $I^2 = 44.4\%$). A fixed effects model was performed as a sensitivity test which changed the pooled adjusted OR to 0.41 (95%CI: 0.08-0.74). We

did not attempt to do an analysis of publication bias or subgroup analyses as there were only 3 studies included in the final analysis.

DISCUSSION

We present the first systematic review and meta-analysis of the effects of NA-NSAIDs and aspirin for CRC chemoprevention in patients with IBD to our knowledge. It is important to synthesize the available literature on this subject as CRC remains an important complication of IBD and NA-NSAIDs including aspirin have been consistently shown to have a protective effect in sporadic colorectal cancer[47,48]. We found 9 retrospective studies fitting the inclusion criteria, but unfortunately there have been no prospective randomized trials.

There were only 3 studies that reported on aspirin use in patients with IBD associated cancer. We found no significant potential protective effect for NA-NSAIDs or aspirin against the development of CRC in IBD patients.

There are several limitations to this meta-analysis. All the included studies are retrospective and are therefore subject to inherent biases and confounding. Publication bias is another possible limitation as negative studies are less likely to be published and therefore not included in the analyses. However we have attempted to reduce the possibility of publication bias by conducting an exhaustive search of the literature and did not limit inclusion of studies based on language. Most of the studies included in our analysis reported NA-NSAID and aspirin use as a secondary outcome measure and results from a multivariate analysis was provided only by 3 studies[39,41,42]. The study with the most robust methodology from Velayos *et al*[39] reported a significant chemopreventative role for both NA-NSAIDs and aspirin. There were differences in the studies related to the definition of drug exposure and as these studies were all retrospective it was not possible to check compliance with the medication. A further limitation with studies of this type is confounding by indication. Aspirin and NA-NSAID use could be associated with another factor, such as another medical condition, that is associated with colorectal cancer. It is not possible to adjust or correct for all such factors so this always must be born in mind when interpreting such studies.

The dose and duration of medication exposure was not consistently recorded. An important consideration of chemoprevention against colorectal cancer is the duration of exposure to the medication. In the evidence for aspirin protecting against sporadic CRC a duration of > 5 years conferred a 34% reduction in CRC risk[49]. The only study included here which took this into consideration was Eaden *et al*[38] where a prescription in the preceding 5-10 years before diagnosis was required for inclusion as positive exposure (Table 1). The dose of aspirin used was not stated in most of the studies but it is likely to have been low dose as used in routine clinical practice in patients with cardiovascular risk factors, 75 mg in the United Kingdom and 81 mg in the United States. It is possible that a higher dose may be needed for chemoprevention of colitis-associated CRC. For example, a recent trial in patients with Lynch syndrome, a hereditary condition associated with high risk of CRC, demonstrated that high dose (600 mg daily) aspirin conferred protection against CRC[50]. Little information was provided about the timing and duration of exposure to aspirin and NA-NSAID's in any of the included studies. Aspirin and NA-NSAIDs may be unable to prevent the progression from dysplasia to cancer and could therefore be chemopreventative only in those with exposure to the drug from soon after onset of IBD and those with longer duration of exposure to the medication. Unfortunately none of the studies included in this meta-analysis provided data on the timing of exposure to NA-NSAID/aspirin and duration of IBD, to determine if early or long-term exposure was chemopreventative. The main outcome of interest was the development of CRC and not dysplasia which could support the argument that in some of the patients, CRC may have developed in those exposed to aspirin or NA-NSAIDs only after they had already developed colorectal neoplasia.

Adverse effects of NSAIDs on the gastrointestinal tract need to be considered in future studies as there is a potential increased incidence of disease flares with the use of NSAIDs, including aspirin[51]. This issue is still under debate as NSAIDs are often used for treatment of arthralgia and abdominal pain and it may be that NSAIDs are used after the flare develops rather than being the potential cause of the flare.

Colorectal cancer remains a serious complication of IBD. Current methods to reduce CRC in IBD are the use of colonoscopic surveillance or by prophylactic proctocolectomy. British Society of Gastroenterology guidelines advocate screening and surveillance colonoscopy which can result in annual tests for high risk patients[52]. Chemoprevention is therefore an attractive proposition for these patients. NA-NSAIDs and aspirin remain biologically plausible targets for chemoprevention in IBD. As we have shown the clinical evidence is limited. The available data is hampered as most of the studies include small numbers of patients and do not include adequate information on medication dose and duration. Potential chemoprevention agents are likely to take several years to display a protective effect as in the sporadic CRC population and this should be borne in mind in future studies. Prospective randomized chemoprevention trials are unlikely to be done as the sample size required would be too large and therefore well-conducted epidemiological studies using prospective databases are needed to clarify the true effect of aspirin and/or NA-NSAIDs on the risk of CRC in patients with IBD.

ACKNOWLEDGMENTS

COMMENTS

Background

Colorectal cancer is an important complication of inflammatory bowel disease (IBD). Primary prevention is an attractive strategy and aspirin and non-aspirin anti-inflammatory drugs are plausible options. Several studies have investigated the possible use of these medications but the data has not been synthesized.

Research frontiers

These medications have shown promise in preventing colorectal cancer in a non IBD population. It is important to examine this potential effects in the IBD population who are at greater risk of colorectal cancer.

Innovations and breakthroughs

This is the first meta-analysis to investigate this potential effect. From the

available evidence there is no data to support the use of these medications in chemoprevention against colorectal cancer but the study has highlighted the lack of high quality data.

Applications

The study highlights the need for focused studies into the potential protective effect of these medications.

Peer-review

This systematic review and meta-analysis gives useful information to clinicians and patients on the role of these medications. It highlights the lack of data in this area and the need for high quality focused studies.

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CASE REPORT

Ampullary neuroendocrine tumor diagnosed by endoscopic papillectomy in previously confirmed ampullary adenoma

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Institutional review board statement: This case study was reviewed and approved by the Soonchunhyang University College of Medicine, Cheonan Hospital Institutional Review Board.

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Abstract

Ampullary adenoma is a common indication for endoscopic papillectomy. Ampullary neuroendocrine tumor (NET) is a rare disease for which complete surgical resection is the treatment of choice. However, because of the morbidity and mortality associated with surgical resection, endoscopic papillectomy is increasingly used in selected cases of low grade, with no metastasis and no invasion of the pancreatic or bile duct. Also, confirmed and complete endoscopic resection of ampullary NET accompanied by adenoma has not been reported to date. We report herein a rare case of an ampullary NET accompanied with adenoma, which was successfully and completely resected *via* endoscopic papillectomy. Prior to papillectomy, this case was diagnosed as an ampullary adenoma.

Key words: Ampulla of Vater; Neuroendocrine tumor; Adenoma; Papillectomy

Core tip: In selected cases without metastasis or invasion of the pancreatic and bile duct, endoscopic

papillectomy can be a treatment of choice for ampullary neuroendocrine tumor (NET). To the best of our knowledge, the complete cure case of successful endoscopic papillectomy for ampullary NET accompanied with ampullary adenoma has not been reported in the English-language literature. This unusual ampullary NET accompanied with adenoma was successfully treated by endoscopic papillectomy.

INTRODUCTION

The incidence of gastrointestinal neuroendocrine tumor (NET) has increased in recent years due to the increased frequency of healthcare examinations and improvements in diagnostic techniques. However, NET of the ampulla of Vater is extremely rare[1-3]. Pancreaticoduodenectomy is generally considered the procedure of choice for NET of the ampulla of Vater larger than 2 cm and for cases of neuroendocrine carcinoma. Endoscopic local resection and surgical ampullectomy have been considered to be safe for small NET of the ampulla of Vater (less than 2-cm diameter) or in patients with severe comorbidities[2-6]. Endoscopic papillectomy is now commonly indicated for adenomas of the ampulla of Vater[7,8].

In the case presented herein, we first diagnosed ampullary adenoma by routine endoscopic examination. However, subsequent endoscopic papillectomy confirmed combined ampullary NET. Ampullary NET accompanied by adenoma of the ampulla of Vater was resected completely by endoscopic papillectomy, without local recurrence during 2 years.

CASE REPORT

A 53-year-old female patient visited our hospital for dyspepsia of 3-mo duration. She had no specific medical or surgical history. Her vital signs at admission were blood pressure 110/70 mmHg, pulse rate 82/min, respiratory rate 20/min, and body temperature 36.3 ℃. Physical examination revealed no marked features. The laboratory data also showed no abnormalities, including tumour markers (CEA 1.0 ng/mL, CA 19-9 2.0 U/mL). Screening upper gastrointestinal endoscopy showed protruding major papilla and subsequent endoscopic biopsy of the ampulla of Vater revealed low-grade ampullary adenoma. A duodenoscopic image showed an enlarged major papilla with central umbilication and

fine nodularity. Endoscopic ultrasonography (EUS) at the major ampulla revealed a 1.1 × 0.9-cm, slightly hypoechoic round ampullary mass confined to the submucosa without definite wall disruption or adjacent invasion (Figure 1). Abdomen computed tomography (CT) did not show an abnormally dilated pancreatic or biliary duct, ductal invasion, or enlarged lymph nodes.
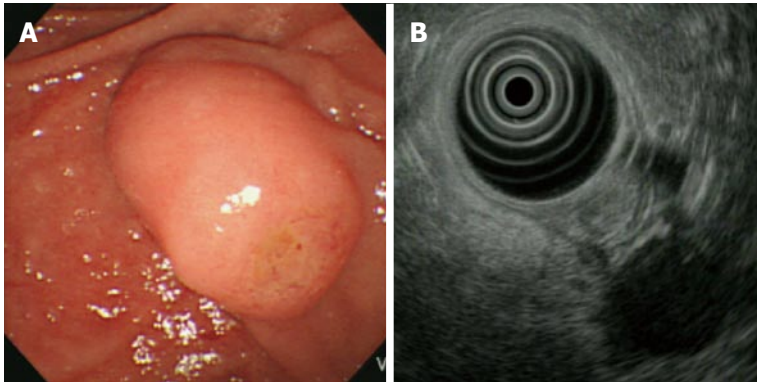
Based on pathology and an imaging study, we planned endoscopic papillectomy for the removal of unexposed ampullary adenoma. Following submucosal injection of 1:10000 diluted epinephrine, snaring papillectomy was performed. However, primary complete resection of major papilla was not achieved. Following the first papillectomy, a remnant, whitish, round mass-like lesion was seen to protrude, and was difficult to differentiate from remnant tumor or a second combined tumor. Therefore, subsequently a second resection was performed successfully in the same manner as the first. An endoscopic image acquired immediately following the two-step papillectomy showed complete resection without complications. Resected tissues showed a papillary roof lesion and a whitish, round, mass-like lesion (Figure 2). Insertion of a prophylactic pancreatic stent failed due to technical difficulties with selective pancreatic duct cannulation. Due to the risk of post-procedure pancreatitis, the number of attempts to cannulate the pancreatic duct was not permitted to exceed five. Post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis did not occur.

Microscopic findings of the resected specimens were as follows. There was no evidence of NET and tubular adenoma in the first primary papillectomy specimen. But, the second resection specimen showed a collision tumor composed of tubular adenoma (closed arrow) and NET [open arrow, hematoxylin-eosin (HE) staining, magnification × 4] and it measured about 1.0 cm. The tubular adenoma component was mainly found in intra-ampullary portion of the ampulla of Vater and exhibited round-to-oval enlarged glands with stratified epithelial cells (HE staining, magnification × 100). The NET component was also found in the second resection specimen, abutting the tubular adenoma and showed cord-like arrangement of monotonous tumor cells (HE staining, magnification × 100). Immunohistochemistry showed that the tumour cells were positive for synaptophysin (magnification × 100) (Figure 3).
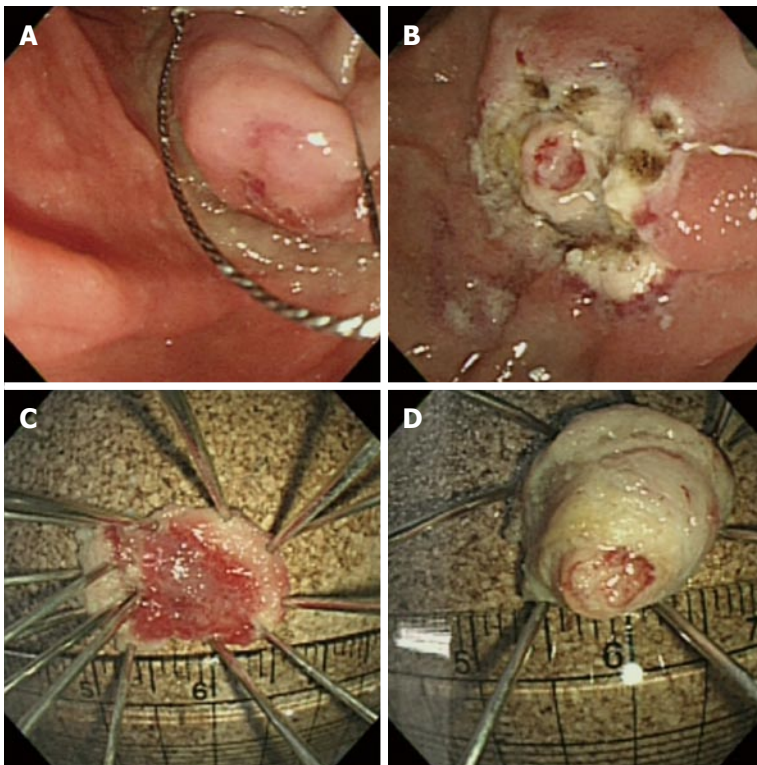
Follow-up endoscopic biopsy of the papillectomy site performed one and three month later did not show remnant tumor except reepithelization (Figure 4). No local recurrence or metastasis of NET or adenoma was detected during 24 mo of follow-up.

DISCUSSION

NET of the ampulla of Vater, formerly known as carcinoid tumors, is extremely rare. It accounts for only about 0.3%-1% of all gastrointestinal NETs,

Figure 1 A duodenoscopic image showed an enlarged major papilla with central umbilication and fine nodularity on ampullary orifice (A), endoscopic ultrasonography at the major ampulla revealed a 1.1 × 0.9-cm, slightly hypoechoic round ampullary mass confined to the submucosa without definite wall disruption or adjacent invasion (B).
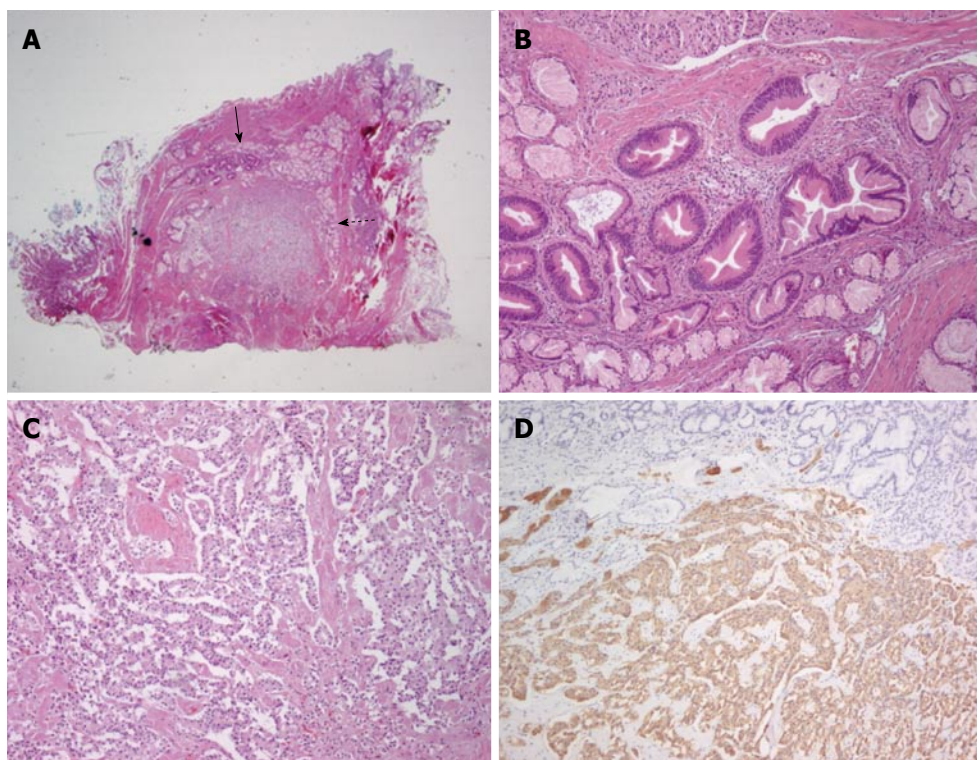

Figure 2 Endoscopic papillectomy. Following submucosal injection of 1:10000 diluted epinephrine, snaring papillectomy was performed (A), finally papillectomy induced ulceration is noted without complications (B), following the first papillectomy, a resected papillary roof is noted (C), A whitish and round mass-like lesion was resected by a subsequent second papillectomy (D).

and less than 2% of all periampullary cancers. The natural history of this disease entity has not been well established[2,9-11]. Well-differentiated (low and intermediate grade) NETs have been variously termed carcinoid tumor (typical and atypical), neuroendocrine tumor (grade 1 and 2), or neuroendocrine carcinoma (low and intermediate grade). The previously used term, carcinoids of the ampulla of Vater, comprises a broad spectrum of morphologically and biologically diverse tumors. In the latest World Health Organization classification, published in 2010, it is recommended to distinguish between (1) neuroendocrine neoplasm, grade 1 (low grade); (2) neuroendocrine neoplasm,
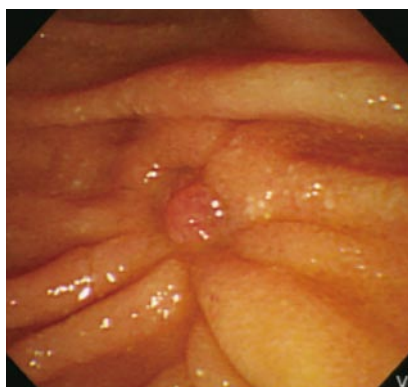
grade 2 (intermediate grade); and (3) neuroendocrine carcinoma, grade 3 (high grade)[6].

The diagnosis of ampullary NET is challenging till now. This tumor frequently originates from the deep mucosa or submucosa, so that cannot easily be detected in biopsy specimens. Most reports describe such lesions as a round or oval mass, with intact overlying duodenal mucosa, with negative biopsies. Also, early lymphatic metastasis is possible despite the small size of the lesions, and an accurate diagnosis is difficult[4,12]. Accuracy rates of biopsy for the preoperative diagnosis of NET range from to 14% to 66%[3,4,13]. Duodenoscopy in combination with ERCP

Figure 3 The protruding whitish mass lesion (second papillectomy tissue, Figure 2D) was composed of two lesions that differed in their histological characteristics. Tubular adenoma (solid arrow) and NET [Dotted arrow, hematoxylin-eosin (HE) staining, magnification × 4; A]. The tubular adenoma lesion exhibited round-to-oval enlarged glands with stratified epithelial cells (HE staining, magnification × 100; B). The NET showed cord-like arrangement of monotonous tumor cells (HE staining, magnification × 100; C). Immunohistochemistry showed that the tumor cells were positive for synaptophysin (magnification × 100; D).

Figure 4 Follow-up endoscopic biopsy of the papillectomy site three month later dose not show remnant tumor.

is the diagnostic method of choice for deeper biopsies with the aim of identifying intrapapillary lesions. EUS, CT or magnetic resonance imaging (MRI) are used for staging and differential diagnosis, together with detection of invasion of the bile or pancreatic duct and metastasis to lymph nodes or other organs[4].

Clinically, NET can cause carcinoid syndrome, which presents as diarrhea or facial flushing, due to increased secretion of serotonin. However, carcinoid syndrome in ampullary NET is rare and the clinical and laboratory findings typical of carcinoid syndrome are frequently absent[14]. Anatomically, ampullary NET develops at the conjugation of the pancreatic and biliary ducts. Jaundice is the predominant symptom (53%) at the time of admission to hospital, followed by pain (24.6%), acute pancreatitis (6%) and weight loss (3.7%)[4,15]. The case presented herein exhibited no specific symptoms due to the tumour, with the exception of mild dyspepsia.

The treatment of choice for NET is complete resection. Metastasis is rare, particularly in ampullary NETs. Therefore, complete resection is required as the primary therapy[1,4,16]. In terms of prognosis, the 5-year survival rate of completely resected NET exceeds 95%[1]. The classical partial pancreaticoduodenectomy (Kausch-Whipple operation) or pylorus-preserving pancreaticoduodenectomy (PPPD) are considered the treatments of choice for ampullary NETs > 2.0 cm in diameter. The mortality and morbidity rates for the two approaches are less than 5% and 15%, respectively[4,17]. Alternatively, in patients with multiple comorbidities or elderly individuals, conservative treatment or minimally invasive endoscopic papillectomy should be considered[5,7]. Local excision may be an option for the treatment of ampullary NET, if the tumor is small and there is no evidence of regional lymph node or distant metastasis. Compared to local surgical excision, endoscopic papillectomy may be less harmful to the patient and reduce the hospital stay duration, and complete resection is possible in selected patients. Similar to the management of an ampullary adenoma, endoscopic papillectomy may be a reasonable alter-

native to surgical resection[7]. When endoscopic papillectomy is decided upon, the differentiation of NET, tumor size, and lymph node metastasis should be considered. Endoscopic papillectomy may be a good alternative in highly differentiated tumours that do not infiltrate the muscularis, tumors < 2 cm in size, and with no distant metastasis. The prognosis is reported to be excellent, with an overall 5-year survival rate of 90%[15].

This presented case was diagnosed initially as an ampullary adenoma (low grade) by endoscopic biopsy during screening endoscopy. Following endoscopic papillectomy for removal of adenoma, the lesion was diagnosed as NET, grade 1 (< 2 mitoses/10 HPF and < 3% Ki67 index) accompanied by low-grade adenoma. Pathologically deep resection margin was not clear for the tumor; however, no evidence of local or distant metastasis was detected by repeated biopsies and radiologic examination during 24 mo of follow-up. Surgical resection was not performed due to successful complete endoscopic resection and imaging studies did not show lymph node or distant metastasis.

To the best of our knowledge, this is the first reported case of endoscopic papillectomy for ampullary NET accompanied by adenoma in the English-language literature. Some cases of ampullary small cell neuroendocrine carcinoma associated with or mixed with adenoma, adenocarcinoma and squamous cell carcinoma have been reported[18-20]. However, pancreaticoduodenectoy were performed in all of the above mentioned cases due to a carcinomatous change. Until now, there is a hypothesis that two different types of tumors arise from common progenitor cell and differentiate different[21,22]. In the case of low grade neuroendocrine tumor with adenoma, the low grade neuroendocrine tumor had good prognosis and no death and no lymph node metastasis were found[23]. However, since the study is with regard to lesion of duodenum and colorectum, further study in necessary to examine a prognosis of adenoma and low grade neuroendocrine tumor confined to ampulla of Vater.

In conclusion, low-grade ampullary adenoma is a common indication for endoscopic papillectomy. Indeed, well-differentiated ampullary NET may be also a good candidate for complete endoscopic resection in selected indications. Even though combined case such as a presented case, may be completely resected by endoscopic papillectomy. The clinical or pathological correlation between NET and adenoma is unclear. Further clinical follow-up is needed to confirm the long term clinical outcome or these combined cases.

COMMENTS

Case characteristics
A 53-year-old female patient visited hospital for dyspepsia of 3-mo duration.

Clinical diagnosis
Based on endoscopic finding and given pathology, clinically the authors diagnosed an unexposed ampullary adenoma.

Differential diagnosis
Unexposed adenocarcinoma arising from the ampulla of Vater.

Laboratory diagnosis
All labs were within normal limits.

Imaging diagnosis
A duodenoscopic image showed an enlarged major papilla with central umbilication and fine nodularity. Endoscopic ultrasonography (EUS) at the major ampulla revealed a 1.1 × 0.9-cm, slightly hypoechoic round ampullary mass confined to the submucosa.

Pathological diagnosis
Low grade neuroendocrine tumor combined with adenoma.

Treatment
Complete endoscopic papillectomy of tumors.

Related reports
Some cases of ampullary small cell neuroendocrine carcinoma associated with or mixed with adenoma, adenocarcinoma and squamous cell carcinoma have been reported, however these cases were managed by surgical resection.

Term explanation
Neuroendocrine tumor (NET) of the ampulla of Vater is formerly known as carcinoid tumors. NET is distinguish between neuroendocrine neoplasm, grade 1 (low grade); neuroendocrine neoplasm, grade 2 (intermediate grade); and neuroendocrine carcinoma, grade 3 (high grade).

Experiences and lessons
Well-differentiated ampullary NET may be a good candidate for complete endoscopic papillectomy in selected indications even though combined case such as an adenoma.

Peer-review
This case report is worthy because of the rarity of the NET in ampulla of Vater accompanied by tubular adenoma treated with endoscopic papillectomy.

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CASE REPORT

Pancreatic perivascular epithelioid cell tumor: A case report with clinicopathological features and a literature review

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Abstract

Perivascular epithelioid cell tumor (PEComa) of the pancreas is an unusual tumor deriving from mesenchyma. This paper described a case of pancreatic PEComa, which was initially suspected as neuroendocrine carcinoma by biopsy, and therefore surgical treatment was recommended due to undetermined diagnosis. Examination of the surgical specimen under a microscope showed that the tumor cell's morphology was epithelioid or spindle-shaped, and ranged in a nested pattern. Additionally, these cells had a large extent of acidophilic cytoplasm, no mitotic figures, and expressed HMB-45, melan-p, and smooth muscle actin immunohistochemically. Pathological examination indicated that PEComa originated from the pancreas, but symptoms related to tuberous sclerosis were absent. Since PEComa is extremely rare in the pancreas, it is likely to be ignored in differential diagnosis. In conclusion, our article highlighted the clinicopathological features of PEComa, and we conducted a literature review focusing on PEComa so as to deepen the understanding of this tumor type.

Key words: Pancreas; Perivascular epithelioid cell tumor; HMB-45; Immunohistochemistry; Clinicopathological feature

Core tip: Perivascular epithelioid cell tumor (PEComa) of the pancreas is an unusual tumor deriving from mesenchyma. Via describing a rare case of pancreatic PEComa, we highlight the clinicopathological features of PEComa and conduct a literature review focusing on this tumor type so as to deepen the understanding of the subject. We also reviewed the biological behavior, prognosis, and therapeutic strategy of PEComa.

INTRODUCTION

Perivascular epithelioid cell tumor (PEComa) is an extremely rare tumor derived from mesenchymal tissue, with characteristics of perivascular epithelioid cells (PEC) in histology and immunohistochemistry[1]. Microscopically, the morphology of PEC is of epithelial origin, containing a bright cytoplasm or fine grained eosinophilic shapes with positive PAS staining. Moreover, the nuclei of PEC are relatively small, and are round or oval shaped with small nucleolus; intranuclear inclusion bodies can be observed occasionally. PEC, distributing in the perivascular region in a radial pattern, is amylase intolerant, shows epithelioid features in nearby vessels, and becomes spindle-shaped when distant from vessels. The proportion of epithelioid cells and spindle cells is different depending on the patient. In immunohistochemistry staining, PEC usually expresses HMB45 and melan-A. PEC is featured with melanosome in ultrastructure, and is rich in glycogen and cytoplasmic filaments[2].

PEComas can be classified as angiomyolipomas, clear cell "sugar" tumors of the lung, lymphangiolei-omyomatoses (LAM), and other PEComas characterized by similar histological and immunohistochemical presentations[3]. Although PEComas are benign in most cases, Bonetti *et al*[4] reported four abdominopelvic sarcomas of PEC in young women, raising concern about malignant PEComas that result in regional tissue infiltration, multiple metastases, and even patient death[5-7]. As for PEComas of the pancreas, only two malignant cases with liver metastasis have been reported in the literature[8,9].

Herein, we report a 50-year-old female with benign PEComas of the pancreas that could not be definitely diagnosed preoperatively. A literature review on pancreatic PEComa, with special consideration of pathological diagnosis, was also performed.
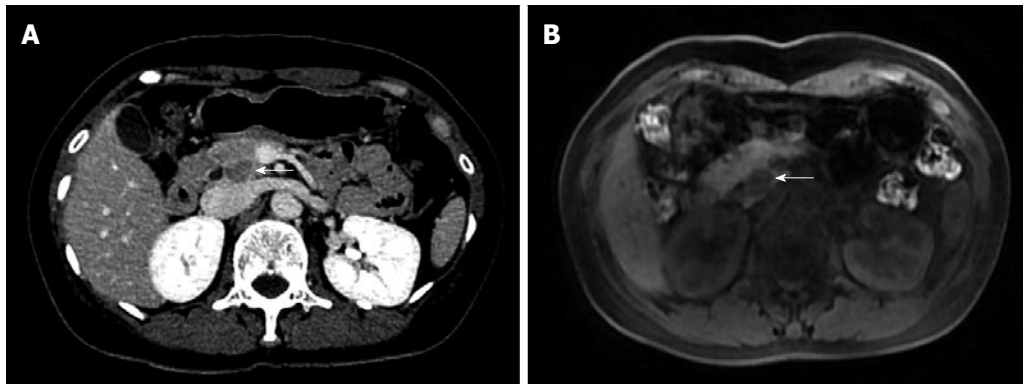
CASE REPORT

Clinical characteristics

A 50-year-old female patient was admitted to our hospital in November 2013 because of abdominal ultrasound (US) findings of a space-occupying lesion in the head of pancreas, which could not be clearly diagnosed. The patient was a lifelong nonsmoker who consumed no alcohol and had no history of family-inherited diseases. The patient denied any history of surgery or trauma. CT examination demonstrated no significant abnormality in the morphology or density of the pancreas, nor was there any pancreatic duct dilatation (Figure 1A). Moreover, the peripancreatic fat space was clearly demarcated and no retroperitoneal lymph nodes were enlarged. On the arterial phase, there was a relatively low density of nodules of approximately 1 cm × 1.4 cm in the uncus of the pancreas. On the portal venous and delayed phases, the nodules enhanced gradually and slightly, with a significantly lower density than the surrounding pancreatic tissue level, whereas the pathologically changed border was well-defined on the delayed phase. Magnetic resonance (MR) imaging (Figure 1B) showed that a round abnormal signal 1.7 cm × 1.4 cm in size was found in the head and uncus of the pancreas; T1WI showed a low signal clearly distinctive of normal pancreatic tissue surrounding a relatively high signal. In T2WI, the mass was difficult to distinguish from the surrounding pancreatic tissue due to the equal signal. Endoscopic ultrasound (EUS) showed a hypoechoic region 1.6 cm × 1.4 cm in size in the uncus of the pancreas. This region had clear boundaries and echo was not equal inside the low echo region. Pancreatic tail shape remained regular and the pancreatic duct was not dilated. There was no dilatation of the intrahepatic bile duct or enlargement of lymph nodes.
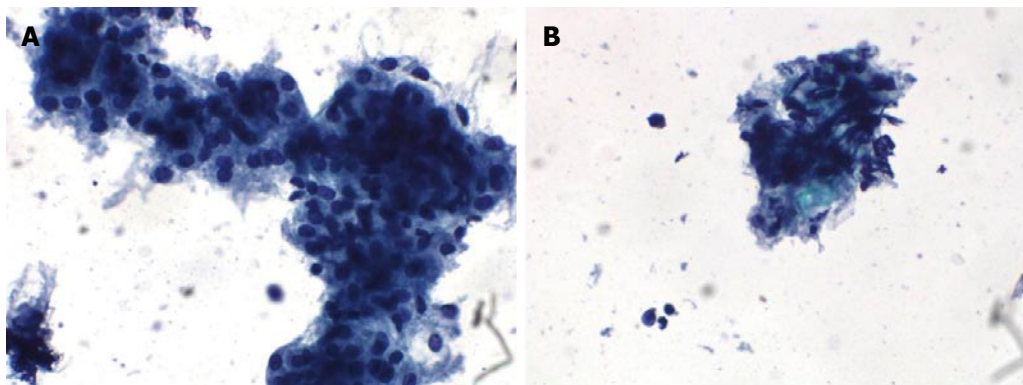
Methods and materials

The patient underwent endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) (ECHO-25G, Cook Company). The subsequent biopsy specimen was fixed in formalin, dehydrated by gradient ethanol, dewaxed by xylol, imbedded in paraffin, cut into sections for microscopic examination, and then stained with hematoxylin-eosin.

All biopsy and surgical specimens underwent immunohistochemical staining by the EliVision™ Plus method. Immunochemical staining was performed for all specimens by using the following markers: CD56, Cam5.2 (cytokeratin), neuron specific enolase (NSE), chromogranin A (CgA), alpha-1-antitrypsin (α-AT), CD34, CD56, S-100, TFE3, estrogen receptor (ER), progesterone receptor (PR), calponin, synaptophysin (Syn), Pax-8, β-catenin, CD117, melan-A, HMB-45, smooth muscle actin (SMA), epithelial membrane

Figure 1 Abdomen computed tomography-scan. A: CT delayed phase: a relatively low density of nodules (arrow) of approximately 1 cm × 1.4 cm in the uncus of the pancreas; B: Round abnormal signal (arrow) of 1.7 cm × 1.4 cm was found in the head and uncus of the pancreas, T1WI showed low signal, clearly contrasting with normal pancreatic tissue surrounding a relatively high signal.

Figure 2 Cytology results. A: Cells were irregular lumps distributed with medium nuclei sizes, arranged in a disorderly manner, and with crowded overlap; B: Tumor cell with abundant cytoplasm and unclear boundary. Messily arranged spindle cell nuclei can be seen in some cell clumps. Scattered single cells were found in the background.

Table 1 Characteristics of antibodies used in immunohisto-chemistry and staining conditions

Antibody	Clone	Source	Dilution
CD56	56C04	Maxim	1:100
CAM5.2	CAM5.2	Maxim	1:50
NSE	E27	Maxim	1:100
CgA	SP12	Maxim	1:100
α-AT	Polyclonal	DAKO	1:100
CD34	QBEnd/10	Maxim	1:100
P53	D0-7	Maxim	1:200
S-100	SP11	Thermo	1:100
TFE3	MRQ-37	Maxim	1:50
ER	1D5	Maxim	1:100
PR	1A6	Maxim	1:100
Calponin	CALP	Maxim	1:100
Syn	Polyclonal	Maxim	1:100
Pax-8	ZR-1	Gene Tech	1:100
β-catenin	CAT-5H10	Maxim	1:100
CD117	YR145, 2E4	Maxim	1:100
Melan-A	A103	Maxim	1:100
HMB-45	HMB45	Maxim	1:50
SMA	1A4	Gene Tech	1:100
EMA	E29	Maxim	1:100
Vimentin	V9	Maxim	1:200
Ki-67	MX006	Maxim	1:200

antigen (EMA), vimentin, p53, and Ki-67. All specimens were heated at 37 ℃ for 1 h and kept at 4 ℃ overnight. Staining intensity was recorded as positive (> 10% of plasma and nucleus stained) or negative. Ki-67 was evaluated by percentage. All information on antibodies used is summarized in Table 1.
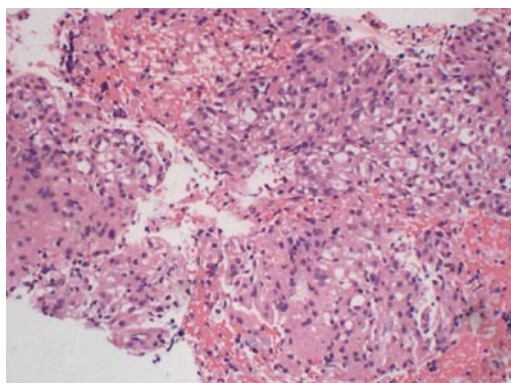
Results
Biopsy specimen: Spindle-shaped nucleus can be found in some cell lumps with sporadically distributed cells in the background, suggesting that they were malignant cells derived from unidentified sources. In DNA ploidy analysis, a few DNA ploidy-abnormal cells were found.

Cytological results: Cytology smear showed that cells were distributed in irregular lumps, with nuclear sizes varying slightly and arranged mussily. Enriched plasma and vaguely defined boundary were identified in the cells (Figure 2).

Tumor cells were epithelioid or spindle-shaped with large size and nested distribution (Figure 3). Tumor cells contained a bright cytoplasm or fine grained eosinophilic

Figure 3 Biopsy specimen. Hematoxylin-eosin staining shows the epithelial tumor cells with bright or slightly eosinophilic granules with nested distribution.

Figure 4 Gross examination. Tumor 2 cm × 2 cm in size in pancreatic head with clear boundaries. Cross-section of gray and solid soft texture. Metastatic lesions were not found.
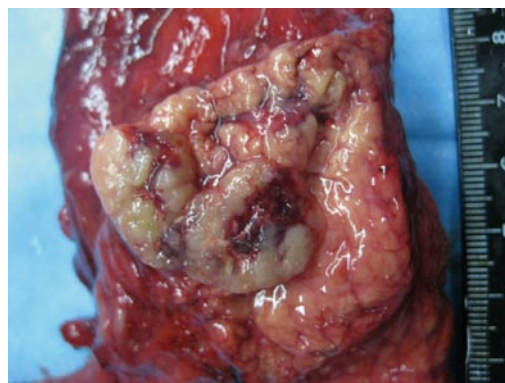
shapes with suspicion of clear cell carcinoma. Therefore, gastrointestinal stromal tumor, neuro-endocrine tumor, acinic cell carcinoma, solid pseudo-papillary tumor, and metastatic clear-cell carcinoma should be excluded. The immunohistochemistry results were as follows: CD56 (+), CAM5.2 (-), P53 (-), NSE (-), vimentin (-), Ki67 (2%), CgA (-), α1-ACT (-), Syn (-), Pax-8 (-), β-catenin (positive in plasma), CD117 (-). Since PEComa of the pancreas had not been diagnosed in our hospital, this tumor was not considered to be PEComa preoperatively. Based on cytological and immunohistochemical results, neuro-endocrine tumor was suspected, but acinic cell carcinoma and solid pseudo-papillary tumor were not excluded, leading to operation being suggested.

Surgical specimen: A mass 2 cm × 2 cm in size in the head of the pancreas was discovered during operation and was of medium texture with a complete capsule. The border was clear between the mass and adjacent tissues, and metastatic lesions were not found.

Gross examination (Figure 4): the pancreas, duodenum, and gall bladder were resected; the size of the pancreas head was 8 cm × 4 cm × 3 cm, with a mass 2 cm × 1 cm × 1 cm in size that was 4 cm away from the surgical margin. The transection of the mass was grayish with a solid soft texture.

Microscopic examination: the boundary between tumor and adjacent tissue was clear. Tumor cells were arranged in nests, with some tumor cells growing around arteries. Mounts of vessel were in mesenchyma with hyaline degeneration (Figure 5B). The morphology of tumor cells was epithelioid or spindle-shaped (Figure 5C and D) and ranging in a nested pattern. These cells had plenty of acidophil cytoplasm and no mitotic figures. There was no fat tissue found in the tumor. Lymphatic invasion was not observed in this patient.

Immunohistochemistry (Figure 6): melan-A (+), HMB45 (+), SMA (+) CD56 (+), Ki67 (1%), CAM5.2 (-), P53 (-), NSE (-), vimentin (-), CgA (-), α-AT (-), Syn (-), Pax-8 (-), β-catenin (positive in plasma), CD117 (-), CD34 (positive in vessel), S-100 (-), TFE3

(-), ER (-), PR (-), calponin (-), and MSA (-).

Diagnosis: PEComa in the head of the pancreas.

Follow-up
No recurrence or distant metastases were observed during follow-up of 14 mo. However, Nagata *et al*[8] reported that benign PEComa could recur after surgery, meaning that it is necessary to follow-up and re-examine patients.
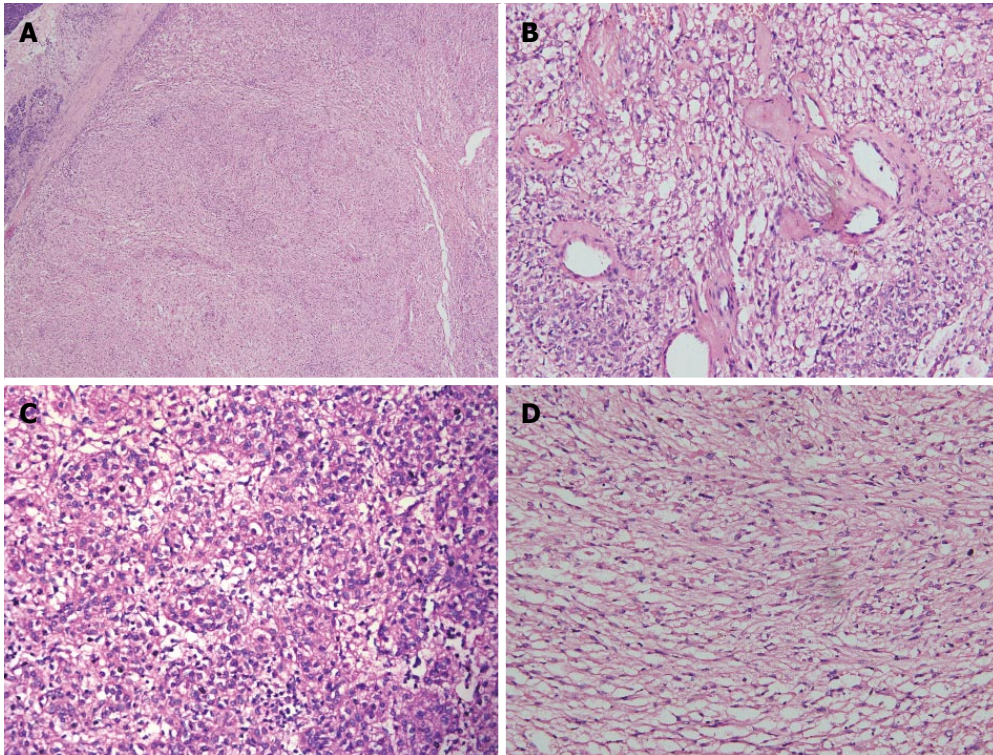
DISCUSSION

PEComa of the pancreas is extremely rare, with only twelve previous cases[2-5,8-17] being reported during the last decade. Combining these previous cases with ours, we found that the patients had a mean age of 52 years (range: 31 to 62 years) and were mostly female (11 females and 2 males). The morbidity of PEComa in females was significantly higher than that in males, suggesting that one risk factor of PEComa is related to sex hormones. Additionally, some studies revealed that progesterone receptors (PR) were expressed in PEComas immunohistochemically, especially in LAM and renal AKL[18]. However, in these thirteen cases, PR was negatively expressed in five PEComas cases and only one PEComa partially expressed ER[15]. Tuberous sclerosis (TSC) containing TSC1 or TSC2 gene deletion can be seen in some PEComas, especially in renal AKL. Located on chromatosome 9q and 16p respectively, TSC1 and TSC2 play a pivotal role in the Rheb-mTOR-P7OS6K pathway[1]. Nevertheless, all the PEComas of the pancreas presented without TSC.

Abdominal pain is the main initial symptom in pancreatic PEComa, although a few asymptomatic cases have been found during health examinations or follow-up examinations for other disease. In PEComa sites, the head of pancreas accounted for six cases, the body for five cases, and the uncus for two. Tumor size varied from 15 mm to 100 mm, with an average of 23 mm. The tumor ruptured in one case.

In pathological morphology, akin to previous

Figure 5 Microscopic examination. A: Microscopy showing clear boundaries between the tumor and adjacent pancreatic tissue; B: Mounts of vessel were in mesenchyma, with hyaline degeneration present; C: Epithelioid tumor cell; D: Spindle-shaped tumor cell with bright or slightly eosinophilic granule. A, B, C and D: Hematoxylin-eosin staining.

Figure 6 Immunohistochemistry. A: Tumor cells expressed HMB-45; B: Tumor cells are immunophenotypically positive for the melanocytic marker melan-A; C: Tumor cells are immunophenotypically positive for the smooth muscle marker SMA. Original magnification × 200. A, B and C: Immunostaining.

cases, the PEComa reported in our case was a solid homogeneous nodule with a mainly clear boundary and that only partially infiltrated to adjacent tissue. Tumor cells that manifested during histological results were mainly made up of clear cells or eosinophilic cells with nested, fascial, or laminar distributions. Tumor cells were epithelioid or spindle-shaped with plenty of glycogen. Nucleolus atypia and small nucleolus can be found in the tumor cells. Mounts of vessel were in mesenchyma, with some tumor cells growing around arteries. Adipose tissue, which would make the diagnosis easier, is rarely presented in PEComa. In the 12 previously reported cases of pancreatic PEComa, adipose tissue was found in 2 cases[8,11]. In one of the two cases, liver metastasis occurred 27 mo

after surgery[8]. Therefore, it seems that the biological behavior of the tumor is not related to the existence of fat tissue. Immunostaining shows that PEComa generally has both smooth muscle cells and evidence of melanoma. Folpe's[19] study indicated that HMB-45 is the most sensitive marker for melanoma cells (96%), followed by melan-A (72%) and microphthalmia-associated transcription factor (MiTF) (50%). In their study, all cases expressed at least one marker of melanoma cells and 80% of cases expressed smooth muscle actin, especially in epithelioid cells. In the reported cases of PEComa of the pancreas, only one of the 13 cases did not express α-SMA[9], which was also the only case of malignant PEComa in the pancreas with epithelioid tumor cells. Therefore, we proposed

that the lack of smooth muscle expression may serve as a malignant indicator of PEComa. All three indicators previously reported in pancreatic PEComa expression are as follows: HMB-45 (13/13), melan-A (8/9), and α-SMA (12/13). Meanwhile, MiTF and CD63 were expressed in tumor cells. CD117 was positive in a few cases. PEComa also expressed TFE3 and cyclin D1, expressed desmin to a lesser extent, and did not generally express S-100 and CK.

To date, the biological behavior and histologic origin of pancreatic PEComas are unknown. Although the pervasive concept is that PEComas are usually benign, increasing reports indicate that PEComas may have malignant potential, despite there being no consensus in how to evaluate PEComa. In 2005, Folpe *et al*[19] suggested that a tumor which meets at least two points of the following criteria should be considered a malignant PEComa based on previous reports about PEComa: (1) tumor diameter >50 mm; (2) tumors with an invasive growth pattern; (3) tumors that possess advanced nucleus and cell richness; (4) mitotic count higher than 1/50 HPF; and (5) necrosis and vascular infiltration. However, it is difficult to confirm the accuracy of Folpe's criteria in distinguishing malignant PEComa from those that are benign, owing to the rare cases of PEComa[5]. In the 13 cases, only one[9] can be regarded as malignant PEComa according to Folpe's criteria, in which liver metastasis occurred 6 mo after surgery. However, this patient had a family history of breast cancer and *BRCA2* gene mutation. The patient undertook radiotherapy and chemotherapy because of breast cancer 10 years prior. Therefore, whether malignant PEComa resulted from *BRCA2* gene mutation or chemotherapy and radiotherapy was unknown. Additionally, invasive PEComa cannot be diagnosed as malignant, whereas multiple liver metastases were found 27 mo after surgery in this patient. More cases need to be analyzed in order to evaluate the recurrent risk of PEComa and develop an effective therapy. If histology results show some malignant features, such as mitosis index, tumor cell pleomorphism, and invasive growth, close patient follow-up is then required.

In diagnosis and differential diagnosis, PEComas, especially the type that mainly present with epithelioid cells or spindle-shaped cells, should be distinguished from pancreatic clear cell neuroendocrine tumor, solid pseudopapillary tumor, metastatic renal clear carcinoma, metastatic gastrointestinal stromal, metastatic melanoma, or soft tissue clear cell sarcoma[13]. (1) Clear cellular neuroendocrine carcinoma: PEComa and clear cellular neuroendocrine carcinoma are similar in morphology and feature plenty of plasma and lipid droplets[20]. They can be distinguished by immunohistochemistry. Chromogranin and synaptophysin are positive in clear cellular neuroendocrine carcinoma, but negative in PEComa; (2) solid pseudopapillary tumor: tumor cells are eosinophilic

and neutrophilic, and papillary structure can be seen under a microscope. Sometimes hemorrhagic necroses are found inside the tumor. Immunohistochemically, CK, CD56, synaptophysin, and β-catenin are positive, while HMB-45 is negative[21]; (3) clear cell carcinoma: cytokeratin is widely expressed in clear cell carcinoma, while melanin is expressed in PEComa; (4) gastrointestinal stromal tumor (GIST): epithelioid cells mixed with spindle-shaped cells in both PEComa and gastrointestinal stromal tumor, but a great deal of vasoganglion and clear and eosinophilic tumor cells exist in PEComa, which lacks fibroblast-like cells. PEComas mostly do not express CD117, but in some cases do express CD117 during immunostaining. Molecular detection shows no c-kit gene mutation. CD34 is positive in GIST, while PEComa does not express CD34[21]; (5) metastatic melanoma: owing to positive expression of melanin in PEComa, differential diagnosis is necessary to distinguish PEComa from metastatic melanoma. In most cases, it is possible to differentiate PEComa with myogenic markers from metastatic melanoma with s-100; and (6) alveolar soft part sarcoma: because of its organ-like clear cell structure, it is also important to differentiate PEComa from alveolar soft part sarcoma. Alveolar soft part sarcoma is a malignant tumor which often contains high levels of nuclear atypia and clear nucleoli. Vascular invasion is common in alveolar soft part sarcoma, which is only found in malignant PEComa. Immunohistochemically, melanoma-derived markers and myogenic markers are not expressed in alveolar soft part sarcoma.

Currently, EUS-FNA serves as one of the most important preoperative diagnosis methods, and is widely used in clinical diagnosis. In the aforementioned 13 pancreatic PEComa cases, 9 performed EUS-FNA examination prior to operation. However, 5 cases could not be diagnosed clearly. Therefore, when a cell arrangement and shape resembling that of neuroendocrine tumor is visible under microscope, we must take into account the possibility of PEComa.

In conclusion, as an unusual tumor deriving from mesenchyma, PEComa of the pancreas is always benign. Cases concomitant with TSC or other syndromes have not yet been reported. Complete surgical resection is the main treatment for PEComa, although the necessity of resection and the timing of surgical treatment are relatively limited. Furthermore, for cases with huge inoperable tumors and multiple metastases, effective treatment is lacking, as the effect of traditional radiotherapy and chemotherapy is poor. Wagner *et al*[22] reported three cases of malignant PEComa that reacted to mTOR inhibitor sirolimus radiological examination, indicating that mTOR inhibitor may serve as a candidate for future targeted chemotherapy drugs, although further study is needed. For benign PEComa, it is recommended that regular patient follow-up be performed, while aggressive therapy is not recommended.

COMMENTS

Case characteristics

A 50-year-old female patient admitted to hospital because of abdominal ultrasound findings of a space-occupying lesion in the head of pancreas, which could not be clearly diagnosed.

Clinical diagnosis

Upon physical examination, the patient had no clinical abnormalities and was diagnosed with a pancreas head mass according to the previous diagnosis.

Differential diagnosis

Pancreatic clear cell neuroendocrine tumor, solid pseudopapillary tumor, metastatic renal clear carcinoma, metastatic gastrointestinal stromal, metastatic melanoma, or soft tissue clear cell sarcoma.

Laboratory diagnosis

Laboratory tests showed no abnormal values.

Imaging diagnosis

Contrast-enhanced computed tomography scan, magnetic resonance imaging, and endoscopic ultrasound all revealed a space-occupying lesion in the uncus of the pancreas.

Pathological diagnosis

By cytological, histological, and immunohistochemical examination, the pathological diagnosis was of perivascular epithelioid cell tumor (PEComa), 2 cm × 2 cm × 1 cm, in the head of the pancreas.

Experiences and lessons

PEComa originating from the pancreas is very rare and we reveal the clinicopathological features of it. Caution is needed in order to differentiate this entity from other pancreatic tumors, especially pancreatic clear cell neuroendocrine tumor, solid pseudopapillary tumor, metastatic renal clear carcinoma, metastatic gastrointestinal stromal, metastatic melanoma, and soft tissue clear cell sarcoma.

Peer-review

This article highlights the clinical characteristics of PEComa of the pancreas and offered an excellent methodology to diagnose the disease. The information in this article is worthwhile to the reader.

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