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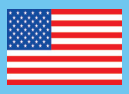

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EDITORIAL

Signaling pathway/molecular targets and new targeted agents under development in hepatocellular carcinoma

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Abstract

Advances in molecular cell biology over the last decade have clarified the mechanisms involved in cancer growth, invasion, and metastasis, and enabled the development of molecular-targeted agents. To date, sorafenib is the only molecular-targeted agent whose survival benefit has been demonstrated in two global phase Ⅲ randomized controlled trials, and has been approved worldwide. Phase Ⅲ clinical trials of other molecular targeted agents comparing them with sorafenib as first-line treatment agents are ongoing. Those agents target the vascular endothelial growth factor, platelet-derived growth factor receptors, as well as target the epidermal growth factor receptor, insulin-like growth factor receptor and mammalian target of rapamycin, in addition to other molecules targeting other components of the signal transduction pathways. In addition, the combination of sorafenib with standard treatment, such as resection, ablation, transarterial embolization, and hepatic arterial infusion chemotherapy are ongoing. This review outlines the main pathways involved in the development and progression of hepatocellular carcinoma and the new agents that target these pathways. Finally, the current statuses of clinical trials of new agents or combination therapy with sorafenib and standard treatment will also be discussed.

Key words: Hepatocellular carcinoma; Molecular targeted agent; Sorafenib; Signaling pathway; Molecular target

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INTRODUCTION

Advances in molecular cell biology over the last decade have clarified the mechanisms involved in cancer growth, invasion and metastasis, and enabled the development of molecular-targeted agents, best represented by trastuzumab for breast cancer, imatinib and rituximab for hematopoietic tumors, and gefitinib and erlotinib for lung cancer. These molecular-targeted agents are broadly classified into two categories: drugs targeting cancer cell-specific molecules, and nonspecific molecular-targeted drugs for molecular biological abnormalities induced in the host stroma or blood vessels by the presence of cancer. Examples of the former approach include trastuzumab, which targets human epidermal growth factor receptor 2 (HER2), the expression of which is a poor prognostic factor for breast cancer; rituximab, which is used to treat B-cell lymphoma, targets CD20 expressed on normal and neoplastic mature B cells; while imatinib binds to the ATP-binding site of Bcr-abl, a protein that causes chronic myelogenous leukemia. However, no critical target molecules responsible for treatment response have been identified in hepatocellular carcinoma (HCC).

In recent years, clinical trials have been conducted for many agents that act on growth factor receptors, such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR), and intracellular signaling pathways. In addition, multi-kinase inhibitors, including sorafenib, have emerged and been evaluated. Clinical trials are ongoing to compare drugs with the same mechanism of action and to test the combined efficacy and relative merits of these drugs with existing drugs for many cancers. Since the main treatment option for metastatic, advanced stage cancers, such as breast and colorectal cancer, is systemic chemotherapy, clinical trials are ongoing to investigate how to combine molecular-targeted agents with standard therapies based on the results of long-term, large-scale clinical trials, and to identify which molecular-targeted agents should be used as initial or second-line therapy.

However, for HCC, background liver damage limits the indication for systemic chemotherapy and no anticancer drugs were found to be effective in large-scale randomized controlled trials except sorafenib. Now that the usefulness of sorafenib has been demonstrated in two large scale randomized clinical trials, the development of new drugs that are effective for poor-prognosis advanced HCC, who are resistant to a standard of care agent, sorafenib.

In this review, the clinical impact of sorafenib and ongoing trials of new agents or combination trials with sorafenib will be described.

SIGNALING PATHWAYS AND MOLECULAR-TARGETED AGENTS IN HCC

As in other cancers, the molecular mechanisms involved in the development and progression of HCC are complex. After hepatitis B virus and hepatitis C virus infection and alcohol or aflatoxin B1 exposure, genetic and epigenetic changes occur, including oncogene activation and tumor-suppressor gene inactivation due to inflammation-induced increase in hepatocyte turnover and oxidative stress-induced DNA damage. Through apoptosis and cell proliferation, these changes lead to the multistep development and progression of a hyperplastic to dysplastic nodule, early HCC, and advanced HCC. A number of studies have reported changes in gene expression, chromosomal amplification, mutations, deletions and copy number alterations (gain/loss), somatic mutations, CpG hypermethylation, and DNA hypomethylation, as well as molecular abnormalities, which can constitute therapeutic targets[1-5].

The binding of growth factors to their receptor proteins activates protein-phosphorylating enzymes, thus activating a cascade of proliferative signaling pathways to transmit proliferative signals into the nucleus. Growth factors, such as EGF, transforming growth factor (TGF)-α/-β, insulin-like growth factor (IGF), and VEGF, also function in liver regeneration after injury, while fibroblast growth factor (FGF) and the platelet-derived growth factor (PDGF) family are involved in liver fibrosis and HCC growth[6-8]. The receptors for these growth factors are broadly classified into G protein-coupled receptors and protein kinases. On ligand binding, these receptors activate their downstream intracellular molecules in a cascade fashion. Many of the growth factor receptors and oncogenes have tyrosine kinase activity, and the tyrosine kinases are classified into transmembrane receptor tyrosine kinases, such as the EGFR and VEGFR, and cytoplasmic non-receptor tyrosine kinases, such as Abl and Src. On the other hand, Raf, mitogen-activated protein kinase (MAPK)/extracellular signaling-regulated kinase (ERK) kinase (MEK), and mammalian target of rapamycin (mTOR) are serine/threonine kinases.

In general, the MAPK, phosphoinositide 3-kinase (PI3K)/Akt/mTOR, c-MET, IGF, Wnt-β-catenin and Hedgehog signaling pathways, and the VEGFR and PDGF receptor (PDGFR) signaling cascades show altered activity in HCC, and agents targeting these pathways are under development (Figures 1-3; Table 1)[9-12]. Many molecular-targeted agents are now under development and the target signaling pathways and growth factors are outlined below.

MAPK pathway (Ras/Raf/MEK/ERK)

The MAPK intracellular signaling pathway, which is mainly involved in cell growth and survival, and regulates cell differentiation, is upregulated in cancer cells. Therefore, this pathway has been extensively studied as a therapeutic target. The MAPK pathway is a common downstream pathway for the EGFR, PDGFR and VEGFR, and is universally used for signal transduction downstream of cytokine receptors, integrin complexes, and G-protein receptors to Ras. The MAPK pathway also plays an important role in HCC, in that its activation is reportedly involved in HCC growth and survival. The downstream ERK is activated by two upstream protein kinases, which are coupled to growth factor receptors by Ras proteins. Ras, which is activated by ligand binding, activates Raf serine/threonine kinases and MEK (MAP kinase/ERK kinase), while MEK phosphorylates and activates ERK, which phosphorylates proteins involved in cell growth, apoptosis resistance, extracellular matrix production and angiogenesis[13-15].

Raf and Ras inhibitors: Raf and Ras are proto-oncogenes. In particular, K-Ras mutations are commonly observed in many cancers, including pancreatic and colorectal cancers. One study reported that 30% of HCCs have Ras mutations[16]. To our knowledge, no agents targeting Ras are planned to enter clinical trials at the present. However, because the binding of Ras protein to the cell membrane and its functional activation require farnesylation, several farnesyltransferase inhibitors are being tested for Ras-related tumors. In addition, vaccine therapy for mutant Ras proteins is currently being tested for solid cancers, including HCC.

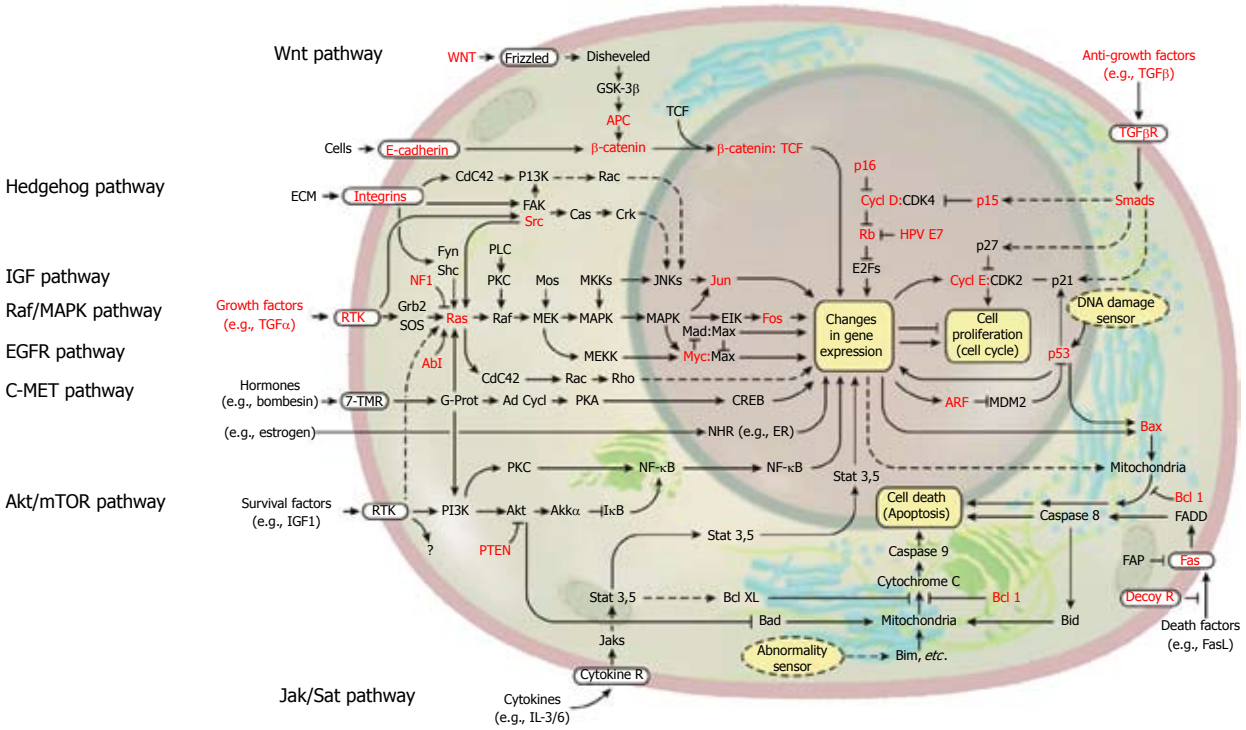

Figure 1 Signal transduction in solid cancer cells including hepatocellular carcinoma. Some of the genes known to be functionally altered are highlighted in red. These signaling pathways, including growth factor pathway, Wnt pathway, Hedgehog pathway, Akt/mammalian target of rapamycin (mTOR) pathway, and Jak/Stat pathway, can be a molecular targets for treatment of hepatocellular carcinoma. (Cited and modified from Hanahan *et al*[10] with permission.) EGFR: Epidermal growth factor receptor; TGF: Transforming growth factor; IGF: Insulin-like growth factor; MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositide 3-kinase; ERK: Extracellular signaling-regulated kinase; NF-κB: Nuclear factor-kappa B; IL: Interleukin.

Table 1 Molecular targeted agents for hepatocellular carcinoma: Targets and development status

Agents	Targets (angiogenesis)			Targets (proliferation)				Positioning	Development status
	VEGFR	PDGFR	FGF	EGFR	Raf	mTOR	TRAIL-R2	DR5	
Sorafenib	•	•			•			1st line	Approved
Sunitinib	•	•						1st line	PⅢ terminated
E7080	•	•	•					1st/2nd line	PII ongoing
Tigatuzumab (CS1008)							•	1st line (sorafenib combination)	P Ⅰ / Ⅱ ongoing
Linifanib (ABT-869)	•	•						1st line	PⅢ ongoing
Brivanib		•			•			1st line, 2nd line, TACE adjuvant	PⅢ ongoing
TSU-68		•	•					TACE combination	PⅢ ongoing
Ramucirumab		•						2nd line	PⅢ ongoing
Everolimus (RAD001)						•		2nd line	PⅢ ongoing
Axitinib		•	•					2nd line	PⅢ ongoing

VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; FGF: Fibroblast growth factor; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin; TACE: Transarterial chemoembolization.

The Raf family consists of three isoforms, A-Raf, B-Raf and C-Raf/Raf-1. Genetic abnormalities, such as point mutations and gene rearrangements, have been reported in various cancers[17]; however, in HCC, *ras/raf* mutations are rare, and no *k-ras* or *b-raf* mutations have been detected[18]. On the other hand, wild-type Raf-1 was reported to be hyperactivated in many cancers, including HCC[19-21]. Sorafenib inhibits Raf, and has multiple characteristics in that it exhibits strong inhibitory activity against Raf-1 (C-Raf) kinase, B-Raf (wild-type B-Raf and mutant V600E B-Raf) serine/threonine kinase, the pro-angiogenic receptor tyrosine kinases VEGFR, PDGFR and FGFR1, and tyrosine kinases, such as c-kit, Flt-3 and RET, which are involved in tumor progression and overall prognosis[22].

MEK: The MEK family consists of MEK1 and MEK2 proteins, which specifically phosphorylate tyrosine and threonine residues, and phosphorylates downstream Erk1 and Erk2[23].

In an immunohistochemical study, MEK1/2 overexpression, ERK1/2 overexpression, and ERK1/2 phosphorylation were observed in 100% (46/46), 91% (42/46), and 69% (32/46) of HCCs, respectively. In ad-

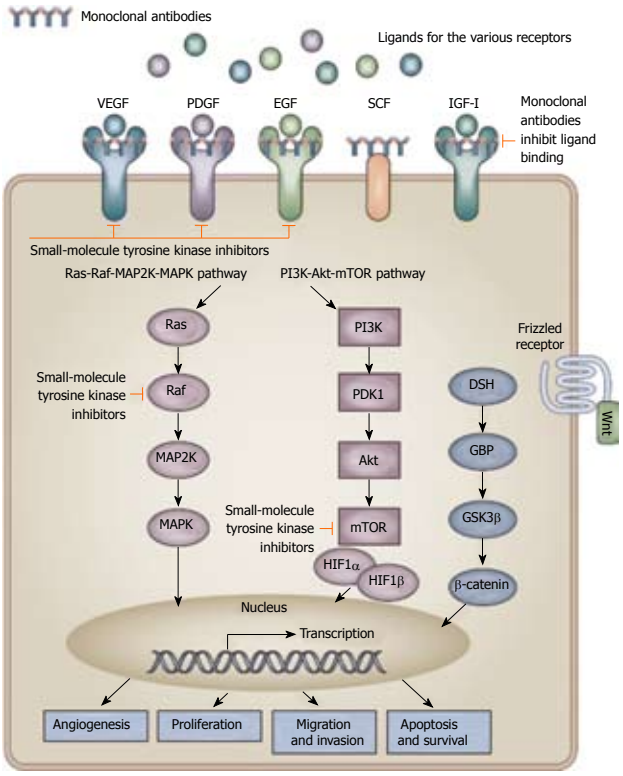

Figure 2 Signaling pathways and potential drug targets to inhibit hepatocarcinogenesis. Activation of receptor tyrosine kinases by their ligands activates downstream signaling pathways with effects on angiogenesis, proliferation, migration and invasion, and apoptosis or survival of cells. Monoclonal antibodies inhibit ligand binding to the receptor and small-molecule tyrosine kinase inhibitors inhibit propagation of the downstream signal. (Cited from Spangenberg *et al*[11] with permission.) IGF: Insulin-like growth factor; MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositide 3-kinase; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; mTOR: Mammalian target of rapamycin; HIF: Hypoxia-inducible factor; SCF: Stem cell factor.

dition, the *in vitro* treatment of HepG2 and Hep3B cells with MEK1/2 inhibitors inhibited cell growth and up-regulated apoptosis[24].

The MEK inhibitors CI-1040, PD0325901, AZD6244, and RDEA119/BAY869766 have been tested in several cancers, including solid tumors such as HCC. Recently, the results of Phase I of AS703026 and E6201 studies against solid tumors were reported in ASCO2010. A phase Ⅱ study of AZD6244 (selumetinib, ARRY-142866) and a phase Ⅰ / Ⅱ study of RDEA119/BAY869766 in combination with sorafenib are being conducted.

PI3K/Akt/mTOR pathway

The PI3K/Akt/mTORpathway also plays an important role in cell growth, survival regulation, metabolism, and anti-apoptosis. The membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is phosphorylated by PI3K into phosphatidylinositol 3,4,5-triphosphate (PIP_3), which binds to and activates the serine/threonine kinase Akt. The tumor suppressor gene product phosphatase and tensin homolog (PTEN) deleted on chromosome is antagonistic to PI3K activity. PTEN is a lipid phosphatase that dephosphorylates inositol phosphates, such as PIP_3. The inactivation of PTEN through gene deletion increases PIP_3 levels, and activates Akt, which inhibits apoptosis, leading to the development of tumors. The serine/threonine kinase mTOR is an important mediator in the PI3K/Akt pathway, which binds intracellularly to a protein called raptor or rictor, and exists as two different complexes, complex 1 and 2 (mTORC1 and mTORC2). mTORC2 (mTOR-rictor) activates Akt, while mTORC1 (mTOR-raptor) is activated downstream of Akt; thus, both molecules regulate protein synthesis (Figures 4 and 5)[25].

Inhibiting mTOR with molecules, such as RAD001, generates additive effects that accompany upstream and downstream target inhibition. Alternatively, upstream receptor inhibition is compensated for by inhibiting the downstream pathway, even if some resistance develops against receptor inhibition regardless of initial or acquired resistance. Therefore, RAD001 is a potential targeted agent for HCC.

Besides the finding that mTOR plays a key role in cell biology, it was also demonstrated that mTOR and S6K are overexpressed in 15%-41% of HCCs. mTOR inhibitors also have antitumor effects in various HCC cell lines and animal models[26-29]. Activation of mTOR is correlated with the development of HCC and recurrence after the excision of early HCC. Regulating this specific intracellular pathway (Ras-Raf pathway) with RAD001 is potentially more effective in suppressing sorafenib-resistant tumors.

A study of 528 HCC samples showed that the expression of pAkt, PTEN, p27 and S6 ribosomal protein (pS6) was a poor prognostic factor for survival[30]. A tissue microarray analysis of HCC samples revealed that the loss of PTEN and overexpression of pAkt and p-mTOR were correlated with tumor grade, intrahepatic metastasis, vascular invasion, TNM stage, Ki-67 labeling index, and matrix metalloproteinase (MMP)-2 and -9 upregulation. Meanwhile, PTEN mRNA expression in the cancerous tissue was downregulated compared with that in the non-cancerous tissue. The levels of PTEN, MMP-2, and MMP-9 mRNA expression were correlated with tumor stage and metastasis, and the levels of PTEN and MMP-9 mRNA expression were inversely correlated[31]. In an extensive analysis of 314 HCC samples in terms of mutation analysis, DNA copy number changes, mRNA levels and immunostaining, Villanueva *et al*[32] found that activation of the IGF pathway, upregulation of EGF, dysregulation of PTEN, and aberrant mTOR signaling were present in half of the samples, and that inhibiting mTOR activity with everolimus was effective in improved survival and suppression of recurrence.

The PI3K inhibitor RG7321 and the Akt inhibitor perifosine target the PI3K/Akt/mTOR pathway and are in early stages of clinical development, while the mTOR inhibitors everolimus (RAD001), sirolimus (Rapamune), and temsirolimus (CCI-779) are at more advanced stages of development. Everolimus is used to treat sorafenib-

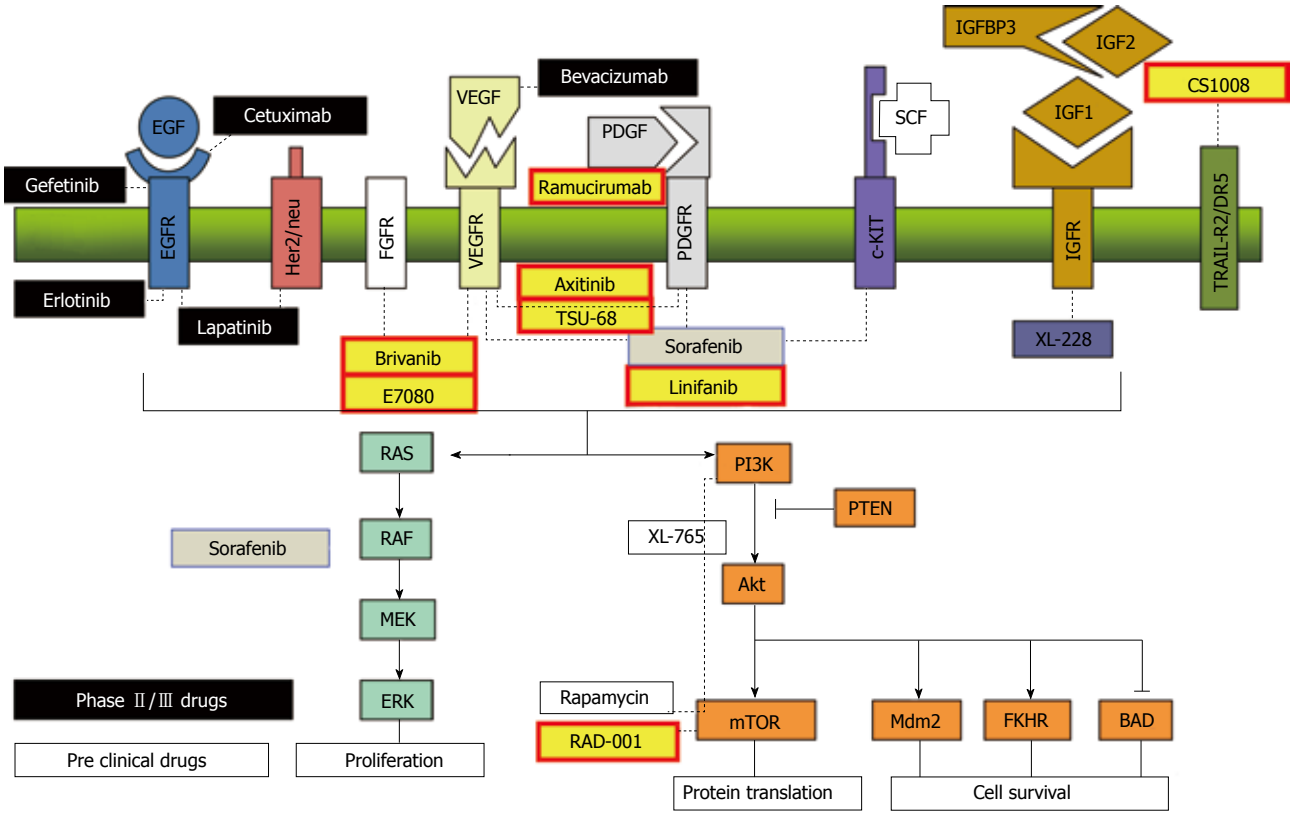

Figure 3 Target molecular and targeted agents under development. Monoclonal antibodies (VEGF: bevacizumab, EGFR: cetuxinab), tyrosine kinase inhibitors (VEGFR: sorafenib, brivanib, linifanib, axitinib, TSU-68; FGFR: E7080, brivanib), EGFR: erlotinib, lapatinib), serine/threonine kinase inhibitors (Raf: sorafenib, mTOR: rapamycin and everolimus) and agonistic ligand of TRAIL-R2/DR5 (CS1008). (Cited and modified from Villanueva *et al*[12] with permission.) IGF: Insulin-like growth factor; PI3K: Phosphoinositide 3-kinase; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; SCF: Stem cell factor; FGFR: Fibroblast growth factor receptor.

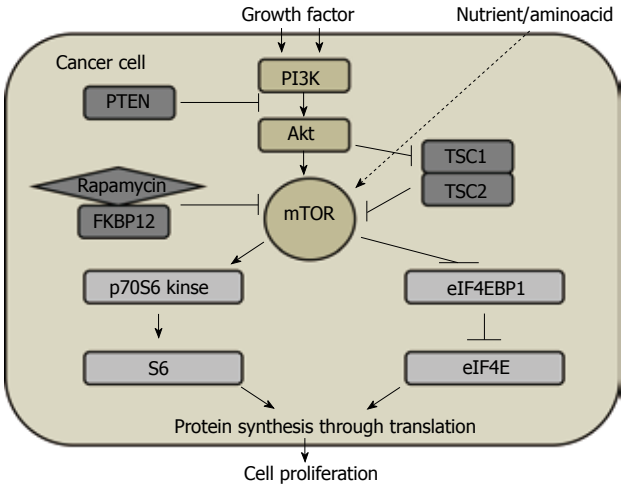

Figure 4 Phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling pathway in cell proliferation in solid cancer. PI3K: Phosphoinositide 3-kinase; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; TSC: Tuberous sclerosis; FKBP12: FK506-binding protein 12.

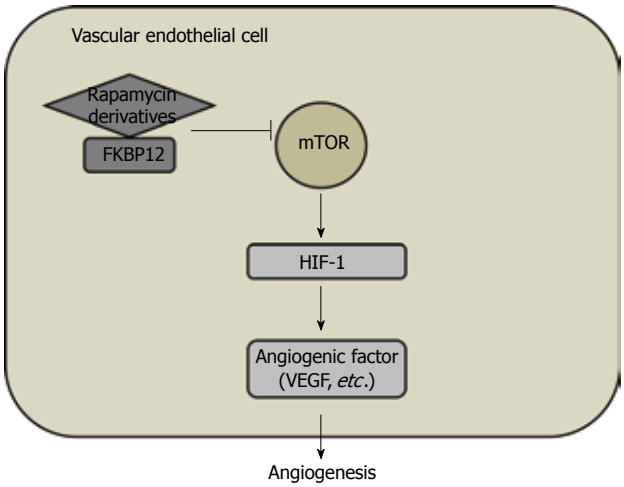

Figure 5 Mammalian target of rapamycin/hypoxia-inducible factor-1/vascular endothelial growth factor signaling pathway in angiogenesis in solid cancer. HIF: Hypoxia-inducible factor; VEGF: Vascular endothelial growth factor; mTOR: Mammalian target of rapamycin; FKBP12: FK506-binding protein 12.

intolerant patients, or for patients showing disease progression after sorafenib administration. A phase Ⅲ study to compare everolimus and a placebo (EVOLVE-1: Advanced Hepatocellular Carcinoma after Disease Progression or Intolerance to Sorafenib EverOlimus for Liver cancer Evaluation) and a phase Ⅰ/randomized phase Ⅱ study (sorafenib + everolimus *vs* sorafenib alone) to test the efficacy and tolerance of sorafenib in combination with everolimus are underway. Since mTOR inhibitors exhibit cytostatic and antiangiogenic effects, they are

expected to be effective in combination with other angiogenesis inhibitors, such as bevacizumab, and may be appropriate for administration after transarterial chemoembolization (TACE). Furthermore, since the mTOR pathway is stimulated by factors such as EGFR, PDGFR, and TGFα, and is closely related to other signaling pathways, including the Ras/Raf/MEK/ERK pathway, they are likely to show promising efficacy when used in combination with other growth factor inhibitors[33].

VEGF/VEGFR, PDGFR, FGFR

Angiogenesis is an important event not only for HCC, but also for cancer growth and metastasis, and occurs because of complex alterations involving promoting factors such as VEGF, angiopoietin, and FGF, and inhibitory factors including thrombospondin and angiostatin, as well as the surrounding tissue. The VEGF family consists of VEGF-A, -B, -C, -D and –E, and placental growth factor (PIGF). The VEGFR family comprises VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), and VEGFR-3 (flt-4). VEGF-A binds to VEGFR-1 and -2 and is involved in angiogenesis and the maintenance of mature blood vessels, while VEGF-C and -D mainly bind to VEGFR-3, are involved in lymphangiogenesis[34,35]. VEGF isoforms, such as $VFGF_{121}$ and $VEGF_{165}$, have been identified, and isoform subtypes also exist, such as $EGF_{166}b$. Thus, it is clear that these growth factors do not exhibit angiogenesis-promoting effects alone, and they have attracted attention as new therapeutic targets[36].

HCC typically exhibits active angiogenesis. During the progression from early to well- and moderately-differentiated HCC, angiogenesis increases and cancer cells acquire the ability to invade vessels and metastasize. Scientific and clinical studies have revealed that, during the progression from hepatitis to cirrhosis, angiogenesis and disruption of the vascular architecture are linked to the progression of HCC, and contribute to increased hepatic vascular resistance and portal hypertension, and decreased hepatocyte perfusion[37]. In addition, a meta-analysis has demonstrated that VEGF expression is a prognostic factor in HCC[38].

Phase Ⅱ studies have been started to test the usefulness of bevacizumab (Avastin®), which directly targets VEGF, in TACE-treated HCC, and the use of bevacizumab in combination with erlotinib (Tarceva®), an EGFR tyrosine kinase inhibitor.

Sunitinib (Sutent®) is a multi-kinase inhibitor that inhibits tyrosine kinases, such as VEGFR-1, -2, -3, PDGFR-α, -β and c-Kit. A phase Ⅱ study of sunitinib in 37 advanced HCC patients showed that the median progression-free survival (PFS) and median overall survival (OS) were 3.7 and 8 mo, respectively. In that study, adverse events included grade 3/4 thrombocytopenia in 37.8% of patients, neutropenia in 24.3%, asthenia in 13.5%, and hand-foot syndrome in 10.8%[39]. Since sunitinib has a lower IC_{50} for each target than sorafenib, it is expected to exhibit greater antitumor activity. However, this factor may be responsible for the higher incidence of adverse events with sunitinib. The main evaluation item in the above phase Ⅱ trial was the response rate, which did not reach the expected value, leading to the conclusion that it was a negative study[40]. In that study, sunitinib was administered at 50 mg/d for four weeks followed by two weeks of rest per cycle[39], whereas Zhu *et al*[40]. used a dosing schedule of 37.5 mg/d for four weeks followed by two weeks of rest per cycle, and reported that the median PFS and OS were 3.9 and 9.8 mo, respectively. An ongoing global cooperative phase Ⅲ controlled clinical trial to compare sorafenib and sunitinib head-to-head, and to seek approval for first-line indications for advanced HCC, adopted a sunitinib dosing schedule of 37.5 mg/d. However, in a "Reflection and Reaction" regarding the above trial results, Forner *et al*[41] cast doubt on whether the drugs at this dose could maintain tolerance and ensure efficacy. Consequently, the trial was terminated on March, 2010 because of the recommendation by data monitoring committee based on interim analysis, showing relatively high toxicity and no superior efficacy to sorafenib.

Brivanib is a kinase inhibitor that selectively inhibits VEGFR-1, -2 and -3, and FGFR-1, -2 and -3. Recent studies suggest that tumor progression following treatment with antiangiogenic agents that target the VEGF signaling pathway alone may result from either evasive or intrinsic resistance[42]. Furthermore, there is strong evidence to support the hypothesis that evasive resistance to anti-VEGF blockade is associated with reactivation of tumor angiogenesis by alternative signaling pathways. One such mechanism of resistance is the activation of the FGF signaling pathway[43,44]. Basic FGF (FGF2) is a potent angiogenic factor. Indeed, expression of FGF2 enhances growth, invasion, and angiogenesis of many tumor types[45,46]. Moreover, recent evidence has shown that FGF is overexpressed and activated in HCC, and that high FGF2 levels may predict a poor clinical outcome among patients with HCC[46].

Considering the proposed importance of FGF signaling in HCC angiogenesis, it is clear that novel antiangiogenic agents that combine inhibition of FGF receptor signaling with inhibition of VEGFR signaling might provide a potential mechanism to overcome anti-VEGF resistance in HCC (Figure 6). With this in mind, it is worthwhile considering the potential future impact of brivanib on the treatment of advanced HCC. Brivanib, a small-molecule tyrosine kinase inhibitor, is the first oral selective dual inhibitor of FGF and VEGF signaling. In multiple preclinical models of human xenograft tumors, including patient-derived HCC xenografts, brivanib has shown potent antitumor activity and no overt toxicity when dosed orally[47,48]. Furthermore, brivanib has demonstrated promising antitumor activity and acceptable tolerability in a phase Ⅱ, open-label study in patients with unresectable locally advanced or metastatic HCC[49,50]. Crucially, within this trial, brivanib showed activity both as first-line therapy (overall survival: 10 mo) or as second-

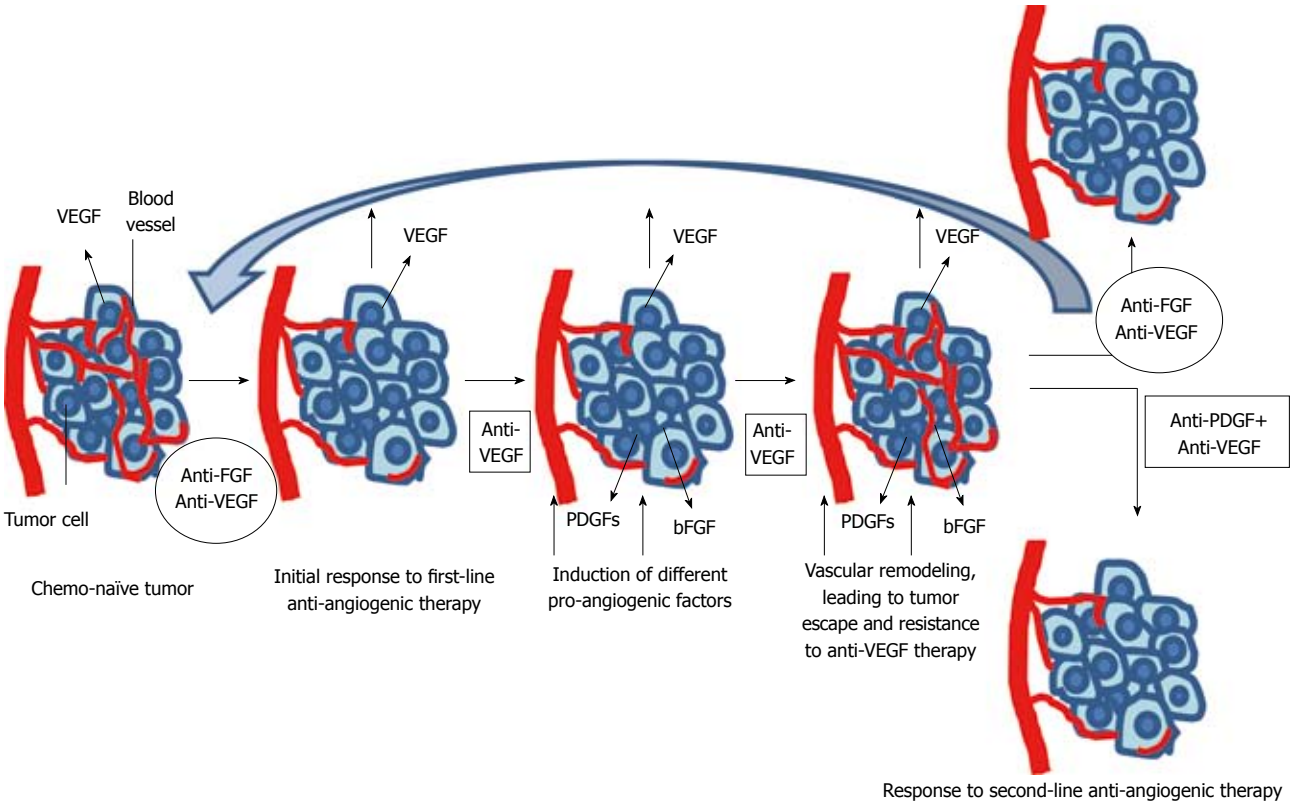

Figure 6 Brivanib may be effective for the failure or resistance of first line anti-angiogenic therapy for vascular endothelial growth factor. In addition, there is a possibility that anti-FGF agents can be first line anti-angiogenic agents. FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor.

line therapy in patients who had failed prior antiangiogenic treatment, primarily with sorafenib (overall survival: 9.5 mo)[50]. Of note, the incidence of all-grade hand-foot syndrome was only 8% in this study.

As for brivanib, an international global phase Ⅲ clinical trial to compare brivanib and sorafenib head-to-head and to seek approval for first-line therapy for advanced HCC has already been started, and the results are eagerly awaited. Since brivanib targets FGF and VEGF, and is associated with relatively mild adverse effects, a second-line study of brivanib in sorafenib-ineffective and -intolerant patients, and a trial to evaluate the use of brivanib in combination with TACE, are underway. Depending on the results of these trials, indications for use in HCC may be obtained; therefore, positive results are eagerly anticipated.

The results have been reported for a phase Ⅱ study of brivanib in 55 patients (cohort A) who had not received systemic therapy for curatively unresectable HCC and 46 patients (cohort B) previously treated with angiogenesis inhibitors, such as sorafenib or thalidomide[49]. The median TTP and OS were 2.8 mo and 10 mo, respectively, in cohort A versus 1.4 mo and 9.8 mo, respectively, in cohort B. Adverse events included fatigue (51.5%), diarrhea (41.6%), hypertension (42.6%), anorexia (41.6%), and nausea/vomiting (40.6%/30.7%) in total. Thus, these results demonstrated the efficacy of brivanib as a second-line treatment. The results of three phase Ⅲ clinical trials, BRISK-PS (sorafenib failure or sorafenib-intolerant patients; brivanib + best supportive care (BSC) *vs* placebo + BSC), BRISK-FL (advanced HCC; brivanib *vs* sorafenib), and BRISK-TA (patients with unresectable HCC, brivanib *vs* placebo as post-TACE adjuvant therapy) are awaited (Table 2).

Linifanib (ABT-869), which strongly inhibits VEGFR and PDGFR, is also under global phase Ⅲ trial.

In a Japanese phase Ⅰ / Ⅱ trial of TSU-68, an oral molecular inhibitor of VEGFR, PDGFR, and FGFR, to test its safety and efficacy in 35 HCC patients, the response rate was 5.6% (CR, PR, SD, PD and NE in 1, 2, 15, 16 and 1 patients, respectively), and the disease control rate was 51.4%[51]. The global phase Ⅲ trial of TACE in combination with TSU-68 has just started on January 2011.

In addition, several phase Ⅰ / Ⅱ trials are being conducted to assess kinase inhibitors such as cediranib (AZD2171), which inhibit VEGFR, PDGFR, CSF-1R (cFms), Kit, and Flt3. Furthermore, a phase Ⅲ global study of axitinib, which is currently being tested in renal cell carcinoma, has also been started as a second line agents on 2011.

EGF/EGFR

EGFR is a member of the HER family, which includes EGFR (erbB1), HER2/neu (erbB3), and HER4 (erb4). All members of this family, except HER3, have an intracellular tyrosine kinase domain, and the binding of a ligand to its extracellular domain triggers signal transduc-

Table 2 Ongoing clinical trials (PⅢ)

First line
Comparison study between sorafenib and single agent (head to head):
Sunitinib → endpoint not met
Brivanib
Linifanib
Combination with sorafenib and another agent:
DXR, erlotinib (SEARCH), everolimus, CS-1008, *etc.*
Second line
Sorafenib failure:
Brivanib, everolimus (RAD001), ramucirumab, axitinib, S-1, *etc.*
Combination with standard therapy
Adjuvant setting after surgery or RFA: STORM
Combination with TACE: SPACE, BRISK-TA, TACTICS, ECOG1208
Combination with HAIC: SILIUS

TACTICS: Phase Ⅱ study: Transcatheter arterial chemoembolization therapy in combination with sorafenib (ClinicalTrials.gov ID: NCT01217034), SILIUS: Randomized controlled trial comparing efficacy of sorafenib *vs* sorafenib in combination with low dose cisplatin/fluorouracil hepatic arterial InfUSion chemotherapy in patients with advanced hepatocellular carcinoma and exploratory study of biomarker predicting its efficacy (ClinicalTrials.gov ID: NCT01214343); HAIC: Hespatic arterial infusion chemotherapy; TACE: Transarterial chemoembolization.

tion through the above-described MAPK and PI3K/Akt/mTOR pathways. Thus, these receptors are involved in cell growth, differentiation, survival, and adhesion[52]. EGFR over expression has been reported in many cancers, and in HCC. For example, Buckley *et al*[53] reported that EGFR, detected by immunohistochemical analysis, was overexpressed in 50 (66%) of 76 HCCs, and that fluorescence *in situ* hybridization showed extra EGFR gene copies in 17 (45%) of 38 HCCs.

EGFR-targeting drugs include anti-EGFR antibodies, such as cetuximab and panitumumab, and small-molecule inhibitors of EGFR tyrosine kinases, such as gefitinib *etc*, and have been used widely for the treatment of several cancers other than HCC. Unfortunately, except for phase Ⅱ trial data, there are little clinical data on the efficacy of these drugs for the treatment of HCC.

Similar to gefitinib (Iressa®), erlotinib (Tarceva®) is an oral EGFR tyrosine kinase inhibitor. Philip *et al*[54] and Yau *et al*[55] have reported the results of phase Ⅱ studies of erlotinib in HCC; the median OSs in their studies were 13 and 10.7 mo, respectively. A phase Ⅲ clinical study (SEARCH study: Sorafenib and Erlotinib, a Randomized Trial Protocol for the Treatment of Patients with Hepatocellular Carcinoma) for sorafenib in combination with erlotinib *vs* sorafenib plus placebo is ongoing. Since erlotinib is associated with a high incidence of skin rash, dry skin and gastrointestinal toxicity, such as diarrhea, the results of the SEARCH study should be evaluated to assess whether this combination therapy can be used in clinical settings. Thomas *et al*[56] conducted a phase Ⅱ clinical study of erlotinib in combination with bevacizumab in 40 advanced HCC patients, and reported promising results; the median PFS and OS were 9 mo and 15.7 mo, respectively. However, they noted frequent treatment-related grade 3/4 toxicities, including fatigue (20%), hypertension (15%), gastrointestinal bleeding (12.5%), wound infection (5%), diarrhea (10%), elevated transaminase levels (10%), and thrombocytopenia (10%), which necessitates further evaluation for drug tolerance. Although a clinical study of erlotinib in combination with bevacizumab (OPTIMOX-3 study) was also conducted in colorectal cancer patients, no tolerance was observed, which led to a change in the protocol[57,58].

After the introduction of a number of molecular-targeted drugs, strategies for the inhibition of similar or different signaling pathways (vertical or horizontal inhibition) with several drugs have been proposed. However, the combined use of molecular-targeted agents has remained largely unsuccessful, including panitumumab in combination with bevacizumab for the treatment of colorectal cancer[59]. Similarly, the results of sorafenib in combination with bevacizumab (vertical inhibition) have been reported[60]. Although some therapeutic responses were obtained, the combination therapy resulted in greater toxicity[60], suggesting the need for detailed evaluation of the dosing regimen.

Lapatinib (Tykerb®) is a dual inhibitor of EGFR and HER-2/neu, and inhibits tumor growth by downregulating MAPK, AKT, and p70S6 kinase[61]. In Japan, lapatinib is indicated for the treatment of breast cancer. In a phase Ⅱ clinical trial of lapatinib in 26 patients with unresectable advanced HCC, the median PFS and OS were 1.9 mo and 12.6 mo, respectively, and adverse events included diarrhea (73%), nausea (54%), and skin rash (42%)[62].

Cetuximab (Erbitux®) is a human/mouse chimeric monoclonal antibody consisting of the variable region of a mouse anti-human EGFR monoclonal antibody and the human immunoglobulin G1 constant region. Cetuximab inhibits the binding of endogenous EGFR ligands, such as EGF and TGFα, to EGFR. In a phase Ⅱ clinical trial of cetuximab in 30 patients with unresectable or metastatic HCC, the median PFS and OS were 1.4 mo and 9.6 mo, respectively, and treatment-related toxicities included grade 3 hypomagnesemia (3.3%) and grade 1/2 acne-like rash (83.3%), which was observed for the duration of anti-EGFR therapy in that study[63].

The EGFR offers a very interesting therapeutic target. As described above, the use of erlotinib in combination with sorafenib is still in the research stage. However, based on the results of phase Ⅱ studies, the efficacy of cetuximab or lapatinib as a monotherapy seems to be limited, and the results of further studies evaluating their efficacy in sorafenib-refractory or -intolerant patients are awaited with interest.

Hepatocyte growth factor/c-Met pathway

Since the hepatocyte growth factor (HGF)/Met pathway is involved in tumor growth, invasion, and angiogenesis in a wide range of neoplasms, HGF and Met have recently attracted attention as therapeutic targets. HGF is a heterodimer consisting of α and β chains bound together by a disulfate bond. The α-chain contains four

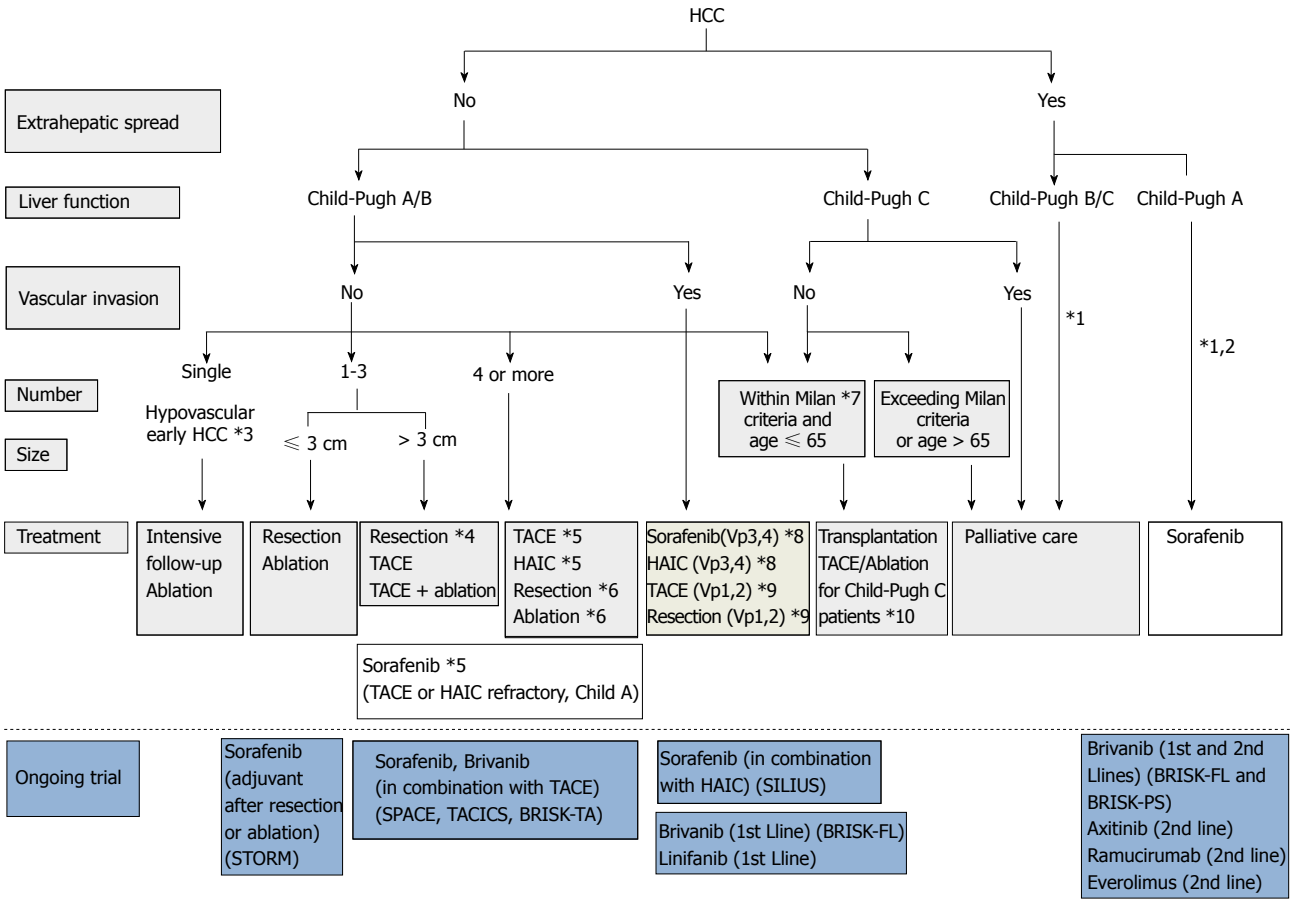

Figure 7 Consensus-based treatment algorithm for hepatocellular carcinoma proposed by Japan Society of Hepatology, revised in 2010. (Cited and modified from Kudo *et al*[67] with permission.) *1: Treatment should be performed as if extrahepatic spread is negative, when extrahepatic spread is not considered as a prognostic factor in Child-Pugh class A/B patients; *2: Sorafenib is the first choice of treatment in this setting as a standard of care; *3: Intensive follow-up observation is recommended for hypovascular nodules by the Japanese Evidence-Based Clinical Practice Guidelines. However, local ablation therapy is frequently performed in the following cases: (1) when the nodule is diagnosed pathologically as early hepatocellular carcinoma (HCC); (2) when the nodules show decreased uptake on Gd-EOB-MRI, or (3) when the nodules show decreased portal flow by CTAP, since these nodules frequently progress to advanced HCC; *4: Even for HCC nodules exceeding 3 cm in diameter, transcatheter arterial chemoembolization (TACE) in combination with ablation is frequently performed when resection is not indicated; *5: TACE is the first choice of treatment in this setting. Hepatic arterial infusion chemotherapy (HAIC) using an implanted port is also recommended for TACE-refractory patients. The regimen for this treatment is usually low-dose FP [5-fluorouracil (5-FU) + CDDP] or intra-arterial 5-FU infusion combined with systemic interferon therapy. Sorafenib is also recommended for TACE or HAIC-refractory patients with Child-Pugh class A liver function; *6: Resection is sometimes performed when more than 4 nodules are detected. Ablation is sometimes performed in combination with TACE; *7: Milan criteria: Tumor size ⩽ 3 cm and tumor number ⩽ 3, or solitary tumor ⩽ 5 cm. Even when liver function is good (Child-Pugh A/B), transplantation is sometimes considered for frequently recurring HCC patients; *8: Sorafenib and HAIC are recommended for HCC patients with major portal invasion such as Vp3 (portal invasion in the 1st portal branch) or Vp4 (portal invasion in the main portal branch); *9: Resection and TACE are frequently performed when portal invasion is minor, such as Vp1 (portal invasion in the 3rd or more peripheral portal branch) or Vp2 (portal invasion in the 2nd portal branch); *10: Local ablation therapy or subsegmental TACE is performed even for Child-Pugh C patients when transplantation is not indicated when there is no hepatic encephalopathy, no uncontrollable ascites, and a low bilirubin level (< 3.0 mg/dL). However, it is regarded as an experimental treatment because there is no evidence of a survival benefit in Child-Pugh C patients. A prospective study is necessary to clarify this issue.

kringle domains, and the β-chain contains a serine protease-like domain. Met is a receptor tyrosine kinase for the HGF ligand, and contains a semaphorin-like domain. HGF or Met overexpression, and *Met* gene mutations and duplications, have been reported in various cancers, and abnormalities due to HGF/Met pathway activation have also been noted[64]. These abnormalities activate the downstream signaling cascade, leading to epithelial-mesenchymal transition and increased proliferative, migratory, invasive, and metastatic potentials of cancer cells[64].

HGF/c-MET-targeted drugs, including kinase inhibitors, HGF inhibitors and decoy c-Met receptor molecules are being developed. Of particular interest is ARQ-197, a c-Met receptor tyrosine kinase inhibitor, which is a non-ATP-competitive molecule that binds near the ATP-binding site. A randomized phase Ⅱ study of ARQ-197 *vs* placebo is ongoing in patients with unresectable HCC after systemic therapy failure. In addition, the results of a phase I study of ARQ-197 in combination with sorafenib was reported in ASCO 2010 (Abstract 3024).

IGF/IGFR

The IGF/IGFR system is involved in cell growth and the chemotherapeutic response. The ligands IGF- I and -Ⅱ bind to their receptors IGF-1R and IGF-2R, and are involved in DNA synthesis and cell growth. Abnormali-

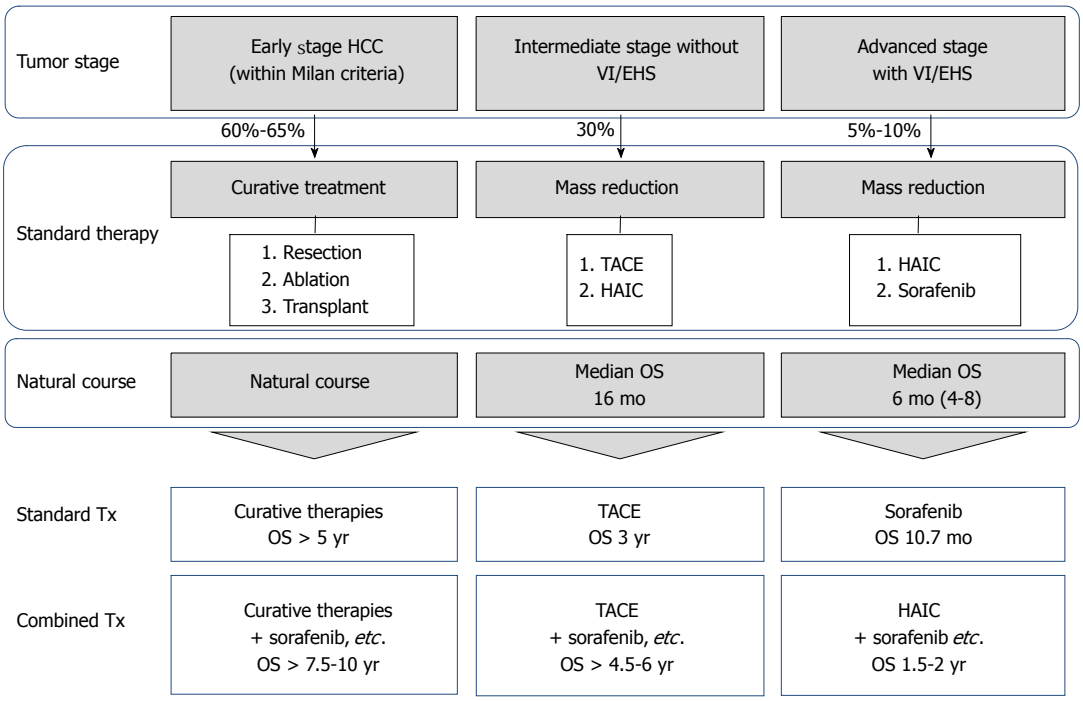

Figure 8 Outcomes of standard treatment modalities and expected future outcomes of combination therapy with molecular-targeted agents. By combining molecular targeted agents with resection or ablation, life expectancy [overall survival (OS)] is expected to be prolonged to 7.5-10 years. In addition, for intermediate stage hepatocellular carcinoma (HCC), the prognosis is expected to be improved to 4.5-6 years by combination with transarterial chemoembolization (TACE). For advanced stage HCC, the prognosis is expected to be improved to 1.5-2 years by combination with hepatic arterial infusion chemotherapy (HAIC).

Table 3 Subanalysis data of the SHARP study

		Advanced HCC with vascular invasion and/or extrahepatic spread	Advanced HCC without vascular invasion and/or extrahepatic spread
Hazard ratio		0.77 (95% CI: 0.60-0.99)	0.52 (95% CI: 0.32-0.85)
Median OS (MST) (mo)	Sorafenib	8.9 (n = 209) (95% CI: 7.6-10.3)	14.5 (n = 90) (95% CI: 14.0-N/E)
	Placebo	6.7 (n = 212) (95% CI: 5.2-8.0)	10.2 (n = 91) (95% CI: 8.6-15.5)

HCC: Hepatocellular carcinoma; OS: Overall survival; MST: Median survival time.

ties in IGF and IGF-1R, or their overexpression, have been reported in various cancers, including HCC. Their associations with disease stage, metastasis, survival[65], and the functions of IGF and IGFR in HCC[66] have been reported.

IGF-targeting drugs are currently being developed, and are mainly anti-IGF-1R antibodies, such as BⅡB022, AVE1642, and cixutumumab (IMC-A12). A phase Ⅱ study of cixutumumab, a phase Ⅰb/Ⅱ study of sorafenib *vs* sorafenib plus BⅡB022, and phase Ⅰ/Ⅱ studies of AVE1642 as monotherapy or in combination with sorafenib or erlotinib are ongoing.

COMBINATION THERAPY OF STANDARD TREATMENT WITH SORAFENIB

In addition to the pharmaceutical-sponsored clinical trials of linifanib and brivanib as first- and second-line therapy in sorafenib-refractory patients, investigator initiated trials (IIT) of sorafenib in combination with hepatic arterial infusion chemotherapy (SILIUS trial: trial number NCT01214343), pharmaceutical and IIT trials of sorafenib in combination with TACE [SPACE, TACICS (trial number: NCT 01217034) and BRISK-TA trials], and a trial to test the inhibitory effect of sorafenibon tumor recurrence after curative treatment (STORM trial) are ongoing. The results of these trials are eagerly awaited (Figure 7)[67,68].

The working hypotheses in these studies can be deduced by extrapolating the median survival time (MST) and hazard ratios in overall survival (OS) calculated in a subanalysis of the SHARP study (Table 3). The results obtained suggest that starting treatment with molecular-targeted drugs at an earlier tumor stage in combination with standard treatment options such as resection, ablation, TACE, or hepatic arterial infusion chemotherapy can improve the prognosis of HCC. Thus, sorafenib has the potential to induce a paradigm shift in the treatment of HCC. For example, in a subanalysis of the SHARP trial, the hazard ratios for OS and MST ratio in intermediate stage HCC without vascular invasion or extrahepatic spread were 0.52 and 1.50, respectively (Table 3). This

suggests that survival of early stage HCC and intermediate stage HCC may be prolonged from 5 years to 7.5-10 years by using sorafenib in an adjuvant setting after curative treatment, and from 3 years to 4.5-6 years by using sorafenib in combination with TACE (Figure 8)[68].

CONCLUSION

Several clinical trials[39,40,49,54,63,69-74] of the molecular-targeted agents are ongoing. Angiogenesis-inhibiting drugs, particularly sorafenib, have been established for HCC, and drugs targeting several molecules are being developed.

Although sorafenib was recently approved, many issues remain to be addressed, including: (1) how to determine and define refractoriness; and (2) whether to continue TACE or hepatic arterial infusion chemotherapy (a de facto standard in Japan) in patients with TACE-refractory HCCs or portal tumor thrombi before starting sorafenib therapy. We strongly recommend that, based on the molecular-targeted agents currently under development, clinical studies (including IITs) should be conducted aggressively, and therapeutic strategies should be devised to resolve the limitations of currently used therapeutic approaches and to improve the therapeutic outcomes.

The introduction of sorafenib to treat HCC in 2007 in Western countries and in 2009 in Japan was undoubtedly the real beginning of a paradigm shift of HCC treatment, representing a significant breakthrough for HCC treatment not previously experienced for this unique tumor. Further development of survival benefit in HCC patients with new targeted agents are greatly expected.

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PNPLA3, the triacylglycerol synthesis/hydrolysis/storage dilemma, and nonalcoholic fatty liver disease

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Abstract

Genome-wide and candidate gene association studies have identified several variants that predispose individuals to developing nonalcoholic fatty liver disease (NAFLD). However, the gene that has been consistently involved in the genetic susceptibility of NAFLD in humans is patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin). A nonsynonymous single nucleotide polymorphism in PNPLA3 (rs738409 C/G, a coding variant that encodes an amino acid substitution I148M) is significantly associated with fatty liver and histological disease severity, not only in adults but also in children. Nevertheless, how *PNPLA3* influences the biology of fatty liver disease is still an open question. A recent article describes new aspects about *PNPLA3* gene/protein function and suggests that the I148M variant promotes hepatic lipid synthesis due to a gain of function. We revise here the published data about the role of the I148M variant in lipogenesis/lipolysis, and suggest putative areas of future research. For instance we explored in silico whether the rs738409 C or G alleles have the ability to modify miRNA binding sites and miRNA gene regulation, and we found that prediction of *PNPLA3* target miRNAs shows two miRNAs potentially interacting in the 3' UTR region (hsa-miR-769-3p and hsa-miR-516a-3p). In addition, interesting unanswered questions remain to be explored. For example, PNPLA3 lies between two CCCTC-binding factor-bound sites that could be tested for insulator activity, and an intronic histone 3 lysine 4 trimethylation peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. Finally, an interaction between *PNPLA3* and glycerol-3-phosphate acyltransferase 2 is suggested by data miming.

Key words: Adiponutrin; Nonalcoholic fatty liver disease; miRNA; Glycerol-3-phosphate acyltransferase 2; Systems biology; Rs738409; Epigenetics

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INVITED COMMENTARY ON HOT ARTICLES

We have read with great interest the recent article by Kumari *et al*[1] describing new aspects about patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin) gene function. Notably, the authors reported that human and murine *PNPLA3* exhibit increased lysophosphatidic acid acyltransferase (LPAAT) activity leading to increased cellular lipid accumulation. Kumari *et al*[1] concluded that the I148M substitution promotes hepatic lipid synthesis due to a gain of function, and that this property provides a plausible biochemical mechanism for the development of liver steatosis in subjects carrying the 148M variant (rs738409, allele G) as discussed below.

These results give new answers to the previous conflicting data around the question of whether the rs738409 is associated with a gain or loss of gene/protein function (Figure 1). For instance, several functional studies were elegantly performed to decipher whether the I148M substitution interferes with hepatic triglyceride (TG) hydrolysis and thus promoting hepatic steatosis[2]. Based on an *in vitro* structural model of the patatin-like domain of PNPLA3 protein, He *et al*[2] showed that the 148M variant interferes with TG hydrolysis, affecting the association of PNPLA3 with the lipid droplets. Thus, the resulting mutant enzyme is inactive against its substrates. In addition, Huang *et al*[3] recently demonstrated that PNPLA3 plays a role in the hydrolysis of glycerolipids, and the I148M substitution causes a loss of function.

Several animal studies were conducted to evaluate the effect of the PNPLA3 loss of function by using knockout models, for instance PNPLA3$^{-/-}$ mice by gene targeting[4]. Surprisingly, loss of PNPLA3 does not cause fatty liver, liver enzyme elevation, or insulin resistance in mice under a standard or a high-sucrose/high-fat diet[4]; a finding replicated by global targeted deletion of the *PNPLA3* gene[5].

Interestingly, a human study demonstrated that the rs738409 PNPLA3 G allele is associated with morphological changes in adipocyte cell size[6], and this concept strongly supports a role of the variants in the liver fat remodeling; unfortunately, these findings were not replicated.

Finally, Kumari *et al*[1] confirmed that PNPLA3 functions as a TG hydrolase in mice and humans but found that the specific TG hydrolase activity is much lower than that of adipose triglyceride lipase (ATGL).

Can a loss or gain of function in a single protein explain such a strong effect in the biology of nonalcoholic fatty liver disease?

The question as to whether the role of the rs738409 in the pathobiology of nonalcoholic fatty liver disease (NAFLD) is associated with a gain or loss of function is hard to answer because the biological function attributed to the PNPLA3 in lipid-related pathways is shared with other related proteins. For example, almost all members of the PNPLA family have established TG hydrolases and phospholipases activities, and PNPLA3 shares, as expected, protein domains with the PNPLA family (PNPLA1, PNPLA2, and PNPLA4-8), and also with PLA2G6 (phospholipase A2, group VI cytosolic, calcium-independent, also known as PNPLA9). In silico prediction of PNPLA3 protein function by imputation of functional association data[7] shows that only PNPLA2, PNPLA3, PNPLA8, and PLA2G6 have carboxylesterase and lipase activities ($P < 4.8$ and 9.4×10^{-5}) and PNPLA3, PNPLA8, and PLA2G6 have phospholipase A2 activity ($P < 1.6 \times 10^{-4}$). As we mentioned recently, these similarities with enzymes with the potential of releasing arachidonic acid as the precursor of potent inflammatory substances such as prostaglandins may have therapeutic implications[8].

In addition, neutral lipid, triglyceride, or glycerolipid catabolic processes are shared by only PNPLA2 and PNPLA3 ($P < 0.05$). This finding is also consistent with the notion that PNPLA3 plays a role in the hydrolysis of glycerolipids as shown by Huang *et al*[3].

Furthermore, *in silico* prediction of the protein network related to PNPLA3 is beyond the PNPLA family (Figure 2), suggesting that some other interesting and still unexplored issue about the putative role of the rs738409 in liver steatosis are waiting for answers. For instance, the putative interaction between PNPLA3 and genes in the related networks, particularly glycerol-3-phosphate acyltransferase 2 (GPAT2, mitochondrial), whose protein esterifies acyl-group from acyl-ACP to the sn-1 position of glycerol-3-phosphate, an essential step in glycerolipid biosynthesis[9]. We wonder to what extent the PNPLA3 and GPAT2 interaction modulates hepatic fat content. We used a data integration and data mining platform to explore the putative interaction between PNPLA3 and GPAT2, and as shown in Figure 3, PNPLA3 and GPAT2 are involved in shared metabolic pathways, including triglyceride biosynthetic process, glycerolipid metabolism pathways, and acyltransferase activity, which may explain an interaction in the pathogenesis of NAFLD. This prediction is biologically plausible as GPAT2 catalyzes the initial and rate-limiting step in glycerolipid synthesis, and overexpression and knock-out studies suggest that GPAT isoforms can play important roles in the development of hepatic steatosis, insulin resistance, and obesity[10].

Impact of rs738409 on genetic risk of NAFLD

The first description about the association between a nonsynonymous single nucleotide polymorphism (SNP) of PNPLA3, the rs738409 C/G, (a coding variant that encodes an amino acid substitution I 148M as described) and fatty liver was reported by Romeo *et al*[11] by performing a genome-wide association study (GWAS) of nonsynonymous sequence variations ($n = 9229$) in a large multiethnic population-based epidemiological study of fatty liver. This initial finding was further replicated in several populations confirming that the G allele in the forward strand is significantly associated with increased risk of hepatic triglyceride accumulation and fatty liver disease, as reviewed recently in a meta-analysis[12]. Likewise, the rs738409 variant was associated with fatty liver in pediatric patients with NAFLD[13-16], and patients with NAFLD related comorbidity, such as type 2 diabetes[17-19] or morbid

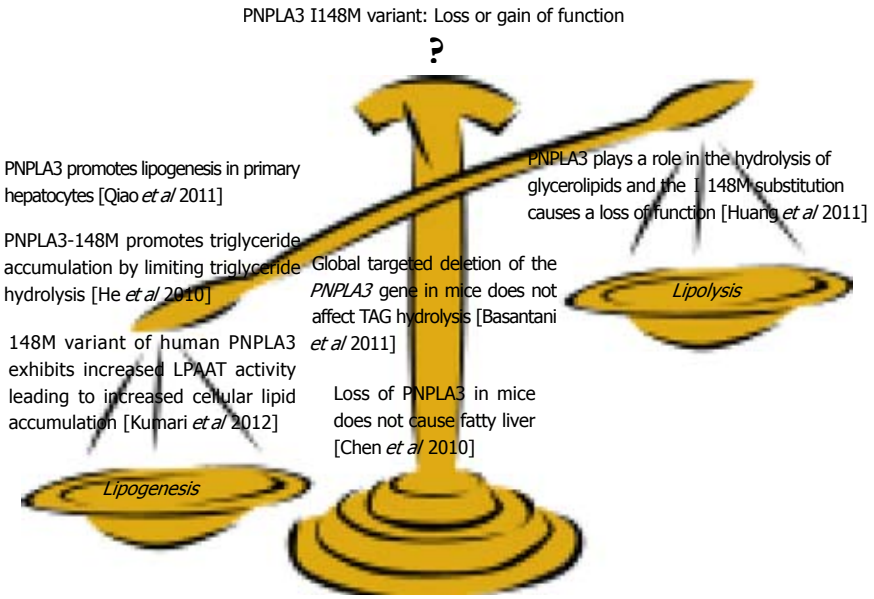

Figure 1 Patatin-like phospholipase domain containing 3 rs738409 and its role in nonalcoholic fatty liver disease. Summary of the evidence from human and rodent studies. PNPLA3: Patatin-like phospholipase domain containing 3.

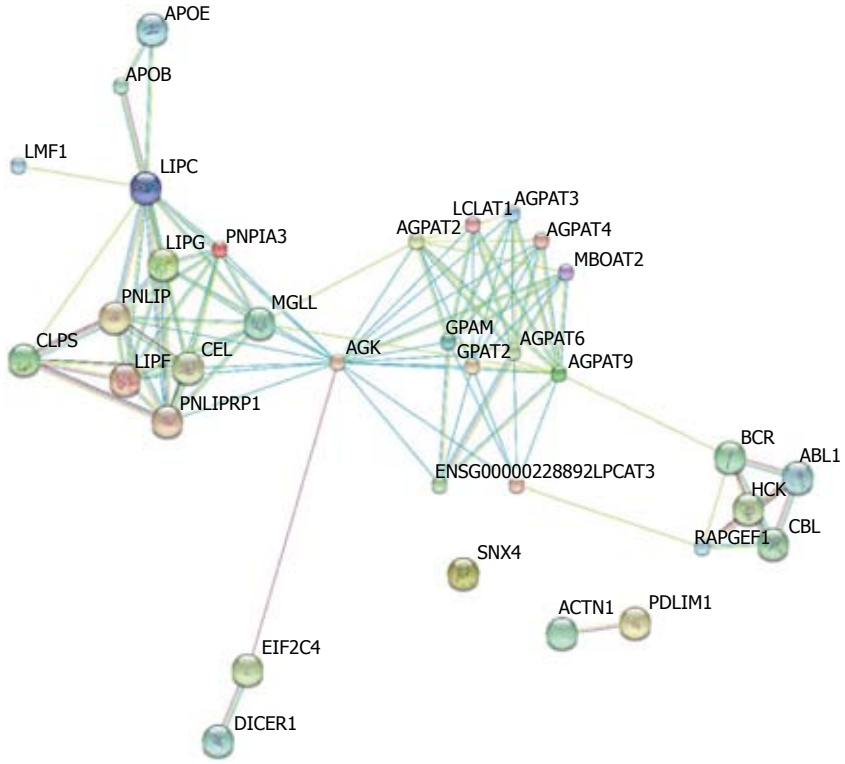

Figure 2 Protein network around patatin-like phospholipase domain containing 3 by the Search Tool for the Retrieval of Interacting Genes/Proteins resource (http://string-db.org/). The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments, Conserved-Coexpression and Previous Knowledge. PNPLA3: Patatin-like phospholipase domain containing 3; LIPF: Lipase, gastric; SNX4: Sorting nexin 4; AGPAT9: 1-acylglycerol-3-phosphate O-acyltransferase 9; MGLL: Monoglyceride lipase; GPAM: Glycerol-3-phosphate acyltransferase, mitochondrial; AGPAT3: 1-acylglycerol-3-phosphate O-acyltransferase 3; LIPC: Lipase, hepatic; MBOAT2: Membrane-bound O-acyltransferase domain containing 2; LCLAT1: Lysocardiolipin acyltransferase 1; AGPAT4: 1-acylglycerol-3-phosphate O-acyltransferase 4; LPCAT3: Lysophosphatidylcholine acyltransferase 3; AGK: Acylglycerol kinase; PNLIPRP1: Pancreatic lipase-related protein 1, GPAT2: Glycerol-3-phosphate acyltransferase 2, mitochondrial; PNLIP: Pancreatic lipase; PDLIM1: PDZ and LIM domain 1; AGPAT2: 1-acylglycerol-3-phosphate O-acyltransferase 2; CEL: Carboxyl ester lipase; EIF2C4: Eukaryotic translation initiation factor 2C, 4; HCK: Hemopoietic cell kinase; AGPAT6: 1-acylglycerol-3-phosphate O-acyltransferase 6; ENSG00000228892: 1-acyl-sn-glycerol-3-phosphate acyltransferase.

obesity[15], although it remained to be explored whether these associations are truly independent of NAFLD[12].

Interestingly, we demonstrated for the first time[20] that the rs738409 was also significantly associated with histological disease severity; a finding that was replicated by others[21-23]. In fact, the G allele significantly increases the risk of progressive liver disease (odds ratio 1.88 per G allele; 95% CI: 1.03-3.43; $P < 0.04$)[20,12].

Overall, the most remarkable conclusion about the impact of the genetic variation in PNPLA3 on NAFLD-related outcomes is the strong effect that the rs738409 has on the susceptibility of fatty liver, because the proportion of the total variation attributed to the SNP genotypes is about 5.3%[20]. This effect is perhaps one of the strongest ever reported for a common variant modifying the genetic susceptibility for a complex disease.

Furthermore, the rs738409 variant not only modifies the biology of NAFLD but also has a considerable impact on the genetic susceptibility to alcoholic liver disease[24-26], and hepatitis-C-virus-induced fatty liver[23], and is a strong predictor of hepatocellular carcinoma occurrence among patients with cirrhosis[27,28], indicating that these diverse liver diseases may share common pathophysiological pathways.

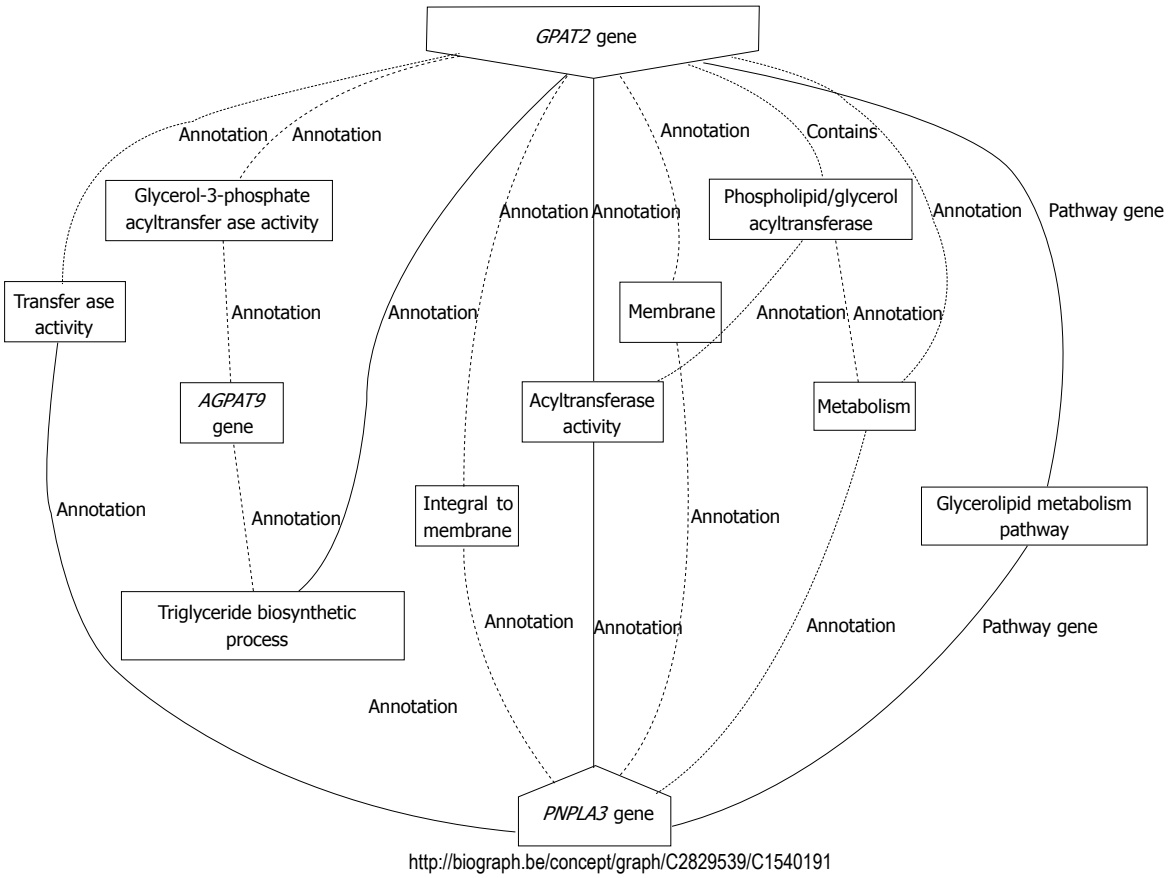

Figure 3 Interaction between patatin-like phospholipase domain containing 3 and glycerol-3-phosphate acyltransferase 2. Prediction was performed by the tool BioGraph (http://biograph.be/about/welcome), a data mining framework that allows for the automated formulation of comprehensible functional hypotheses relating a context to targets. *PNPLA3*: Patatin-like phospholipase domain containing 3; *GAPT2*: Glycerol-3-phosphate acyltransferase 2; *AGAPT9*: Acylglycerol-3-phosphate acyltransferase isoform.

PNPLA3: Short summary about gene structure and variation

PNPLA3 gene is located in chromosome 22 (22q13.31) and has nine exons; its transcript length is 2805 bp and it is translated to a protein of 481 amino acids. There are 34 SNPs with reported frequency and heterozygosity information in the HapMap (www.hapmap.org). Nevertheless, among all known PNPLA3 variants in coding or noncoding regions, there are three SNPs that have shown association with NAFLD-related phenotypes, including the rs738409. One is the rs6006460 (S453I) that after gene resequencing is associated with lower hepatic fat content in African Americans[11]. The other SNP is the nonsynonymous rs2294918 (Lys434Glu) that is significantly associated with serum alanine aminotransferase levels[29]. Haplotype analysis of PNPLA3 shows that the rs738409 is in moderate linkage disequilibrium (LD) (r^2: 0.65) with the other variants, including rs2294918 (Figure 4). Thus, this scenario precludes any imputation across the PNPLA3 locus centered on rs738409, and suggests that the I148M variant might be the casual variant in the susceptibility of fatty liver. However, Figure 5 shows annotation of nearby SNPs in LD (proxies) with rs738409 based on HapMap data and a nearby locus, such as SAMM50 that is a component of the sorting and assembly machinery (SAM) complex of the outer mitochondrial membrane. The SAM complex has a role in integrating β-barrel proteins into the outer mitochondrial membrane, and makes SAMM50 a good candidate because mitochondrial dysfunction may play a major role in NAFLD pathogenesis, as suggested by experimental models[30] and human studies[31]; nevertheless, this issue deserves further investigation.

Another interesting feature to highlight is the population diversity of rs738409. Genome diversity data extracted from http://www.ncbi.nlm.nih.gov/SNP/ shows that the risk allele G is highly prevalent around the word, with an average prevalence close to 0.70 from Caucasian to Yoruban populations. Negative selection of the ancestral allele that seems to be the C allele (http://www.ensembl.org), which is shared by chimpanzees, orangutans, macaques, and other species, suggests that environmental pressures have exerted a strong influence. However, this picture probably reflects a sort of confusion about the reference strand and probably these annotation data refer to the minus strand when the gene is located in the plus strand, and the real frequency of the risk allele G is < 50% in all populations. In fact, published data[12] from rs738409 association studies show that the most prevalent allele is certainly the reference allele C.

PNPLA3: Mechanisms of control of gene and protein expression and unexplored areas of research

PNPLA3 is a multifunctional enzyme that has both triac-

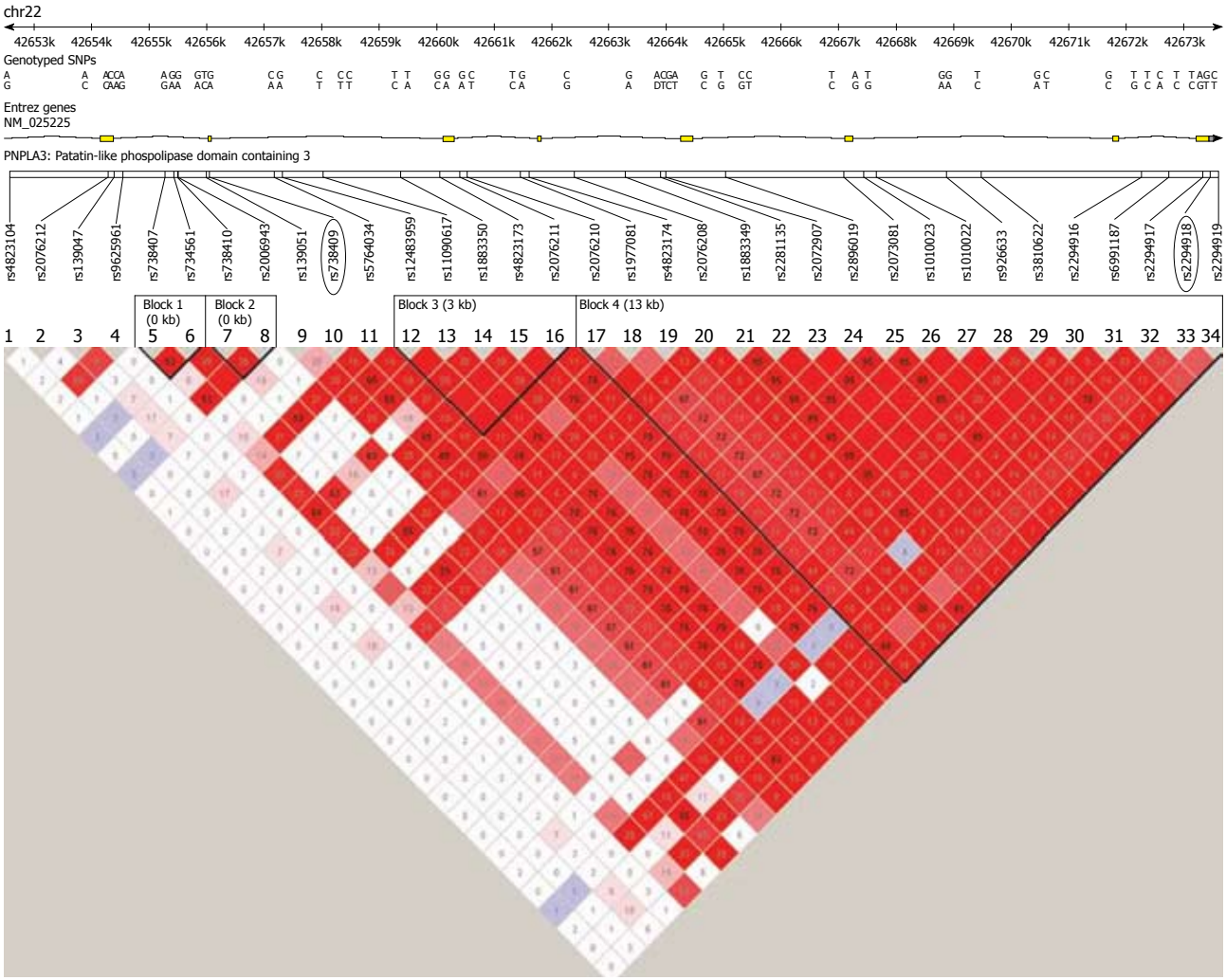

Figure 4 Linkage disequilibrium plot across the patatin-like phospholipase domain containing 3 gene. The horizontal white line depicts the DNA segment of chromosome 22q13.31. The rs738409 location is highlighted in a black ellipse. An LD plot is depicted in the bottom part of the figure: each diamond represents the magnitude of LD for a single pair of markers, with colors indicating strong LD (red r^2 = 1.0) and no LD (white, r^2 = 0) as the extremes (different red tones indicate intermediate LD). Numbers inside the diamonds stand for r^2 values. Haplotype analysis of PNPLA3 shows that the rs738409 is in moderate LD (no more than r^2: 0.65) with other variants. Plot was obtained by the software Haploview 2.0 freely available at www.broad.mit.edu/mpg/haploview/. LD: Linkage disequilibrium; PNPLA3: Patatin-like phospholipase domain containing 3.

Table 1 Patatin-like phospholipase domain containing 3 protein features and functions

PNPLA3 belongs to the IPLA2/lipase family. The protein encoded by *PNPLA3* is a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes. Multifunctional enzyme that has both triacylglycerol lipase and acylglycerol O-acyltransferase activity (http://genatlas.medecine.univ-paris5.fr/)
Lipid hydrolase with an unusual folding topology that differs from classical lipases[38]
The enzyme is highly regulated by changes in energy balance: nutritional control of *PNPLA3* being affected by a feed-forward loop[36]
PNPLA3 mRNA increases during differentiation of rat adipocytes in an insulin-dependent manner[39]
PNPLA3 mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat WAT[39]
PNPLA3 mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat white adipose tissue[40]
Promoter region of the human adiponutrin/*PNPLA3* gene is regulated by glucose and insulin[33]
Fasting significantly downregulates *PNPLA3* mRNA expression in liver and adipose tissue[35]
Feeding significantly upregulates *PNPLA3* mRNA in liver and fat[34]
Liver *PNPLA3* mRNA is expressed in human liver in higher levels compared with adipose tissue[36]
PNPLA3 mRNA is highly expressed in liver of b/ob mice[40] and visceral and subcutaneous adipose issue in obese humans[41]
PNPLA3 mRNA is expressed in hepatocytes but not in Kupffer cells[35,36]
PNPLA3 is expressed in hepatocytes but not in liver endothelial and Kupffer cells; microarray-based gene profiling showed that the expression level of *PNPLA3* in hepatocytes is correlated with that of genes associated with the lipogenic pathway such as ME1, SPOT14, and SCD1[35]
PNPLA3 is regulated in human hepatocytes by glucose *via* ChREBP[42]
SREBP1c is able to induce *PNPLA3* expression in human immortalized hepatocytes and in HepG2 hepatoma cells[37]

PNPLA3: Patatin-like phospholipase domain containing 3; SREBP1c: Sterol regulatory element binding protein 1; ChREBP: Carbohydrate-responsive element-binding protein; ME1: Malic enzyme; SCD1: Stearoyl-CoA desaturase 1.

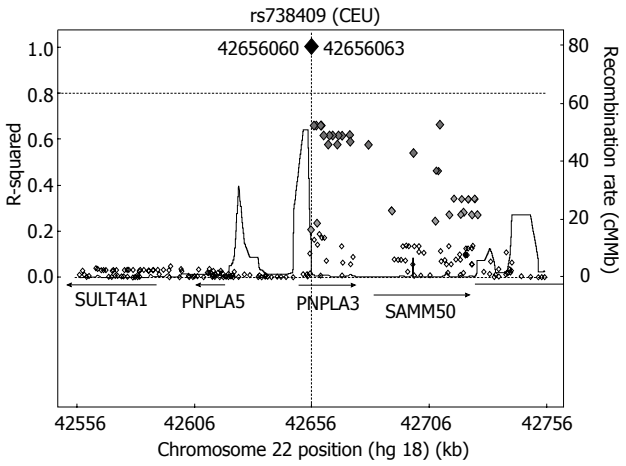

Figure 5 Regional Linkage disequilibrium plot for the rs738409 at chromosome 22 (22q13.31). The single nucleotide polymorphisms are plotted with their proxies (showed as diamonds) (based on HapMap data for CEU) as a function of genomic location, annotated by the recombination rate across the locus and nearby genes. The regional association plot was performed by the SNAP server, available at http://www.broad.mit.edu/mpg/snap/. SULT4A1: Sulfotransferase-4A1; PNPLA5: Patatin-like phospholipase domain containing 5; PNPLA3: Patatin-like phospholipase domain containing 3; SAMM50: Sorting and assembly machinery component 50 homolog.

ylglycerol lipase and acylglycerol O-acyltransferase activity[32]; subfamily hierarchy of PNPLA3 protein is shown in Figure 6. A summary of PNPLA3 protein characteristics and mechanisms of gene regulation is shown in Table 1. A very interesting feature of PNPLA3 protein is that is induced by several nutritional factors, such as oleic acid, C18:2 fatty acid, palmitic acid, glucose, insulin and lactone. Moreover, it was shown that the promoter activity of PNPLA3 is upregulated by glucose concentrations in a dose-dependent manner[33]. Adiponutrin was initially regarded as a dietary and obesity-linked protein[34]. Furthermore, Hoekstra *et al*[35] suggested that PNPLA3 plays an important role in hepatic lipid metabolism under conditions of lipid excess. In the light of these findings, subsequent studies were conducted to evaluate how *PNPLA3* is transcriptionally regulated, and most of the experiments were focused to answer the question whether the lipogenic transcription factor sterol regulatory element binding protein 1 (SREBP-1) was orchestrating these events. Interestingly, Huang *et al*[36] demonstrated in mice that *PNPLA3* mRNA levels are regulated by SREBP-1c as they found a SREBP1c binding site mapped to intron 1 of PNPLA3. In particular, Huang *et al*[36] observed robust transcriptional upregulation of PNPLA3 expression with carbohydrate feeding mediated by activation of liver X receptor/retinoic acid receptor (RXR) and increased levels of SREBP1c. In agreement with this finding, we observed in a high-fat-induced rat NAFLD model that liver transcript levels of RXR-α were significantly upregulated in fatty liver in comparison with normal liver (RXR-α mRNA/TATA box binding protein mRNA ratio: 11.71 ± 1.11 *vs* 6.48 ± 1.15, respectively, $P = 0.008$), data adjusted by HOMA-IR (homeostatic model assessment-insulin resistance) using analysis of covariance, (unpublished data).

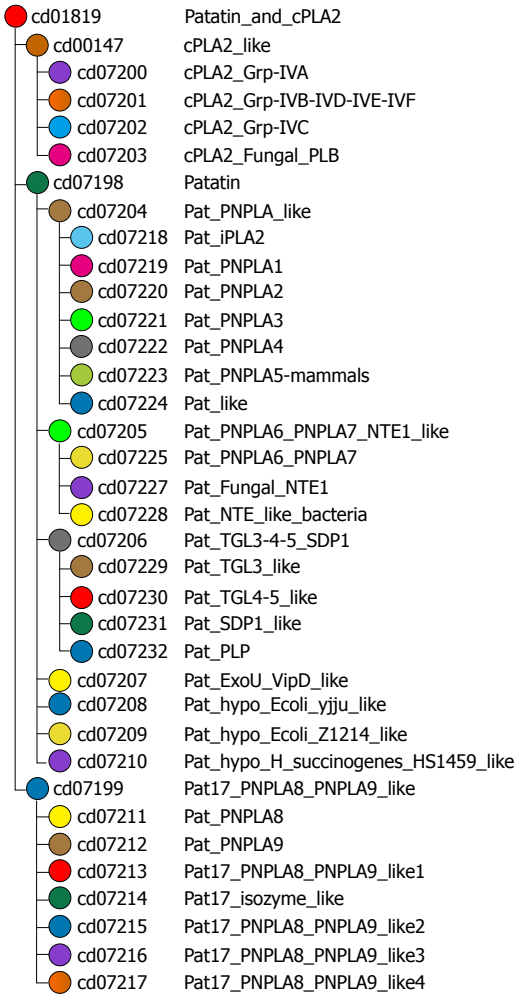

Figure 6 Domain family hierarchy of patatin-like phospholipase domain containing 3 protein. Data extracted from NCBI-curated domains at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. This picture provides data about how patterns of residue conservation and divergence in a family relate to functional properties. In this particular case, picture shows the patatin-like phospholipase family. Patatin is a storage protein, but it also has the enzymatic activity of a lipid acyl hydrolase, catalyzing the cleavage of fatty acids from membrane lipids. Members of this family have also been found in vertebrates. This family also includes the catalytic domain of cytosolic phospholipase A2 (PLA2; EC 3.1.1.4) hydrolyzes the sn-2-acyl ester bond of phospholipids to release arachidonic acid. At the active site, cPLA2 contains a serine nucleophile through which the catalytic mechanism is initiated. PNPLA3: Patatin-like phospholipase domain containing 3; TGL: Triglyceride lipase; PLP: Pyridoxal phosphate.

In addition, *in vitro* data show that mouse but not human *PNPLA3* gene expression is under the transcriptional control of carbohydrate-responsive element-binding protein (ChREBP)[37].

Unexplored mechanism of gene expression regulation

As shown in Figure 7, the promoter of *PNPLA3* gene has a typical chromatin structure [a peak of histone 3 lysine 4 trimethylation (H3K4me3) between the bimodal peaks of H3K4me1] and is DNase hypersensitive. The gene lies between two CCCTC-binding factor (CTCF)-bound sites that could be tested for insulator activity. An intronic H3K4me1 peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. The presence of a CpG island is typical of an active

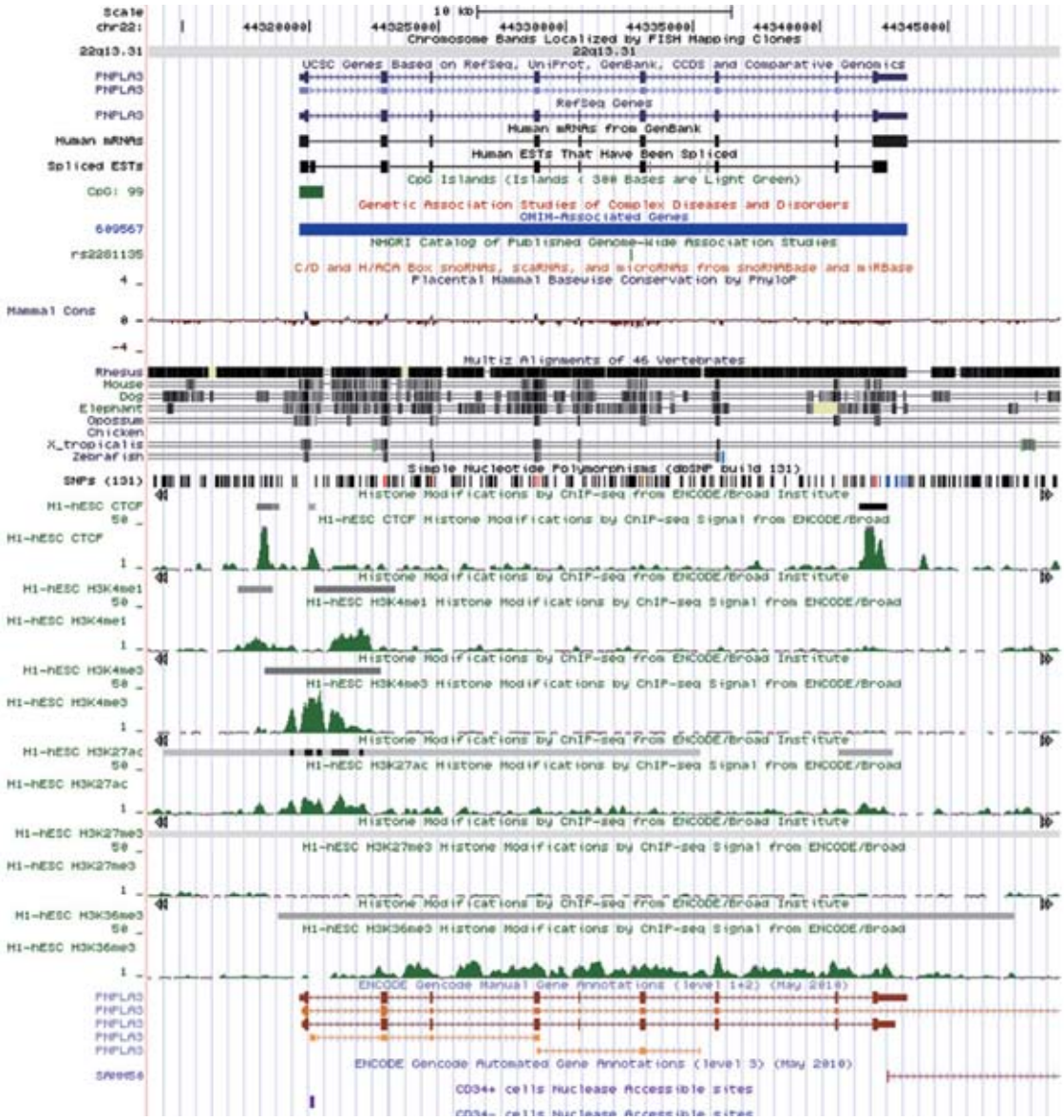

Figure 7 Prediction of patatin-like phospholipase domain containing 3 gene structure extracted from The University of California-Santa Cruz (UCSC) Genome Browser. http://www.genome.ucsc.edu/.

Table 2 *In silico* prediction of rs738409 C or G alleles as putative and differential target sites of microRNA

rs738409-C miRNA	rs738409-G miRNA
hsa-miR-1233	hsa-miR-1181, hsa-miR-1295, hsa-miR-1298, hsa-miR-133a, hsa-miR-133b
hsa-miR-1249	hsa-miR-135a, hsa-miR-136, hsa-miR-139-5p, hsa-miR-190b, hsa-miR-222
hsa-miR-129-5p	hsa-miR-297, hsa-miR-324-5p, hsa-miR-331-5p, hsa-miR-34c-5p, hsa-miR-362-5p
hsa-miR-155	hsa-miR-370, hsa-miR-370, hsa-miR-376b, hsa-miR-384, hsa-miR-412, hsa-miR-432
hsa-miR-365	hsa-miR-449a, hsa-miR-449b, hsa-miR-452, hsa-miR-455-5p, hsa-miR-509-3-5p
hsa-miR-433	hsa-miR-511, hsa-miR-513a-5p, hsa-miR-513b, hsa-miR-515-3p, hsa-miR-516a-3p
hsa-miR-498	hsa-miR-516b, hsa-miR-518d-5p, hsa-miR-519b-5p, hsa-miR-519c-5p, hsa-miR-519e
hsa-miR-517a	hsa-miR-526a, hsa-miR-550, hsa-miR-552, hsa-miR-554, hsa-miR-557, hsa-miR-579
hsa-miR-517c	hsa-miR-582-5p, hsa-miR-584, hsa-miR-593, hsa-miR-600, hsa-miR-600, hsa-miR-601
hsa-miR-578	hsa-miR-609, hsa-miR-613, hsa-miR-623, hsa-miR-623, hsa-miR-623, hsa-miR-629
hsa-miR-586	hsa-miR-632, hsa-miR-636, hsa-miR-642, hsa-miR-642, hsa-miR-654-5p, hsa-miR-659
hsa-miR-874	hsa-miR-661, hsa-miR-663b, hsa-miR-664, hsa-miR-668, hsa-miR-760, hsa-miR-875-5p

Prediction was assessed by PITA miRNA prediction tool. Each allele was represented by at least 60 bp around the polymorphic base using a seed of 7 bp.

promoter. The coding sequence is well conserved across species and there is no known alternative splicing of the

Table 3 Results of search for miRNA target sites on the 3' untranslated region of the patatin-like phospholipase domain containing 3 gene by DIANA-microT v3.0 prediction tool

miRNA name	Binding category	UTR start	UTR stop	UTR binding NTs
hsa-miR-769-3p	8mer (pos 1)	89	117	GCC GAC U G GGAUCCCAG
hsa-miR-769-3p	7mer (pos 2)	389	417	GAGA GAUCCCA
hsa-miR-769-3p	7mer (pos 1)	699	727	G CAG GGC CGG AUCCCAG
hsa-miR-769-3p	7mer (pos 1)	834	862	G CA CCUG AUCCCAG
hsa-miR-516a-3p	8mer (pos 1)	128	156	UGAA AGGAAGCA
hsa-miR-516a-3p	7mer (pos 2)	216	244	GCU UUGGG AGGAAGC
hsa-miR-516a-3p	8mer (pos 2)	591	619	UG AAGGAAGC

mRNA. PNPLA3 is an OMIM-related gene (number 609567). These data were extracted from The University of California-Santa Cruz (UCSC) Genome Browser.

Concluding remarks and future research directions: Possible role for rs738409 alleles in modifying miRNA target sites

SNPs associated with polygenetic disorders, such as NAFLD, can destroy or create miRNA binding sites. We hypothesize that disruption of miRNA target binding by rs738409 alleles may play a role in the effect of the gene variant on fatty liver disease susceptibility. To test this, we analyzed *in silico* whether the *rs738409 C* or *G alleles* have the ability to modify miRNA binding sites and miRNA gene regulation by using the PITA microRNA prediction tool (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html). We observed that rs738409 alleles show potentially different miRNA binding sites (Table 2), suggesting a putative different role in regulation of gene regulation; a hypothesis that has to be proven experimentally. Finally, although these data do not explain the association of the rs738409 variant with NAFLD, it is worth noting that prediction of PNPLA3 target miRNA by DIANA-microT v3.0 (http://diana.cslab.ece.ntua.gr/microT/) shows two miRNAs potentially interacting in the 3'UTR region (hsa-miR-769-3p and hsa-miR-516a-3p) (Table 3).

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Human endogenous retroviruses and cancer: Causality and therapeutic possibilities

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Abstract

A substantial part of the human genome is derived from transposable elements; remnants of ancient retroviral infections. Conservative estimates set the percentage of human endogenous retroviruses (HERVs) in the genome at 8%. For the most part, the interplay between mutations, epigenetic mechanisms and post-transcriptional regulations silence HERVs in somatic cells. We first highlight mechanisms by which activation of members of several HERV families may be associated with tumor development before discussing the arising chances for both diagnosis and therapy. It has been shown that at least in some cases, tumor cells expressing HERV open reading frames (ORFs) thus gain tumor-promoting functions. However, since these proteins are not expressed in healthy tissues, they become prime target structures. Of potential pharmacological interest are the prevention of HERV transposition, the inhibition of HERV-encoded protein expression and the interference with these proteins' activities. Evidence from recent studies unequivocally proves that HERV ORFs represent a very interesting source of novel tumor-specific antigens with even the potential to surpass entity boundaries. The development of new tumor (immune-) therapies is a very active field and true tumor-specific targets are of outstanding interest since they minimize the risk of autoimmunity and could reduce side effects. Finally, we postulate on main future research streams in order to stimulate discussion on this hot topic.

Key words: Human endogenous retroviruses; Gastrointestinal cancer; Therapeutic targets; Tumor-specific antigens; Tumorigenesis

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HUMAN ENDOGENOUS RETROVIRUSES: AN INTRODUCTION

Human endogenous retroviruses (HERVs) are remnants of ancient retroviral infections. Many insertions into the genome have taken place tens of millions of years ago[1,2]. Since the first set of data on the human genome project has been published in 2001, it is well established that about 8% of the genome consists of HERVs and their total number is approximately 3×10^5 copies[3]. Generally, HERVs are classified into three groups:

class I [gamma (like) retroviruses], class II [beta (like) retroviruses] and class III [spuma (like) retroviruses][1]. The most common nomenclature utilizes the single-letter amino acid code corresponding to the tRNA primer that is used for reverse transcription of the HERV genome[4]. For the most part, their canonical structure of a single open reading frame (ORF) consists of the *gag*, *pol* and *env* genes flanked by 5' and 3' long terminal repeats (LTR)[2,5]. The latter features are what endogenous and exogenous retroviruses have in common.

ENDOGENIZATION

Following a retroviral infection, the fate of the host depends on the pathogenicity of the infectious virus. Highly pathogenic ones will kill the host whereas ones with only weak pathogenicity may manage to infect many different cell types, including reproductive tissue cells. Subsequently, an endogenous retrovirus will establish if virus and host proceed to fixation of the virus' sequences in the host genome. This process has been termed endogenization or molecular domestication[6]. In most cases, HERV activities have been silenced by a variety of mechanisms. Mutational inactivation includes deletions as well as point mutations and probably has been triggered by specific regulatory proteins such as APOBEC[7,8]. Furthermore, epigenetic mechanisms including methylation and histone modification contributed to HERV inactivation[9]. Besides directly silencing the expression, posttranscriptional regulation further protects the host's genome[10]. It has been argued that the presence of such a high number of HERV copies must be advantageous for the host, too[11]. An amazing idea suggests that over time of evolution, retroelements like HERVs actively contribute to the development of novel physiological capacities[12,13]. It is for example easily imaginable that a *de novo* ORF which basically encodes a membrane protein may give rise to a protein with novel functions when mutated. If such a protein is beneficial to the host, it will be fixed. Thus, HERVs are together with other mobile genetic elements drivers of the (human) evolution by providing material for genomic evolution, variation and natural selection[6,12,14]. This argumentation adds another level of complexity to the relationship of humans (and all other vertebrates) and retroviruses[15]. Consequently, HERVs must not be considered as parasites but as true symbionts - on the population level. However, the individual risk for *de novo* insertions is rather low with estimated rates of only 1 in 100 births[16].

PRESERVED FUNCTIONS

Although the vast majority of HERV sequences have been inactivated over time as outlined above, there are some examples of HERVs with potentially useful functional modules; comparable to the proviruses of their exogenous counterparts. Among the cellular functions influenced by HERVs are enhancement and promotion of gene expression. In a study on primate evolution ERV-9 LTR sequences were found in higher primates and humans. In the latter, tissue specific enhancer activity could be detected in hematopoietic cells and even stronger in embryonic cells[17]. HERV-E LTR functions as enhancer for endothelin B receptor and apolipoprotein C- I genes in humans[18]. Furthermore, HERV sequences also give rise to novel or alternative splicing and polyadenylation sites[19]. They also can be involved in membrane fusion, with Syncytin in the placenta being the most prominent representative here of[20].

HERV AND CANCER

A variety of oncogenic mechanisms have been attributed to animal oncogenic retroviruses[21,22]. Moreover, it has been suggested, that failures and errors in single somatic cells' efficiency to control HERV activity potentially results in genome damage and may thus contribute to the formation of cancer[14]. The possible oncogenic mechanisms of HERVs include (Figure 1): (1) the general or more specific (re)activation of HERV sequences by hypomethylation[23-25]; (2) the expression of HERV encoded oncogenes such as Rec and NP9[26]; (3) the inactivation of tumor suppressor genes by *de novo* insertion or translocation of retroelements within the genome[26]; (4) the regulation of nearby (proto-) oncogenes or growth factors by the regulatory sequences of LTRs[27,28]; and (5) the potential of Env proteins to induce cell fusions, which may contribute to tumor progression or even aid in metastasizing processes[29]. Far from being complete, this is already a quite impressive list. An additional aspect comes from the observation that Env proteins of the mouse leukemia virus, the Mason-Pfizer monkey virus and also of HERV-K have strong immunosuppressive properties and may thus help tumor cells evade an antitumoral immune response[26,30,31].

Methylation

As a general rule, all human regulatory genomic sequences become methylated unless specific factors prevent methylation[23]. In addition, methylated sites are more prone to mutations[23] and by this means, virus inactivation is further strengthened. Demethylation of regulatory regions is possible in the context of normal physiological processes by strong transcriptional activators. Re-expression of methylated sites is also possible during cell stress dependent on chromatin remodeling as a reaction to this stress[32]. Obviously, the maintenance of methylation patterns and status must play a central role in HERV transcriptional control. In healthy somatic and mature germ cells HERV sequences are generally (hyper-) methylated. Thus, HERV transcriptional activity is mainly restricted to germ cell development or the desensitization of check-point activation in meiotic cells. This mechanism may also be responsible for a high(er) retroelement expression in germ cell tumors[23]. In somatic cells, severe global hypomethylation

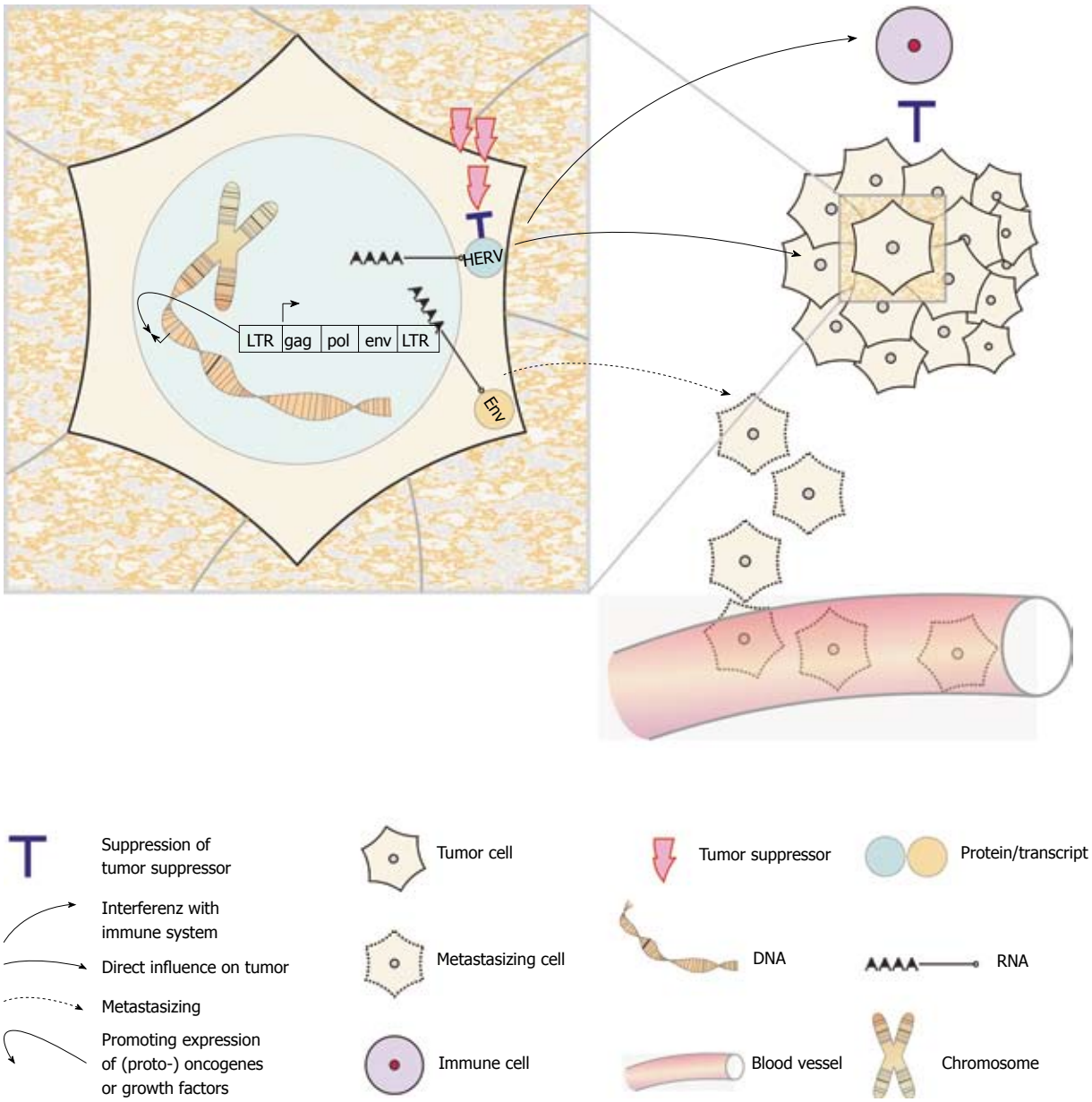

Figure 1 Possible mechanisms by which human endogenous retroviruses contribute to oncogenesis. Human endogenous retroviruses (HERVs) transcripts or proteins may directly have tumor promoting properties. The long terminal repeat (LTR) elements can function as promotors or enhancers for nearby (proto-) oncogenes or growth factors. Especially Env proteins might attract regulatory immune cells and thus provide an immunosuppressive microenvironment. And finally, the Env proteins may be directly involved in the metastasizing process.

leads to apoptosis induction mediated by TP53 and other tumor suppressive factors[33]. Premalignant and malignant cells are typically insensitive to apoptosis induction[34] and aberrant expression from normally methylated promoters is a main oncogenic force. In line with this, a general hypomethylation of HERV sequences can be found in the cancer cells of different entities, including testicular germ cell cancer, teratocarcinomas, colorectal, breast and ovarian cancer[35-39]. However, methylation analyses are biased by a lack of accuracy of the bisulfide sequencing technique[40]. When not highly standardized, this may account for a number of false positive or negative results in methylation analyses. Thus, it is always recommended to combine methylation analyses together with an investigation of mRNA or superior protein expression.

HERV ORF expression

Of interest, Syncytin-1 is the only expressed HERV sequence with a presumable physiological function. Syncytin-1 expression takes place in the placenta in the context of syncytiotrophoblast generation by cellular fusion of precursor cells, the cytotrophoblasts[41,42]. This expression follows after a general hypomethylation of a HERV-W *env* sequence and the Env protein is considered to contribute to this cellular fusion process. It may be a coincidence, but for many tumor entities, naturally occurring cellular fusions have been described[43-45] and this may hint towards the expression of similar HERV Env proteins.

Expression of HERV sequences has been described for several tumor entities including melanoma, breast, ovarian, prostate and colon cancer[37-39,46,47]. Active retrotranspositions cause DNA strand-breaks and will thus lead to an activation of check-point signaling, e.g., TP53. Thus, transpositions as another mechanism for HERV re-expression may consequently occur especially in tumors with defect check-points and TP53 mutations[23].

Tumor induction/promotion

Beside sheer tumor specific expression, HERVs have repeatedly been discussed to induce or promote tumorigenesis. Potential mechanisms have been outlined in the preceding paragraphs. Here we want to gather the bits of evidence that have been obtained so far.

Several groups could show the production of HERV-derived proteins or even of viral particles in tumor cells[48-54]. In a mouse study, Howard and coworkers could directly link genome hypomethylation to ERV up-regulation[55]. Further research could make the connection between hypomethylation of (H)ERVs and chromosomal instability; it is by mediating ectopic recombination[56]. These HERV-induced recombination events have been found to produce large scale chromosomal anomalies[57], a hallmark of most tumors[34]. Finally, Lamprecht and colleagues could link the deregulated expression of the colony-stimulating factor 1 receptor (CSF1R) in B cell-derived Hodgkin's lymphoma cells to hypomethylation of an up-stream HERV-derived LTR, which promotes ectopic expression of the CSF1R proto-oncogene[58]. However, the question if the reactivation of a (pro-) virus could actively promote cellular transformation or at least contribute to tumor progression is formally unsolved for human cancer. Similarly, it is unknown, if HERV activation is an early or a late step in tumor formation. Still, when considering the above listed bits of evidence, it seems reasonable to conclude that HERVs' contribution to the multi-step process of tumor development in humans is very likely[14].

Immune responses towards HERV sequences

The human immune system's capability to recognize HERV sequences has so far only scarcely been analyzed. However, some examples can be found in the literature. In patients with kidney cancer, cytotoxic T lymphocytes (CTLs) reactive to a HERV-E sequence encoded on chromosome 6q were found[59]. Serological responses and CTLs reactive to HERV-K sequences were detected in melanoma patients[60,61]. Anti-Env antibodies for HERV-K, -E and ERV3 were present in sera of patients with ovarian cancer[38] and in male patients with germ cell tumors[62]. Similarly, in breast cancer patients, anti-HERV-K serum antibodies were detected together with HERV-K-specific CTLs[63]. The orchestrated activation of both arms of the adaptive immune system in the latter cases is a strong indicator of HERV sequences' high immunogenicity. Consequently, one may conclude that at least no strong tolerance towards HERV encoded sequences is induced during lymphocyte development. Future studies will have to analyze whether the immunological recognition of HERV sequences is executed by highly avid or only by intermediate avid T cells and antibodies. Also, it must be carefully analyzed, which HERV sequences give rise to strong immune responses when aberrantly expressed in tumor cells. We would like to state that immune recognition is a strong indicator for endogenous expression of a given HERV protein, as has been shown for other tumor antigens[64]. Of note, T cell reactions against HERVs, such as HERV-K, HERV-L and HERV-H, were associated with successful control of human immunodeficiency virus (HIV) in a subset of HIV patients[65]. It can be anticipated that this association of successful HIV control by HERV specific immune reactions will be translated into the tumor field. One of the major questions with clinical relevance is whether HERV-specific immune signatures can be associated with better prognosis or not.

THERAPEUTIC STRATEGIES

Assuming that in the normal physiology of adult tissues, HERVs do not play a vital role and following the line of evidence that HERV sequences are of significance in tumor formation, development and metastasis, HERVs recommend themselves as prime targets for tumor therapy. Several targeting strategies have been suggested (Figure 2 for an overview) and first experimental results can be found in the literature.

Inhibition

In the light of the tremendous success in HIV control with infected people treated by antiretroviral combination therapies, it would make sense to simply reverse the expression of HERV sequences in human tumor cells. The group of Carlini analyzed the effect of a reverse transcription inhibitor (Abcavir) on prostate cancer cell lines[66]. It showed a strong anti-proliferative capacity and even triggered senescence in the cancer cells. Interestingly, the authors found an up-regulation of transcripts from LINE elements in the treated cells but unfortunately, they did not analyze HERV expression[66].

A direct targeting of HERV proteins by small molecular inhibitors or via RNA interference would also be worth trying. However, this has not yet been done. Therapeutical use of natural inhibitors of retroviruses such as APOBEC[67,68] or TRIM5[69] would be another possible future option. First, detail knowledge on how and when such retroviral restriction elements act on HERVs must be build up.

Passive immune therapy

Only very recently, Wang-Johanning and coworkers designed a monoclonal antibody (mAb) recognizing a HERV-K Env protein. They described that HERV-K Env protein expression was substantially higher in malignant breast cancer cell lines than in non-malignant breast cells. Furthermore, HERV-K expression was detected in 148

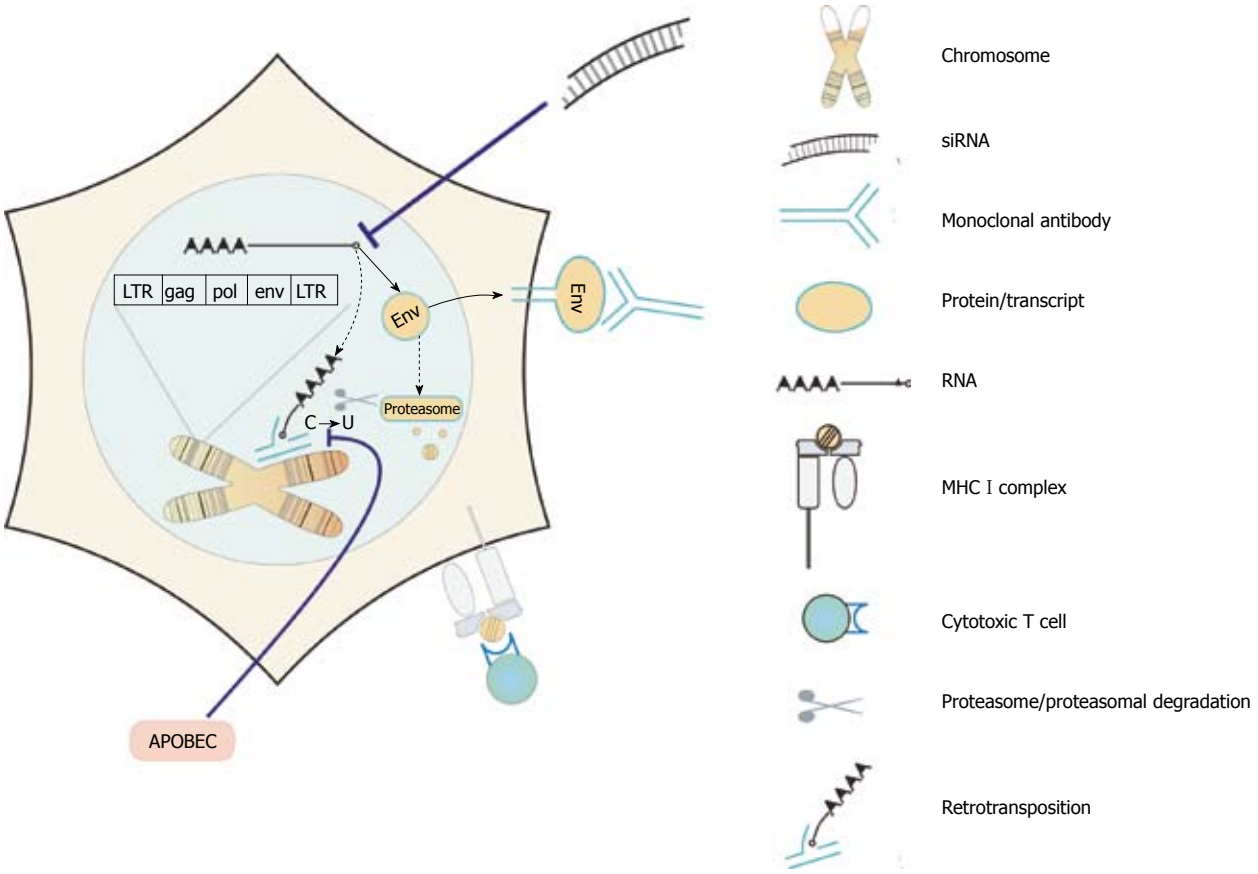

Figure 2 Therapeutic possibilities to target tumor cells with active human endogenous retroviruses. Expressed Env proteins may be targeted by therapeutic monoclonal antibodies. (Re-) activation of retroelement-controlling proteins may help to reduce human endogenous retrovirus (HERV) activities. Small molecule inhibitors of HERV proteins or inhibitory targeting of expressed HERV sequences potentially will prevent oncogenic properties of HERV and harm or kill tumor cells with activated HERV oncogenesis. HERV proteins with tumor-specific antigen properties can be targeted by specific T cells. LTR: Long terminal repeat; MHC: Major histocompatibility complex.

(66%) of 223 primary breast tumors. And a higher rate of lymph node metastasis was associated with HERV-K-positive tumors. Anti-HERV-K-specific mAbs inhibited tumor growth and induced apoptosis of breast cancer cells *in vitro*. Mice treated with these mAbs showed significantly reduced growth of xenograft tumors. *In vitro*, this treatment resulted in an over-expression of several proteins involved in the apoptotic signaling pathways in malignant breast cells[70]. In principle, targeting HERV Env proteins by therapeutical antibodies should be exploitable to all individual tumors expressing HERV Env. Moreover, passive immune therapies may well be applied in combination with active immune therapies.

Active immune therapy

The ideal cancer therapeutic agent should be able to discriminate between cancer and normal cells (i.e., specificity) and be potent enough to kill small or large numbers of tumor cells (i.e., sensitivity). A feature that makes immunotherapies unique is that an ideal cancer immunotherapy should be able to prevent recurrence of the tumor (i.e., durability). In the last decades it became increasingly apparent that this durability in prevention of tumor recurrences is due to persistent recognition of tumor antigens by lymphocytes.

Researchers distinguish between tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). TAAs are antigens that are expressed in normal tissue but to a much higher extent in malignant cells. Contrary to this, TSA are truly specifically expressed in tumor cells alone. Beside specific point[71] or frameshift mutations[72], proteins from tumor-inducing viruses[73] for the most part form this class of tumor antigens. Most of the features an ideal TSA should possess have been assigned to HERV encoded proteins. This being beside exclusivity also the necessity of expression for maintenance of the cancer cells' transformed state. Thus, immune-escape by simple down-regulation of expression is prevented[74]. Moreover, to ease therapy development, ideal TSA expression should be not only present in single tumors but shared between individual tumors of a given entity or even superior between tumors of different entities[75,76]. Finally, the immune system should be able to mount both a cellular and a humoral response[63]. When summing up these desired properties of TSAs attributable to (at least some) HERV-encoded proteins, one may conclude that they might indeed be ideal targets for tumor immunotherapy. Because of the multitude of HERV-

encoded sequences one can even expect that the development of a polyvalent (i.e., containing many epitopes) vaccine basing only on HERV epitopes may be possible. Even more visionary, actual bioinformatics approaches will allow the identification of immunogenic core epitopes shared between different HERV copy ORFs active in different tumor entities in order to design a universal HERV-based vaccine. As a first step in that direction, we recently described two $CD8^+$ T cell epitopes encoded by a HERV-H copy located on Xp22.3[77].

FUTURE PERSPECTIVES

At the moment, in the field of HERVs more questions are open than answered. Are there human (tumor-) cells producing virus particles? If so, are those particles infectious? Further analyses on expression of HERV sequences and proteins - and in especially of Env proteins - would add to the full picture and understanding of the relationship of tumors and endogenous retroviruses. In a first step the mutual interaction between HERV Env and the immune system, also in a suppressive manner, has been addressed[26,30,31]. These analyses should be expanded. Especially a broader knowledge on tumor infiltrating cells specific for HERV epitopes and their prognostic value would be interesting. Furthermore, it would be very beneficial to know if there is a correlation between tumor grade, stage, progression or outcome and the expression of HERV sequences.

CONCLUSION

Our understanding of HERVs has come a long way. They must be considered as domesticated retroviruses with even main functions in evolution. On the level of an individual human being, however, their activities most likely are tightly controlled. Heavy genetic disorders, as present in tumor cells, generally seem to be linked with HERV activities. The tumor-specific expression of HERV-encoded proteins opens the way to diagnostically and therapeutically interesting opportunities: (1) The targeting of HERV proteins either biochemically or immunologically as TSAs; (2) Immune recognition of tumor cells takes place already early in tumor development. HERV-encoded ORF-derived proteins are likely candidates of this early recognition. Consequently, they may be ideal for screening people at risk to develop cancer as we suggested for frameshift mutations in lynch syndrome[72,78]; (3) the recognition of expressed HERV sequences by the adaptive immune system is likely to result in a better prognosis for patients raising to-be-defined minimum levels of immune responses. Such HERV-specific responses may well be suited for prognostic purposes.

POSTULATES

We would like to take the chance and hypothesize on some of the open questions and obvious tasks in the HERV/tumor field: APOBEC and other retroelement controlling factors are likely to be inactivated in cancer cells with active HERV-driven oncogenesis. If this is frequently the case, they must be considered tumor suppressor genes and screening for their inactivation would possibly hint towards specific HERV activation.

HERV-encoded TSAs are released into the circulation[79] and thus screening of HERV-TSA blood levels will become an interesting field of investigation. Similarly, HERV-specific (immune-) therapies will be developed in the near future for several tumor entities. For these immunotherapies, beside knowledge about expression in different tumors, the level of tolerance towards HERV-TSAs will guide the decision on which candidates to investigate in clinical trials.

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REVIEW

Celiac disease: Prevalence, diagnosis, pathogenesis and treatment

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Abstract

Celiac disease (CD) is one of the most common diseases, resulting from both environmental (gluten) and genetic factors [human leukocyte antigen (HLA) and non-HLA genes]. The prevalence of CD has been estimated to approximate 0.5%-1% in different parts of the world. However, the population with diabetes, autoimmune disorder or relatives of CD individuals have even higher risk for the development of CD, at least in part, because of shared HLA typing. Gliadin gains access to the basal surface of the epithelium, and interact directly with the immune system, *via* both trans- and para-cellular routes. From a diagnostic perspective, symptoms may be viewed as either "typical" or "atypical". In both positive serological screening results suggestive of CD, should lead to small bowel biopsy followed by a favourable clinical and serological response to the gluten-free diet (GFD) to confirm the diagnosis. Positive anti-tissue transglutaminase antibody or anti-endomysial antibody during the clinical course helps to confirm the diagnosis of CD because of their over 99% specificities when small bowel villous atrophy is present on biopsy. Currently, the only treatment available for CD individuals is a strict life-long GFD. A greater understanding of the pathogenesis of CD allows alternative future CD treatments to hydrolyse toxic gliadin peptide, prevent toxic gliadin peptide absorption, blockage of selective deamidation of specific glutamine residues by tissue, restore immune tolerance towards gluten, modulation of immune response to dietary gliadin, and restoration of intestinal architecture.

Key words: Celiac disease; Demography; Diagnosis; Pathogenesis; Treatment

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INTRODUCTION

Celiac disease (CD) is a life-long gluten-sensitive autoimmune disease of the small intestine affecting genetically susceptible individuals worldwide. CD individuals may present gastrointestinal symptoms, extraintestinal symptoms or no signs of symptoms. The classical symptoms include gastrointestinal-related symptoms such as diarrhea, steatorrhea and weight loss due to malabsorption. About 50% of CD patients present extraintestinal or

atypical symptoms, such as anemia, osteoporosis, dermatitis herpetiformis, neurological problems and dental enamel hypoplasia[1-3]. The variable clinical picture of CD is due to having both genetical and immunological bases, with age of onset, extent of mucosal injury, dietary habits, and gender[4], affecting the clinical manifestation of the disease.

CD diagnosis is based on presence of predisposing genetic factor human leukocyte antigen (HLA) DQ2/8, with positive biopsy and serological antibodies upon gluten contained diet. The spectrum of CD may present in different forms[5]. The classical form may be diagnosed at any age of life and is often characterized by crypt hyperplasia and villous atrophy along with features of malabsorption. The atypical form is characterized by positive celiac serology, limited abnormalities of the small intestinal mucosa or no intestinal symptoms, but associated extraintestinal conditions such as osteoporosis, peripheral neuropathy, anemia and infertility. The latent form is defined by presence of predisposing gene HLA-DQ2 and/or HLA-DQ8, normal intestinal mucosa and, possible positive serology. Extraintestinal features and biopsies of the small bowel show alterations with gluten intake (i.e., gluten-sensitive). Rarely, usually after age 50 years[6], some that have initially responded to a gluten-free diet (GFD), develop recurrent symptoms and biopsy changes despite a GFD. This is the refractory form[7]. If no response to GFD was initially documented, however, the use of sprue-like intestinal disease or unclassified sprue has been used.

PREVALENCE

CD originally thought to almost exclusively affect white Europeans, is now known to be widely distributed worldwide[8]. Epidemiological studies conducted in areas supposedly free of CD, including Africa, the Middle East, Asia, and South America, show that the disease was previously underdiagnosed[9]. This provides evidence that CD is one of the most common genetic diseases, resulting from both environmental (gluten) and genetic (HLA and non-HLA genes) factors.

The world distribution of CD seems to have followed the mankind wheat consumption and the migratory flows. Man originally fed on meat, fruit and vegetables, with no exposer to gluten-containing cereals. It was only about 10 000 years ago in a small region called the "Fertile Crescent" of the Middle-East (including Anatolia (Southern Turkey), Lebanon, Syria, Palestine and Iraq) where wild wheat and barley grains successfully cultivated due to favorable environmental conditions. In the Fertile Crescent some tribes changed from nomadic to stable settlement style of living because land cultivation permitted food storage, and later migrated westwards to obtain new lands for cultivation. These persons spread through the Mediterranean area (Northern Africa, Southern Europe) and Central Europe. The expansion continued from 9000 to 4000 BC by which time the cultivation of wheat and barley had spread all over the Old Continent, also reaching Northern Europe (Ireland, Denmark and the Scandinavian countries). This expansion in farming was due to the diffusion of agricultural practices and replacement of local inhabitants by descendants from the Middle-East[10]. Hence, the European and North-African populations share genetic background with the peoples of Middle-East origin.

In the last few years a number of studies in different populations have been carried out using molecular genetics methods to identify genes causing CD. CD-predisposing genetic loci are *CELIAC1* on chromosome 6, *CELIAC2* on chromosome 5q31-33[11], *CELIAC3* on chromosome 2q33[12], and *CELIAC4* on chromosome 19p13.1[13]. *PARD3* and *MAGI2* tight junction genes associations have also been reported in Dutch CD or ulcerative colitis patients, suggesting a common intestinal defect in these two conditions[14]. Another gene expressed in major histocompatibility complex (MHC) I antigen presenting cell is *HLA B8*, was found to be associated with CD in Algeria[15], Iraq[16,17] and Turkey[18]. Moreover, atypical CD Saharawi patients were found to over-express the MHC class I chain-related gene A (MICA) allele 5.1[15], which have also been reported in Western countries[19]. Increased prevalence of HLA-A25 in Turkish children with CD was also reported, suggesting that this genotype is particularly encountered among this population[18], with no association described in Western countries.

Useful Background: Genes causing CD CELIAC1 on chromosome 6 (*HLA-DQ2* and *HLA-DQ8*); CELIAC2 on chromosome 5q31-33[11]; CELIAC3 on chromosome 2q33 (containing T lymphocyte regulatory genes *CD28*, *CTLA4* and *ICOS*)[12]; and CELIAC4 (myosin IXB gene, *MYO9XB*) on chromosome 19p13.1[13].

HLA genotype contributes to the genetic risk for CD at 30%-50%[20,21]. Non-HLA genes contribute more evidence to the CD genetic background than the HLA genes, but each by itself contributes only a modest to the disease development. Hence, it is reasonable to assume that the susceptibility to CD involves with polymorphic genes that influence the immune response to gluten, as shown for the HLA-linked genes[22].

Ninety percent of European patients with CD carry the HLA-DQ2 molecule, encoded either in cis on the HLA-DQA1*0501-DQB1*0201 haplotype (HLA-DQ2.5cis) or in trans on the HLA-DQA1*0505 DQB1*0301/DQA1*0201-DQB1*0202 haplotypes (HLA-DQ2.5trans). Approximately 5% express HLA-DQ8, encoded by HLA-DQA1*0301-DQB1*0302. The majority of the remainder carry the HLA-DQA1*0201-DQB1*0202 haplotype[20]. With genetic testing, DQ2 is almost synonymous with DQB1*02, a gene with two common alleles designated DQB1*0201 and DQB1*0202. The DQ2 frequency in Caucasian in Western Europe populations has been estimated at 20%-30%, and relatively high frequencies also occur in Northern and Western Africa, the Middle East and central Asia[23]. Thereafter, the overall frequency of DQ2 declines from West to East with low frequencies in

Table 1 Frequency of human leukocyte antigen-DQ2, encoded by human leukocyte antigen-DQB1*02 and human leukocyte antigen-DQ8, encoded by human leukocyte antigen-DQA1*0301-DQB1*0302

< 5%	5%-20%	20%
HLA-DQ2		
Albania	Belarus Algeria	Algeria
Canada BC (Athabaskans)	Cameroon	Australia
Cook Islands	Congo	Belgium
Indonesia	Costa Rica	Central African Republic
Japan	China	Croatia
Jordan	Cuba	England
Papua New Guinea	Ecuador Africans	Equatorial Guinea Bioko Island
Philippines	France	Ethiopia
Samoa	India	Germany
	Malaysia	Greece
	Mexico	Iran
	Poland	Ireland South
	Russia	Israel
	Singapore	Italy
	South Korea	Mongolia
	Spain	New Zealand
	Sri Lanka	Pakistan
	Sweden	Saudi Arabia
	Taiwan, China	Slovenia
	Thailand	Tunesia
	Turkey	United States
	Uganda	
	Ukraine	
	Vietnam	
HLA-DQ8		
Australia	Algeria	Argentina
China	Belgium	Ecuador
Georgia	Brazil	Ethiopia
Greece	Canada BC (Athabaskans)	Mexico
North India	Croatia	Venezuela
Spain	England Caucasoid	
Uganda	France	
	South India	
	Israel	
	Italy	
	Japan	
	Russia	
	South Korea	
	Tunisia	
	Turkey	
	Ukraine	
	United States	
	European American	

Estimates are based on studies included in a comprehensive Internet website[24]. In several countries, the frequency is not known. HLA: Human leukocyte antigen.

populations in South-East Asia and the virtual absence of DQ2 in Japan (Table 1). DQ8 frequency has a worldwide distribution, whereas DQ2.5, is common in South and Central America; approximately 90% of Amerindian populations carry DQ8 and may display the celiac phenotype[24,25]. The frequency of DQ8 population is shown in Table 1.

In the past, the prevalence of CD had been underestimated, but it is now regarded one of the most common genetic disorders in the West with 1% prevalence[26-28]. In-

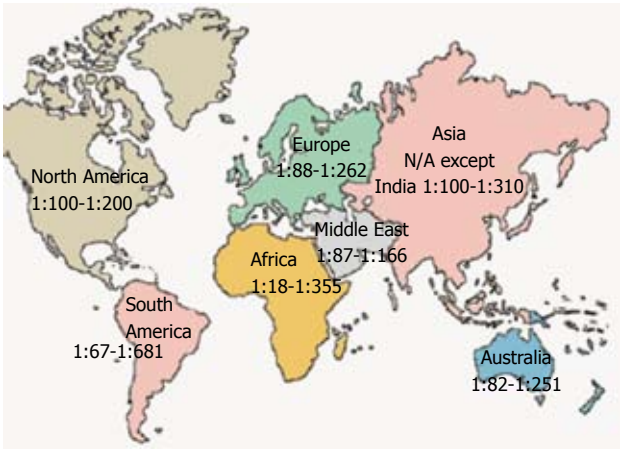

Figure 1 Prevalence of celiac disease worldwide. N/A: Not available.

terestingly, there is increased prevalence of CD amongst women compared to men with male:female ratio of 1:2.8[29]. This could be due to the finding that men with CD were diagnosed at an older age[30]. Indeed, there have been reported CD cases among immigrant children native of Eastern Europe, Northern, West and East Africa, the Middle East and Southern Asia, according to their acquisition of Western dietary practices (i.e., short period or lack of breast feeding and early weaning with a great amount of gluten intake)[31]. This suggests that many persons may have the genetic predisposition to CD but the clinical presentation only occurs when there is sufficient gluten in the diet.

Normal at-risk persons

In several parts of the world, the presence of the combination of antibody (serum tissue transglutaminase and endomysial autoantibodies) positivity and an HLA haplotype associated with CD is predictive of small-bowel abnormalities indicative of CD. For the majority of countries, the CD prevalence is unknown. Figure 1 shows a range of estimated normal at-risk CD prevalence in continents and nations around the globe. It must be noted that some studies report prevalence of CD based on serology, others on celiac compatible small bowel biopsies and a few on serology, biopsy and response to gluten challenge.

North America

CD prevalence in North American and Europe were found to be similar in symptomatic patients and not-at-risk subjects. In the United States, CD is believed to affect 0.5%-1.0% of the general population[32]. The study by Fasano *et al*[28] on serum antibody and biopsy screening were performed for a total of 13 145 United States subjects: first-degree (n = 4508) and second-degree relatives of patients (n = 1275) with biopsy-proven CD, symptomatic patients (n = 3236) (with either gastrointestinal symptoms or a disorder associated with CD), and not-at-risk individuals (n = 4126). The overall CD prevalence is 1:133 in the not-at-risk groups, whereas in the at-

Table 2 Prevalence of celiac disease in Europeans based on unselected population serological screenings[36-38] (adapted)

Countries	Prevalence
Czechoslovakia	0.193
Estonia	0.103
Finland	0.110
Hungary	0.101
Ireland	0.126
Italy	0.115
Norway	0.224
Portugal	0.135
Spain	0.124
Sweden	0.174
Switzerland	0.133
Netherlands	0.179
United Kingdom	0.111

risk group, the prevalence is 1:22 in first-degree relatives, 1:39 in second-degree relatives, and 1:56 in symptomatic patients[28].

South America

In South America, CD had been historically considered a rare disorder and the prevalence investigations have not been extensively studied. However, during the last few years studies in Brazil disclosed a prevalence of 1:681 in healthy blood donors[33] and 1:473 among adult outpatients attending a clinical laboratory for routine blood testing[34]. In an urban area of Argentina, the overall prevalence of CD, among 2000 adults from the general population (996 women; median age 29 year, range 16-79 year) was 1:167, with prevalence in women double that for men[35]. The high CD prevalence in Argentina could be correlated with HLA DQ8 (> 20%) in the Argentina population.

Europe

The overall prevalence of CD in Western populations is close to 1% (1:100) and may be higher in Northern European countries (Table 2)[36-38]. The Scandinavian countries, Ireland, and the United Kingdom population tended to show a higher prevalence of CD of approximately 1.0%-1.5%, although there also were studies that showed a lower prevalence in these countries. A study of small-intestinal biopsy obtained from healthy Dutch blood donors at Arnhem and Nijmegen Blood Donation Centers shows that the prevalence of CD-compatible biopsies of 1:330[39]. The prevalence of CD among 3654 children (age range, 7-16 years) in Finland was at least 1:99 based on serum autoantibodies and small-bowel abnormalities[40]. The prevalence of CD in Northern Spain in the general population was 1:389[41], 1:132 (0.75%) in Eastern Switzerland adolescents[42].

Africa

In Northern African populations (including Morocco, Algeria, Tunisia, Libya and Egypt) higher incidences of 0.28%-5.6% of CD have recently been reported in the general population[43-46]. The prevalence of CD in asymptomatic Tunisian school children was estimated to be about 1:157, which is close to the European prevalence. In this respect, the highest world frequency of 16.4% is reported in the CD associated with Insulin Dependent Diabetes Mellitus[47], in Oran (Algeria). A recent serological screening in 2500 Tunisian healthy blood donors[45], showed similar that the prevalence of anti-endomysium antibodies in the general population of 1:355, to that of Europeans. Due to high wheat and barley consumption in the North American countries[45], as well as high frequency of CD predisposing DR3-DQ2 haplotypes in these populations[48-50], these high CD frequencies are not surprising.

Saharawi population in North Africa, who are of Arabian and Berberian origin, having a high degree of cognation and live as refugees in the Sahara desert (Algeria), has the highest prevalence of CD (5.6%) known in the world today[43,49]. This elevated prevalence in this population may be explained both by genetic factors: very high frequency of the DR3-DQ2 haplotype, and by environmental factors: changed of dietary habits in the last few decades. The reduced rates and duration of breast-feeding and increased consumption of gluten in early life as part of the staple diet, supplied by Western countries as humanitarian aids[51], may have played a role in this elevated CD prevalence. However, there are other unknown genetic and environmental factors that explain such a high frequency of CD in the Saharawi people, because there is a much lower prevalence of CD in Sardinia population with similar staple diet consumption and frequencies of DR3-DQ2[52].

Australia and New Zealand

Australia and New Zealand are the two countries having the highest proportion of individuals from Caucasian background, with high prevalence of HLA DQ2 and per capita wheat consumption of > 150 and 75-150 kg per person per year, respectively[23]. Only two prevalence studies have been carried out in these two countries. From a random population of 1064 adults in Christchurch, New Zealand (96% Caucasian), CD was confirmed histologically in all patients with positive serology giving an overall prevalence of 1:82 (1.2%)[53]. A larger study in 3011 adults from a large Caucasian community in Western Australia, revealed an overall prevalence of CD of 1:251 (0.4%) of the population[54].

Asia

CD is likely to be rare in Indonesia, South Korea, Philippines and many smaller Pacific islands because of their low wheat consumption and a low frequency of HLA-DQB1*02. In South-East Asia, HLA-DQB1*02 is often present in more than 5% of the population but CD is predicted to be rare, as staple diets are based on rice. In contrast, prevalence rates that are similar to those in Europe are likely to apply from Pakistan in the South to Kazakhstan in the North. Ancient migration patterns

Table 3 High risk populations for celiac disease[73] (adapted)

Relatives, especially first-degree
Anemia, especially iron deficiency
Osteopenic bone disease
Insulin-dependent diabetes (type 1), especially children
Liver disorders, especially Autoimmune hepatitis and primary biliary cirrhosis
Genetic disorders, including down and Turner's syndrome
Autoimmune endocrinopathy, especially thyroid disease
Skin disorders, particularly dermatitis herpetiformis
Neurological disorders, including ataxia, seizures, myasthenia gravis
Others, including immunoglobulin A nephropathy

that determine the frequency of DQB1*02 would also predict more patients with CD in Western China than in Eastern China[23]. Interestingly, there is one report of CD in three adult descendants of Chinese and Japanese families who migrated to Canada[55].

In genetic studies of CD in India, the appearance of HLA associations is similar to those in Western countries with a frequency of HLA-DQB1*02 of close to 100%[56]. This association is more frequent in the population of Northern and North-Eastern India (16%-27%)[57], than in groups of adults in the Southern state of Tamil Nadu (9%-14%)[58]. The prevalence of CD in India is nearly the same as that in Western Caucasian populations[59]. In Punjab (North-west India) school children, CD frequency was estimated to be 0.3%[60]. This prevalence is probably an underestimation. A retrospective analysis of confirmed cases of CD between 1995 and 2000 in Dayanand Medical College and Hospital (Ludhiana, Punjab) from a total of 202 cases showed an initial of 10 positive cases with a significant growth rate of 79.43% annually with a trend equation increase of 15.49 cases/year[61]. These studies showed that CD is relatively common in Northern India where there has been a history of wheat cultivation from before 1000 BC[62]. Hence, the relative rarity of CD in Southern India reflects the effect of both genetic and environmental factors.

The prevalence in first-degree relatives of North Indian children with CD diagnosed as per the European Society for Pediatric Gastroenterology and Nutrition criteria is 4.4% of the first-degree relatives (85% positive for HLA DQ2/DQ8), which is 14 times higher than that of the general population[63]. There have been reports on clinical experience of biopsy-defined CD in 10 North Indian Immigrants or descendants born in Canada out of 14 Asians diagnosed since 1988 in a single Canadian teaching hospital[55]. Several studies, particularly from Northern and North-West India, have also documented the presence of CD in children presenting with chronic diarrhea[64].

Middle East

It seems likely that the prevalence of CD in the Middle East is similar to that of Europe[65]. CD is a relatively common cause of chronic diarrhea in Iran, Iraq and Kuwait and has been diagnosed in 2%-8% of patients with type 1 diabetes in Iran, Israel and Saudi Arabia[39]. Many of these countries have a per capita wheat consumption that ranks among the highest in the world (> 150 kg per person per year)[23]. Although only a limited number of genetic studies have been carried out, the population of countries such as Iran, Israel, Saudi Arabia and Turkey have a high frequency of HLA-DQB1*02. The prevalence of CD in adult blood donors in Iran, Israel, Syria, Turkey and Anatolia are 1:166[66], 1:157[67], 1:62[68], 1:87[69], 1:100[70], respectively. Similar prevalence rates were determined in surveys of Iranian children (1:165, 0.6%)[71], and Turkish children (1:115, 0.9%)[72].

The prevalence of CD is approximately 0.5%-1% in all parts of the world, except for populations with very low and very high intake of gluten in their diet.

High at-risk persons

In the general celiac population (without classical CD symptoms, e.g., diarrhea or weight loss), there are high risk groups that may have higher CD prevalence rates (Table 3). Among factors that denote a higher risk for CD, the most important factor is familial history of biopsy proven CD with an estimate of 20% or more of first-degree relatives having CD[73]. Some authors observed a higher prevalence in CD siblings as compared to parents[74-76]. A study in Swedish youth (< 20 years old) diagnosed with type 1 diabetes (T1D) confirmed the low prevalence (0.7%) of diagnosed symptomatic CD at initial onset of clinical diagnosis, but document by screening an increasing prevalence of silent CD during a 5-year follow-up to reached an overall prevalence of 10%[77]. Thus, the prevalence of an association with CD in high risk groups may increase over time.

The overall prevalence of CD is highly dependent on the HLA DQ2/DQ8 typing and gluten consumption. The population with positive HLA typing for celiac have high chances of developing celiac symptoms when on high gluten consumption. However, the population with diabetes, autoimmune disorder or relatives of CD individuals) have even higher risk for the development of CD, since they share the same HLA typing.

PATHOGENESIS

CD is an intestinal enteropathy triggered by the ingestion of gliadin and of other related prolamins in genetically predisposed individuals[22,78]. Wheat and related species such as barley and rye also induce CD[79]. A small minority of CD patients also react to oat[80]. Gliadin peptides exert damaging effects since they are resistant to gastrointestinal enzymes[81], they have amino acid sequences that are specific for HLA-DQ2, which is a class Ⅱ major histocompatibility complex, they also have preferred glutamine residues for tissue transglutaminase (tTG)-mediated deamidation[82], and lastly, they affect intestinal permeability[83]. Hence the pathogenesis of CD is dependent on genetic and environmental factors. The environmental factor is mainly ingestion of gluten, while several genes contribute to the genetic predisposition[13]. CD commonly

Table 4 Possible clinical manifestations of celiac disease[8] (printed with permission)

Typical symptoms	Atypical symptoms	Associated conditions
Chronic diarrhea	Secondary to malabsorption	Possible gluten dependent
Failure to thrive	Sideropenic anemia	IDDM
Abdominal distention	Short stature	Autoimmue thyroiditis
	Osteopenia	Autoimmune hepatitis
	Recurrent abortions	Sjogren syndrome
	Hepatic steatosis	Addison disease
	Recurrent abdominal pain	Autoimmune atrophic gastritis
	Gaseousness	Autoimmune emocytopenic diseases
	Independent of malabsorption	Gluten independent
	Dermatitis herpetiformis	Down syndrome
	Dental enamel hypoplasia	Turner syndrome
	Ataxia	Williams syndrome
	Alopecia	Congenital heart defects
	Primary biliary cirrhosis	IgA deficiency
	Isolated hypertransaminasemia	
	Recurrent aphthous stomatitis	
	Myasthenia gravis	
	Recurrent pericarditis	
	Psoriasis	
	Polyneuropathy	
	Epilepsy	
	Vasculitis	
	Dilatative cardiomyopathy	
	Hypo/hyperthyroidism	

IgA: Immunoglobulin A; IDDM: Insulin dependent diabetes mellitus.

appears in early childhood, with severe symptoms including chronic diarrhea, abdominal distension, and failure to thrive. In many patients, symptoms may not develop until later in life, when the disease symptoms include fatigue, diarrhea, and weight loss due to malabsorption, anemia, and neurological symptoms (Table 4). Celiac disease is a life-long disorder, and if untreated, it is associated with increased morbidity and mortality[84, 85].

Possible triggers

Genetic predisposition association (HLA, MYO9B), exogenous trigger (gluten), pro-autoimmune genetic background, viral infections, tissue damage, early termination of breastfeeding and gender contribute to the development of CD (Table 5)[86].

Apart from introduction of gluten during the first year of life, infectious agents may play a role in development of CD. Several studies have implicated infections with Adenovirus type 12[87-89], hepatitis C virus[90,91], *Campylobacter jejuni*[92], *Giardia lamblia*[93], Rotavirus[94] and Enterovirus infection[95] as triggers for the development of CD. The immunologic response in persons genetically susceptible

Table 5 The most important factors contributing to the development of celiac disease[86] (printed with permission)

Factors contributing to the onset of celiac disease	Mechanism
Gluten	Elicit T cell responses Induces cytokine production and intestinal lesion
Age of introduction of gluten	Weak gut immune during early childhood
HLA-DQ2 or HLA-DQ8	Gluten presentation
MYO9Bo	Increased permeability of the intestine
Pro-autoimmune genetic background	Shift in Th1/Th2 balance towards Th1
Viral infections	Defect in generation of active tolerance (e.g., regulatory T cells) IFN production
Tissue damage	Tissue damage Increased level of tTG Danger signals
Early termination of breastfeeding	Decreased protection against infections
Gender	Hormone-related pro-autoimmune status

Th1: T helper 1; Th2: T helper 2; tTG: Tissue transglutaminase; HLA: Human leukocyte antigen; IFN: Interferon.

CD may be triggered due shared viral sequence of 8 to 12 amino acids with the toxic gliadin fraction[89]. Other factors such as timing of gluten ingestion and breast feeding cessation may involve in the pathogenesis and disease development of CD[96]. Some initiating factors, such as gluten overload, gastric surgery "unmasking", giving up smoking, and infections can also trigger the disease, which can become apparent in an abrupt manner[97,98].

Prolamin trigger

Gluten is a protein that appears in wheat, barley, rye and oat, compositing of prolamin and glutelin. The majority of the proteins in food responsible for the immune reaction in CD are the prolamins. Prolamins is found in several grains, such as wheat (gliadin), barley (hordein) and rye (secalin), corn (zein) and as a minor protein, avenin in oats. Because of their high glutamine content and specific sequence patterns, prolamins are resistant to gastrointestinal proteolytic enzymes and are excellent substrates for deamidation by tissue transglutaminase.

The incomplete gastrointestinal digestion of gluten leads to the appearance of gluten-derived gliadin peptides such as 33mer (LQLQPFPQPQLPYPQPLPYPQPQL PYPQPQPF) with a variety of characteristics[81]. It contains overlapping T-cell epitopes, and its deamidated form is a potent T-cell stimulator, generating the glutamic acid residues essential for binding to HLA-DQ2 in celiac patients[99]. The ingestion of prolamins from wheat, barley, rye and possibly oats causes histological changes in the small intestine mucosa of celiac patients, leading to a malabsorption syndrome[100]. Clinical symptoms of an autoimmune attack after ingestion of the gluten containing food include digestive symptoms and skin reactions.

Gliadin peptides cause stimulation of the innate and adaptive immune system[83,101,102]. The prototype of pep-

tides effective on innate response is peptide 31-43/49, which has been proved both *in vitro* and *in vivo* to be toxic for CD patients[103,104]. Peptide 31-43 (p31-43) stimulates the synthesis and release of interleukin (IL)-15, a proinflammatory cytokine, that promotes the adaptive immune response[101], involving CD4+ T cells that recognize several deamidated gliadin peptides[82]. Unlike p31-43 which is not immunogenic for T cells, peptide 57-68 (p57-68), which binds to HLA-DQ2/8 molecules, is one of the dominant epitopes recognized by T cells isolated from the intestine of CD patients[82]. The so-called toxic peptides, of which p31-43 is probably the most fully studied, modulate the small-intestinal mucosal biology *via* an innate immune mechanism.

Time of trigger

Several studies related the rise in childhood CD to infant feeding practices[96,105]. Consumption of wheat, barley, and rye in the first 3 mo children have significantly increased the risk of developing CD-associated autoantibodies, as compared with exposure during first 4 to 6 mo[105,106].

Although CD can be diagnosed at any time of life, it is present mostly in either early childhood (between 9 and 24 mo) or in the third or fourth decade of life[8,85,107,108]. In contrast to the 1/1 sex ratio in children, in adulthood it is diagnosed twice in females. Interestingly, celiac disease is also becoming more frequently recognized in the elderly, and in this population, a 1/1 sex ratio has also been noted[109]. Although the "classical" gastrointestinal malabsorption syndrome characterized by diarrhea, steatorrhoea, weight loss, fatigue, and anemia may occur in severe cases, most patients have a milder symptoms such as abdominal discomfort, bloating, indigestion, or non-gastrointestinal symptoms (or no symptoms at all)[8,85,107,108]. Mäki *et al*[2] reported a shift of 5-6 years of age at diagnosis in Finland, with less than 50% of new cases presenting typical gastrointestinal symptoms. In England[110], Scotland[111], Canada[112], and the United States[28], reports have also shown that almost 50% of newly diagnosed CD patients do not present with gastrointestinal symptoms.

GENETICS

Genetics play a strong role in CD, indicated by the high disease concordance in monozygotic twin[113]. The CD prevalence rose to 17.6% in sisters, 10.8% in brothers, and 3.4% in parents[21]. CD is associated with HLA alleles as well as more than 250 other MHC and non-MHC genes. The main genetic factor is the given HLA-DQ genes, i.e., the genes encoding DQ2 or DQ8 in the HLA complex on 6p21. Approximately 95% of celiac have a DQ2 comprised of DQB1*302 and DQA1*03. A small number of individuals lacking either of those heterodimers have DQB1*02 and DQA1*05 alone[20,114]. Gene dosage also affect CD susceptibility; individuals with the heterodimer comprised of DQB1*02 and DQA1*05 and most of the remaining 5% have a DQ8 heterodimer. Homozygous individuals who carry DQB1*02 and DQA1*05 in cis on both chromosomes have a great risk developing complicated forms of CD[115]. A significant higher risk for CD of 1:7 for DQ2 and DQ8 individuals and 1:2518 for subjects lacking all predisposing factors have been determined[21].

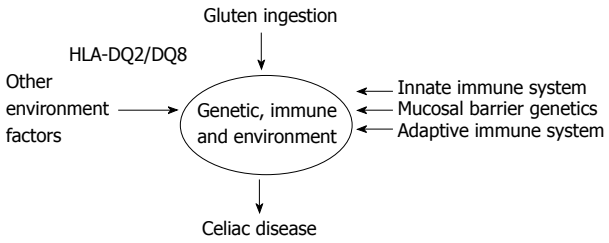

Figure 2 Factors necessary for celiac disease development[96] (adapted). HLA: Human leukocyte antigen.

It is found that 30% of the Caucasian populations carry HLA-DQ2 and most will eat wheat, while only 1 in 100 will develop disease[116]. The remaining susceptibility is thought to be due to a combination of genetic and environmental factors (Figure 2).

HLA-DQ2.5 carriage is necessary for disease development, but it is not sufficient by itself. A combination of other genetic factors influencing the mucosal barrier, the adaptive and the innate immune system also impact the likelihood of disease development. Wheat ingestion is a known environment factor that is necessary for disease development but on top of this, a number of factors such as timing of gluten ingestion and breast feeding cessation may influence disease development[96].

Studies using twins, which are assumed to share environmental factors, have estimated the percentage of non-HLA genetic variants which predispose to disease as approximately 60%[117]. To date a large list of variants have been suggested to predispose to CD through a combination of linkage and association studies, a large number of variants, however, do not stand up to further scrutiny. Only those that have been validated with convincing evidence in multiple populations are mentioned here.

In CD, like many common diseases, this genome wide linkage approach has been fairly unsuccessful at locating variants. Linkage was found to various regions including 5q and 19p, however, the only genomic region that was replicated with some reliability in other populations was 2q33, a region that contains the *CTLA4*[118], *ICOS* and *CD28* genes[117]. *CTLA4* is an excellent candidate gene for involvement in CD not only due to its integral involvement in the suppression of immune responses but also because it has been implicated as a genetic variant that increases susceptibility to T1D.

The prevalence of CD in patients diagnosed with T1D has been estimated at up to 15% in children and 6% in adults[119]. The reason for this association has never been fully elucidated, but common mechanisms within the pathogenesis and genetics of the two conditions may provide some insights. IL-21 region displays CD associations to T1D[120], rheumatoid arthritis[121], Grave's

disease[122] and psoriatic arthritis[123]; but genetic involvement in all these conditions is not currently understood. There is possibility of shared genetic susceptibility to autoimmunity through *IL-2*, *IL-21* locus, both inside and outside of the HLA region, with almost no function identified thus far[116]. Like the studies associated with the HLA-DQ2.5 variant, further identification of the causal variant and its function will provide a unique insight into CD and other autoimmune disease biology.

Immune function

CD is an autoimmune disease associated with the genetic predisposition HLA and tTG autoantigen. tTG is a calcium dependent enzyme that plays a crucial role in CD pathogenesis[124]. tTG mediates ordered and specific deamidation of gliadins, creating an epitope that binds efficiently to DQ2 and is recognized by gut-derived T cells[125]. During gluten consumption, these tTG autoantibodies are produced by the mucosa of the small intestine, and detected in patients' serum but disappear slowly from the patient's circulation on a GFD[126]. Extraintestinal CD symptoms may be associated with immunoglobulin A (IgA) deposits on extracellular tTG in the liver, kidney, lymph nodes and muscles of CD patients[126].

The toxic peptides, such as the 19-mer, trigger an innate immune response[101], characterized by the production of IL-15 by epithelial cells and lamina propria dendritic cells[127]. There is some evidence that this response is a generalized response in all individuals, but is amplified in CD patients (possibly due to a lower threshold to IL-15) who only get disease as a result of adaptive immune system involvement[128]. IL-15 affects the epithelial barrier, both by increasing the permeability through disruption of the tight junctions[129,130] and acting on intraepithelial lymphocytes (IELs) promoting interferon γ (IFN-γ) production as well as a potent cytotoxic activity particularly by $NKG2D^+$ cells[131,132]. Therefore, immunoadaptive peptides, like the 33-mer, can now reach the lamina propria, where they are presented by dendritic cells to gluten-specific T cells[133,134].

Other autoantigens that are normally "cryptic" can be unmasked and cause a self-aggressive immunologic response following the gliadin-initiated inflammatory process[8]. In fact, persistent stimulation by some proinflammatory cytokines (IFN-γ and tumor necrosis factor α) can cause further processing of autoantigens and their presentation to T lymphocytes by antigen-presenting cells. The mucosa is expanded by increased numbers of lymphoid cells both in the intraepithelial compartment, in which there is an increase in γδ T cells, and in the lamina propria, which is expanded by lymphocytes and plasma cells. The intestinal crypts are elongated because of an increase in dividing epithelial cells, and villi are shortened or even completely absent because of rapid loss of mature epithelial cells from the villus tip.

Intestinal epithelium function

Intestinal epithelium plays a central role in CD disease pathogenesis. It modulates the intestinal immune system that is acutely altered by gliadin. This indicates that gliadin can gain access to the basal surface of the epithelium, and therefore interact directly with the immune system, *via* both trans- and paracellular routes of absorption (Figure 3).

Retrotranscytosis

The protected retrotransport of secretory IgA into the intestinal lumen *via* the transferrin receptor CD71, allows the entry of intact and thus harmful gliadin peptides into the intestinal mucosa by a transcellular route. The overexpression of the transferring receptor CD71 in patients with active CD by transportation of gliadin across the intestinal mucosa through retrotranscytosis of secretory immunoglobulin-gliadin complexes is shown in Figure 4[135,136].

Transcytosis of α2-gliadin-33mer (an important trigger of CD) by apical-to-basal is stimulated by IFN-γ, which is a key cytokine involved in CD immunopathogenesis[137].

Paracellular route

There have been recent hypothesis associated with non-digested gliadin absorption in the intestinal lumen during the early event in CD pathogenesis by stimulation of the innate and adaptive immune system[83,101,102]. Zonulins provide information on the regulation of intercellular tight junctions (TJs) and increased intestinal permeability[138-142]. It is released by the enterocyte upon apical exposure to α-gliadin digests[129,143]. Lammers *et al*[143] have identified that MyD88 induces release of zonulin upon gliadin binding to CXCR3 on enterocytes, as a result inducing greater epithelial permeability and subsequent paracellular gliadin passage to the gut mucosa.

After binding to its surface receptor, gluten is internalized[144] and subsequently triggers a series of intracellular events including phospholipase C and Protein kinase Ca activation and actin polymerization, which lead to the opening of TJs[139,145] through Zot/Zonulin receptor (Figure 5).

Other pathways

There are several pathways including cellular signals that may be involved in the mucosal damage in CD. Deamidation of gluten peptides by tissue tTG reinforces presentation of gluten peptides by HLA-DQ2 or HLA-DQ8 molecules of plasmacytoid dendritic cells (pDCs) to T cells, which activate gluten-reactive Th1 cells and produce high levels of proinflammatory cytokines. IL-21 is overproduced in the mucosa of CD patients, where it helps sustain T-bet expression and IFN-γ production[146]. Th1 cytokines promote increased cytotoxicity of IELs and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand system, or IL-15-induced perforin/granzyme and NKG2D-MICA signaling pathways. IFN-α released by activated pDCs perpetuates the inflammatory reaction by inducing Th1

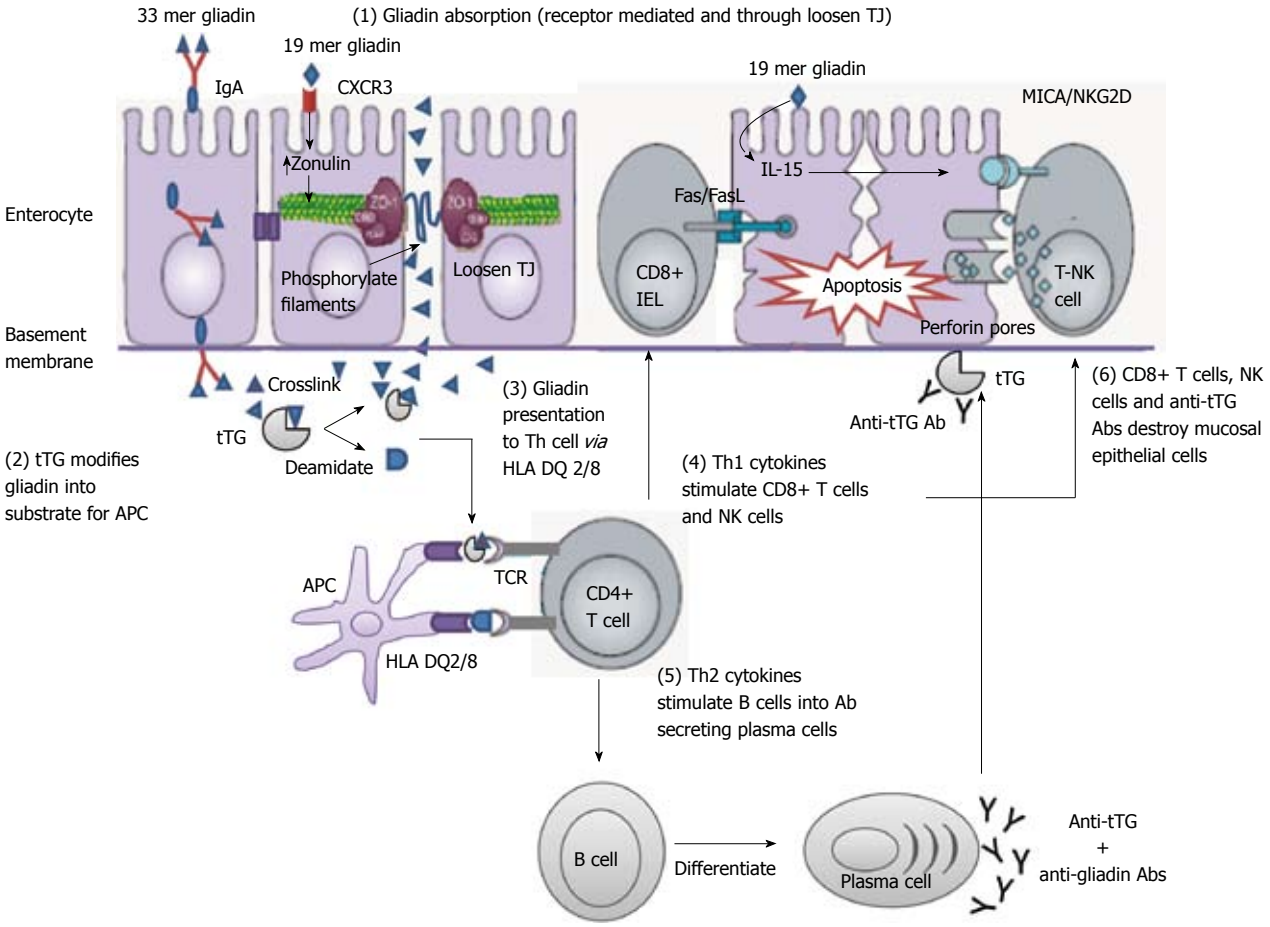

Figure 3 Mechanisms of mucosal damage in celiac disease[80] (adapted). Gliadin peptides crosses the enterocyte by paracellular tight junctions (TJ) as a consequence of increased release of zonulin leading to impaired mucosal integrity upon 19 mer gliadin binding to chemokine (C-X-C motif) receptor 3 (CXCR3) receptor, or *via* transcytosis, or retrotranscytosis of secretory immunoglobulin A (IgA) through transferrin receptor CD71. Tissue transglutaminase (tTG) deamidates or crosslinks 33 mer gliadin which is then recognized by human leukocyte antigen (HLA)-DQ2 or -DQ8 molecules of antigen presenting cell (APC). APC presents the toxic peptide to CD4+ T cells. Activated gluten-reactive CD4+ T-cells produce high levels of pro-inflammatory cytokines. T helper 1 (Th1) cytokines promote increased cytotoxicity of intraepithelial lymphocytes (IELs) and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand (FasL) system, or interleukin 15 (IL-15)-induced perforin/granzyme and homodimeric natural killer-activating receptor-major histocompatibility-class I chain-related gene A comple (NKG2D–MICA) signaling pathways. The production of T-helper2 (Th2) cytokines activate and induce clonal expansion of B cells, which differentiate into (antigliadin and anti-tTG) antibody secreting plasma cells. Interaction between with the extracellular tTG and anti-tTG-autoantibody may induce epithelial damage. TCR: T cell receptor.

cells to produce IFN-γ. IL-21 and IL-15 produced by DCs and intraepithelial cells also inhibit transforming growth factor beta signaling and regulatory T cells (Tregs) function. Additionally, the production of Th2 cytokines, Th2 cells drives the activation and clonal expansion and differentiate of B cells into plasma cells secreting anti-gliadin and anti-tissue transglutaminase antibodies[147], which interact with extracellular tTG, and may induce epithelial damage.

Hence in CD, there is impaired suppressor activity of Tregs. This defect in Tregs function could play a role in the pathogenesis of CD and in CD autoimmunity[148].

DIAGNOSIS

Approach to initial CD diagnosis

In 1970, the European Society of Paediatric Gastroenterology laid down criteria for the diagnosis of CD in children, entailing three biopsies of an initial flat mucosa in the upper small intestine, restoration of the mucosa to normal on a GFD, and a deterioration of the mucosa after gluten challenge[149]. Given the current availability of serological tests being highly sensitive and specific, the European Society of Paediatric Gastroenterology, Hepatology, and Nutrition has proposed a revised CD diagnostic protocol[150]. Based on this protocol, if the symptoms (either "classical" or "atypical") and serological tests are suggestive of CD, small bowel biopsy followed by a favourable clinical and serological response to the GFD is now considered sufficient to definitely confirm the diagnosis. In asymptomatic patients improvement in mucosal appearance may be required to confirm the diagnosis, but in majority symptomatic patients, continual abnormality of mucosa at the second biopsy is more likely to indicate slow /partial mucosal recovery[151]. This may also reflect that the site of re-biopsy (proximal small intestine) is often the last site to improve.

The current approach to evaluating CD has been modified by the advent of highly sensitive and specific serological tests. An algorithm for diagnosing CD is

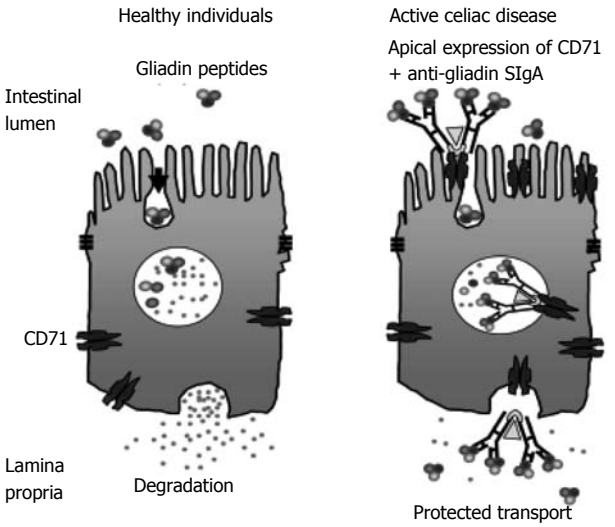

Figure 4 CD71 receptor-mediated transport of immunoglobulin A-gliadin complexes in celiac disease[136] (adapted). Gliadin bound to apically expressed CD71 receptor in active celiac disease individual allows protected transport of gliadin into the lamina propria. SIgA: Secretory immunoglobulin A.

given in Figure 6. Assays for IgA anti-tissue transglutaminase (TGA) and IgA anti-endomysial (EMA) have both the highest specificities and sensitivities, and are therefore regarded as being superior serological screening tools for diagnosis of CD[152].

Initial CD evaluation is based on a combination of positive CD-specific serological tests, histological findings in the intestinal biopsy, CD-predisposing gene encoding HLA DQ2 or DQ8, family and medical history of CD, and clinical or histological response to GFD[26,80]. However, CD diagnosis can be challenging in some non-responsive patients to GFD[7]. Practically all patients with CD carry HLA-DQ2 or HLA-DQ8. Thus the absence of these gene pairs reflects a very high negative predictive value for CD and should prompt consideration of other causes of small bowel-related symptoms and pathological changes[153,154]. Positive TGA or EMA at initial diagnosis of CD or at any time in the clinical course of the disease helps to confirm the diagnosis of CD because of their excellent specificities of over 99% when small bowel villous atrophy is present on biopsy[155].

However, false positive serological assays may also occur[156], in liver disease and small-bowel inflammation[157], so documentation of gluten sensitivity is important. A combination of biopsy and serological antibody can also be used to support diagnosis to reduce false positive results. A validated subjective Celiac Dietary Adherence Test, a patient-completed tool, can also be used in conjunction with biological markers to assess dietary adherence and disease activity in individuals with CD[158].

Diagnosis of refractory CD

The influence of noncompliance to a GFD and the substantial number of patients being undiagnosed are of greatest concern, as these factors could possibly contribute to the refractory form of CD and to the development of malignancies. These patients' CD symptoms do not revert on GFD. The first evaluation step of a potential RCD case is to confirm correct initial diagnosis of CD[7]. Sometimes neglected in this determination is the documentation of an initial and convincing response to a GFD, i.e., demonstration that the disease was truly a "gluten-sensitive" small bowel disorder. Otherwise, it may be difficult to ascertain if CD was initially present. More precise terms in this clinical setting include "sprue-like intestinal disease" or "unclassified sprue".

Some patients with developed RCD are likely to have negative TGA and EMA[6,159], demonstrating that negative CD specific serology does not exclude the diagnosis of CD. A family history of CD in first-degree relatives (especially siblings) further supports the diagnosis of CD in patients, having 14% positive tTG test and 10% positive EMA with an estimated prevalence of 11% where 54% had "silent" disease, most with severe intestinal villous atrophy[76]. The diagnosis of CD by histological findings or clinical improvement after GFD without confirmation with other diagnostic criteria may not be entirely reliable because CD is just one of many causes of villous atrophy. Clinical response to GFD or exacerbation after gluten re-introduction have low sensitivity of 59% and specificity of 92% for CD[160], which account to a positive likelihood ratio of 7.37 (means that individuals with positive histology upon gluten re-introduction are 7.37 times more likely to have CD than those with negative histology upon gluten re-introduction) and a negative likelihood ratio of 0.44 (means that individuals with positive histology upon gluten re-introduction are 0.44 times less likely to lack CD than those with negative histology upon gluten re-introduction). Thus, a critical review of prior tests and villous histology is crucial to determine the accuracy of a prior diagnosis of CD. Ideally, documented normalization of biopsies after a GFD and then demonstration of recurrent symptoms with histological relapse best defines refractory CD (RCD). Obviously, this is not always possible.

RCD is believed to affect approximately 5% of patients with CD. It is subdivided into types I and Ⅱ, with normal and aberrant (expressing cytoplasmic) CD3, but lacking surface expression of the T-cell markers CD3, CD4, CD8[161], and the T-cell receptor, intraepithelial T lymphocytes in the small intestinal mucosa, respectively[162]. Enteropathy-associated T-cell lymphoma (EATL) occurs in more than half of patients with RCD Ⅱ within 4-6 years after RCD Ⅱ diagnosis, and is the main cause of death in this group of patients[76,163]. RCD type 1 only rarely evolves into EATL. Recent data indicate a relative risk for patients with (untreated) CD to develop EATL[164,165].

DIAGNOSTIC TESTS

Serological tests

HLA typing: The contribution of HLA type to the genetic risk for CD has been variously estimated at

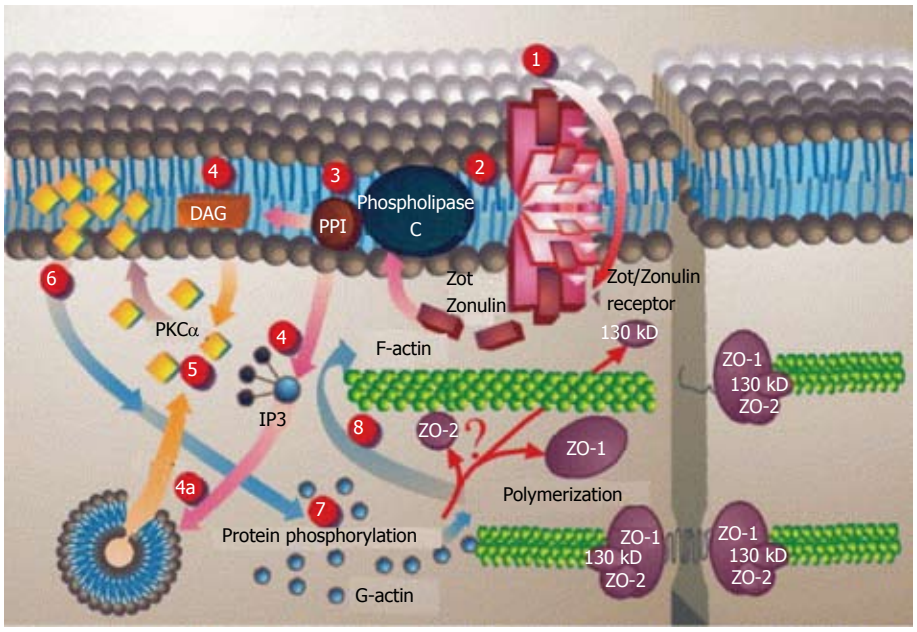

Figure 5 Proposed Zot intracellular signal mediated opening of intestinal tight junctions[146] (printed with permission). 1: Zot interacts with a specific Zot/Zonulin intestinal surface receptor; 2: Leading to protein internalization; 3: Activation of phospholipase C; 4: Hydrolyzes phosphatidyl inositol to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG), either *via* DAG or (4a) through the release of intracellular Ca^{2+} *via* PPI-3; 5: Protein kinase C alpha (PKCα) is then activated; 6: Membrane-associated, activated PKCα catalyzes the phosphorylation of target protein(s); 7: With subsequent polymerization of soluble G-actin in F-actin; 8: This polymerization causes the rearrangement of the tight junctions (TJ) filaments and displacement of proteins [including zonula occludens-1 (ZO-1)]. As a result, intestinal TJ becomes loosened. IP3: Inositol trisphosphate.

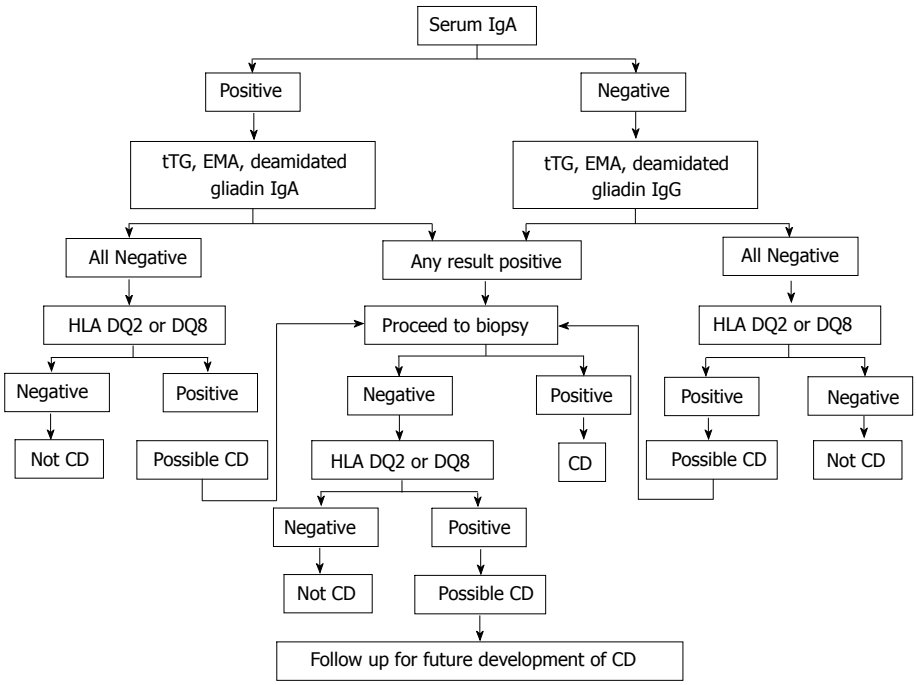

Figure 6 Celiac disease diagnostic testing algorithm (adapted from Mayo Medical Laboratories, Mayo Foundation for Medical Education and Research). IgA: Immunoglobulin A; IgG: Immunoglobulin G; tTG: Tissue transglutaminase; EMA: Endomysial; HLA: Human leukocyte antigen; CD: Celiac disease.

30%-50%[20,21]. Many of the polymorphic genes are involved in susceptibility to CD encode products that influence the immune response upon gluten ingestion, as shown for the HLA-linked genes[22]. Although Non-HLA genes contribute more than HLA genes to the genetic background of CD, each of them adds only a minor contribution to the disease development.

There is strong association between CD and the presence of HLA DQA1*0501-DQB1*02 (DQ2) and DQA1*0301-DQB1 [0302 (DQ8) haplotypes. Approximately 90% to 95% of patients with CD carry DQ2 and those patients that are negative for HLA-DQ2 are usually positive for HLA-DQ8[166,167], indicating a strong genetic risk for the disease[100]. Several studies also have confirmed that the absence of HLA-DQ2, HLA-DQ8, or both virtually excludes the diagnosis of CD[168-170]. However, the modest sensitivity (HLA-DQ2, 70%-99.8%; HLA-DQ8, 1.6%-38%) and specificity (HLA-DQ2, 69%-77%; HLA-DQ8, 77%-85%) of the test means that a positive result is not sufficient to diagnose the disease[having a low positive predictive values (HLA-DQ2, 6.3-18; HLA-DQ8, 0.28-8.1) and likelihood ratios (HLA-DQ2, 2.25-4.33; HLA-DQ8, 0.07-2.53)][171]. Even the presence of HLA-DQ2 or HLADQ8 in patients with positive serologic test results is strongly suggestive but not pathognomonic for CD. Antibody screening to identify participants with preclinical CD may be reduced by preselecting HLA risk group from the large populations with long-term follow-up for CD[172]. Hence HLA-DQ genotyping could be included in the algorithm of selecting large populations prospec-

Table 6 Operating characteristics of serological markers to detect the celiac disease in adults[178] (adapted)

Serological tests	Sensitivity	Specificity	Predictive value		Likelihood ratio	
	95% CI (%)	95% CI (%)	Positive	Negative	Positive	Negative
IgG AGA	57-78	71-87	0.2-0.9	0.4-0.9	1.96-6	0.25-0.61
IgA AGA	55-100	71-100	0.3-1.0	0.7-1.0	1.89-∞	0-0.63
IgA EMA	86-100	98-100	0.98-1.0	0.8-0.95	43-∞	0-0.14
IgA TGA	77-100	91-100	> 0.9	> 0.95	8.55-∞	0-0.25
IgA TGA and EMA	98-100	98-100	> 0.9	> 0.95	49-∞	0-0.02

IgG: Immunoglobulin G; IgA: Immunoglobulin A; AGA: Anti-gliadin antibodies; EMA: Endomysial; TGA: Transglutaminase.

tively screened for CD.

Antibody level: Several serum antibodies have been used to initially evaluate patients with suspected CD, monitor adherence and response to GFD, and screen asymptomatic individuals. Anti-gliadin antibodies (AGA) detection has low sensitivity and specificity, leading to high false-positive rate in patients[173]. Recent reports of deamidated gliadin peptide AGA (DGP-AGA) have suggested a much improved accuracy[174]. The sensitivity and specificity for IgA DGP-AGA is 84.3% and 79.8%, whereas for IgG DGP-AGA the sensitivity and specificity are 82.3% and 98.9%, respectively[175]. As shown in Table 6, EMA and TGA have been found to be superior to AGA and gives highest sensitivity and specificity of greater than 95% when used in combination[173,176,177]. EMA testing, however, produces a subjective and highly observer-dependent result, whereas TGA testing is quantitative.

Small intestinal mucosal biopsy

Histopathological analysis: Although the diagnosis of CD can be suspected on clinical or laboratory grounds, or as a result of serological tests, histology of the proximal small intestinal mucosa is still the diagnostic gold standard and must always be performed. Small intestinal histopathology of CD biopsy samples are characterized by typical architectural abnormalities. Marsh[178] has pioneered the theory of a sequence of progression of the CD lesion in the small intestinal mucosa.

According to the modified Marsh classification: normal mucosa is classified Marsh 0, intraepithelial lymphocytosis as Marsh Ⅰ, intraepithelial lymphocytosis and crypt hyperplasia as Marsh Ⅱ, and intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy as Marsh Ⅲ[179]. Later the Marsh-Oberhuger system was developed, where stage 3 was split into three sub stages (a, b and c)[178,180]. The Marsh-Oberhuber classification was based on a 6-stage grading, namely (1) type 1 infiltrative lesions, characterized by normal mucosal architecture with an increased number of IELs; (2) type 2 hyperplastic lesions, characterized by an increase in crypt depth without villous flattening; (3) type 3a, 3b, and 3c destructive lesion, characterized by mild, marked, and complete villous flattening, respectively; and (4) type 4 hypoplastic lesions, characterized by villous atrophy with normal crypt height and IEL count.

Considering the broad spectrum of lesions possibly present in CD, the Marsh-Oberhuber system is undoubtedly valid under optimal clinical conditions, but the considerable number of diagnostic categories involved makes it prone to a low inter-observer and intra-observer agreement.

False-positive and false negative test results may occur due to patchy mucosal damage, inter-observer variability, low-grade histopathological abnormalities and technical limitations. Hence, reliance on standard histological findings alone may result in failure to diagnose CD[181]. Several other limitations may be evident in high-volume, service-oriented laboratories with limited attention to quality control. Poorly oriented biopsies fixed in the endoscopy suite may be prone to difficult interpretation. Inter-observer variation in pathological interpretation may occur, especially if there is limited access to a pathologist with expertise focused on interpretation of small intestinal biopsies. Some patients with low-grade histopathological abnormalities (Marsh Ⅰ/Marsh Ⅱ) can present with gluten-dependent symptoms or disorders before overt villous atrophy occurs. Furthermore, some patients with isolated intraepithelial lymphocytosis (Marsh Ⅰ), who are not clinically suspected of having CD, may develop CD during follow-up[182]. Although the mucosal changes in CD are thought to develop gradually, whether minor mucosal lesions in asymptomatic patients indicate CD in an early stage is not yet clear[183].

In case of strong clinical suspicion of CD, duodenal biopsy must be performed regardless of serological analysis[184]; in cases of low suspicion of disease or screening, duodenal biopsy probably only needs to be performed in seropositive patients. Hence, the new system for routine use of simplified grading system with uniform diagnosis and increase validity of the pathologic diagnosis of CD was developed by using only three categories (A, B1 or B2) with A representing normal villous with lymphocytic infiltration and B1 and B2 representing partial and complete villous atrophy, respectively[185]. The new proposed grading system classified the CD lesions into non-atrophic (grade A) and atrophic (grade B)[186]. Grade A was characterized by the isolated increase of IELs (> 25/100 enterocytes)[187], whereas grade B was split into B1, in which the villous/crypt ratio is less than 3/1, with still detectable villi, and B2, in which the villi are no longer detectable. A comparison between the Marsh-Oberhuber and the new

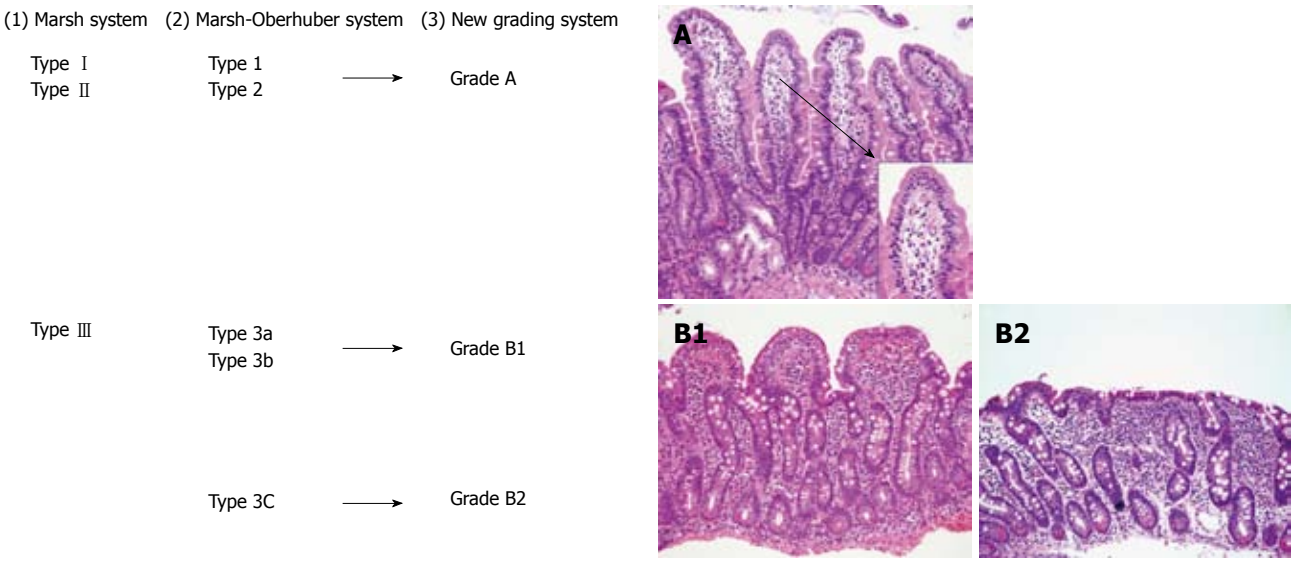

Figure 7 A comparison between the Marsh classification for celiac disease. 1: Marsh-Oberhuber; 2: Grading system for celiac disease, and the new grading system; 3: Representative pictures of the grades A (original magnification, 20×; insert, 60×), B1 (20×), and B2 (20×), proposed in the new grading system. An alternative classification may simply describe "mild", "moderate" or "severe (flat)" architectural changes[186] (printed with permission).

Table 7 Factors that support the diagnosis of celiac disease in patients with an increased density of intraepithelial lymphocytes but no villous shortening[194] (printed with permission)

Family history of celiac disease	15% of first-degree relatives are affected
Concomitant autoimmune conditions	Risk of coeliac disease approximately 5-fold
Increased density of γδ+ IELs	Sensitivity 0.84, specificity 0.91
Increased density of villous tip IELs	Sensitivity 0.84, specificity 0.95
HLA DQ2 or DQ8	High sensitivity, low specificity Negative predictive value high
Gluten dependence	Should be ascertained by gluten challenge or gluten-free diet

IELs: Intraepithelial lymphocytes; HLA: Human leukocyte antigen.

grading criteria is shown in Figure 7. Figure 7C represents pictures of the grades proposed in the new histologic grading criteria.

Recently, quantitative measurements of villous height, apical and basal villous widths, and crypt length (morphometry) have been used to determine changes in duodenal morphology, particularly after the introduction of a GFD, in correlation with Marsh grade, self-reported adherence to GFD, and changes in serology. GFD resulted in increase in villous area and a progressive decrease in crypt length, with a plateau after 6-12 mo and mean villous area half that of control subjects[188].

Intraepithelial lymphocyte: The presence of aberrant IELs appears to be a reliable prognostic marker to differentiate between RCD type I and type Ⅱ patients, with characteristic normal and aberrant IELs, respectively. IELs are considered aberrant when there is cytoplasmic CD3 expression, but no expression of surface CD3, CD4 and CD8 T-cell markers[189,190]. The current methods for double CD3/CD8 T cell receptor clonal from intestinal tissue can be done by immunohistochemistry, polymerase chain reaction or flow cytometry[161,162,189]. The presence of these IELs is associated with a significant increase in EATL development[161,163,191,192]. Increased IEL's may be used to support or exclude diagnosis of CD, and may be useful for follow up as mentioned in Table 7[193].

In 95% of non-refractory CD and control patients, the highest percentage of aberrant T-cells in duodenal biopsy specimens is in agreement with the cut-off of the % T cells which are aberrant. Such a cut-off has been previously suggested in the RCD group based on the clinical observation that none of the RCD patients with less than 20% aberrant T-cells eventually developed EATL[163]. Clonal T-cell population can be found in the intestinal mucosa of RCD patients, which relates to the development of EATL[161,194]. Immunophenotyping using flow cytometry[162], gives significant higher negative predictive value and sensitivity (both 100%) for aberrant T-cells were found with regard to EATL development in RCD, when compared to clonality in a duodenal biopsy specimen (75% and 78%, respectively). The positive predictive values (59%) and likelihood ratios (1.85) of these tests for EATL development in RCD are almost comparable.

Aberrant T-cells is quantified by flow cytometry is well suited to identify RCD patients at risk for EATL as it has a higher predictive value and sensitivity than T-cell clonality analysis of duodenal biopsy specimens. A cut-off value of 20% appears reliable for early risk stratification[159], and targeted therapeutic options in RCD patients[6,27,195]. This is particularly important since once overt T-cell lymphoma has developed, treatment outcome and survival are very poor[159,196]. Additionally, quantification of aberrant T-cells is useful for the subsequent follow-up of treated RCD Ⅱ patients[27].

Table 8 Future therapeutic approach for celiac disease treatment

Mechanism		Therapeutic agent	Stage of study
Hydrolysis of toxic gliadin	ALV003	Glutenenases and endoprotease	Phase Ⅱ
	AN-PEP	Prolyl endopeptidase	Phase Ⅱ
		Lactobacilli	Discovery
	VSL#3	*Lyophilised bacteria*, including *Bifidobacteria*, *Lactobacilli* and *Streptococcus salivarius*	Discovery
Prevention of gliadin absorption	Larazotide	Hexapeptide derived from zonula occludens toxin of *Vibrio cholera*	Phase Ⅱ
		Synthetic polymer poly (hydroxyethylmethacrylate-co-styrene sulfonate)	Discovery
		Anti-gliadin IgY	Discovery
tTG2 inhibitor		Dihydroisoxazoles	Discovery
		Cinnamoyltriazole	Discovery
		Aryl β-aminoethyl ketones	Discovery
Peptide vaccination	Nexvax2	Three deamidated peptides derived from wheat α-gliadin, ω-gliadin and β-hordein	Phase Ⅰ
		Human hookworm (Necator americanus) inoculation	Phase Ⅱ
Modulate immune response		HLA-DQ2 blocker	Discovery
		Interleukin blocker	Discovery
		NKG2D antagonist	Discovery
Restore intestinal architecture		R-spondin-1	Discovery

tTG2: Tissue transglutaminase 2; PEP: Prolyl endopeptidases; NKG2D: Homodimeric natural killer-activating receptor; HLA: Human leukocyte antigen; IgY: Immunoglobulin Y.

Useful background for the diagnosis of CD: The HLA class Ⅱ molecules DQ2 and DQ8 are required for but are not sufficient for the development of CD: 50% of Americans are positive of one of those molecules, but only 1% develop CD. Negative HLA DQ2 or DQ8 may rule out CD as a cause of the enteropathy; IgA TGA serology is > 95% sensitive for CD, especially when there is a high titre, but false positive tests can occur; Anti-gliadin antibodies have a relationship high false negative rate, and have been replaced by IgG DGP assays that appear to have a sensitivity compared to TGA; The endoscopic features of CD (scalloping of mucosal folds, less prominent folds, fissules, and a nodular/mosaic pattern) are 59% sensitive but 92% specific for CD. For example, other small bowel disorders, including Crohn disease in the duodenum, may cause mucosal scalloping and other endoscopic features of CD.

TREATMENT

Existing treatment

GFD: Currently, the only effective treatment available for CD individuals is a strict life-long GFD[197]. In reality, total avoidance of gluten intake is extremely difficult, due to hidden gluten from food contamination[198]. For safety purposes, United States Food and Drug Administration has set the limit (August 2011) of < 20 ppm gluten (equivalent to 10 ppm gliadin) for gluten-free foods. The total daily consumption of gluten-free foods must be taken into account as it may exceed the tolerable limit of each celiac individual. It has been estimated that the threshold of prolonged gluten ingestion in some CD individuals may be lower than 50 mg/d[199]. However, some CD individuals can conceivably be more sensitive. The presence of hidden gliadin in contaminated food products represents an imminent risk for celiac consumers, because of long-term effect of regular ingestion of small amounts of gliadin[200], on causing positive tTG and characteristic small bowel biopsy.

Gluten modification: Approaches to modify dietary gluten have been made to render gliadin non-toxic, since it is a non-invasive approach to CD patients. This approach has been less appealing due to loss of baking characteristic, public refusal for genetically modified crops, contamination of genetically modified crops with gluten contained crops grown nearby and heterogeneous uncharacterised immunostimulatory epitopes in gluten, and difference among patients response to epitopes and gluten levels[201].

A greater understanding of the pathogenesis of CD allows alternative future treatments to be designed. A number of preliminary studies have been published that illustrate from a conceptual perspective future possible approaches that may be pursued in more detail (Table 8).

FUTURE TREATMENT APPROACHES

Hydrolysis of toxic gliadin peptide

Prolyl endopeptidases: Prolyl endopeptidases (PEPs) are endoproteolytic enzymes expressed in micro-organisms and plants. These enzymes cleave proline-rich gluten to smaller peptides that are ready for digestion by intestinal brush-border enzymes (aminopeptidases and carboxypeptidases). Limited efficiency was found, since PEP required 3 h preincubation with gluten containing foods to achieve full detoxification of peptides and to prevent intestinal transport of active gluten fragments[202]. This is unlikely to be achieved by co-administration of PEP and gluten-containing diet.

A two-stages cross-over phase Ⅱ clinical trial was performed using asymptomatic CD patients eating, a slice of bread daily and a slice of bread pre-treated with PEP daily[203]. After 2 wk of PEP treated gluten challenge, majority of patients did not develop malabsorption, measured by faecal fat excretion and D-xylose

malabsorption tests. The tests likely lacked the necessary sensitivity to assess minor malabsorption resulting from active CD, since no histological confirmation was performed to determine deterioration in the Marsh grading[201]. When PEPs were consumed as jam spread on a slice of gluten-containing bread by CD patients, villous blunting was seen in small bowel biopsy histological evaluation in most patients[204]. Further studies are needed to determine the appropriate dose of enzyme and time of administration relative to the quantity of ingested gluten.

ALV003: ALV003, a mixture of two glutenases, an endoprotease from germinating barley and PEP, was pretreated with wheat flour and tested in CD patients[205]. Symptoms typically associated with gluten ingestion were not significantly reduced by ALV003 pre-treatment, but ALV003 abolished immune responses induced by gluten in CD patients. A randomized controlled phase Ⅱa clinical trial has been performed where CD patients received either ALV003 or placebo daily for 6 wk at the time of 2 g gluten contained bread. This proof-of-concept study demonstrated that ALV003 can attenuate gluten-induced small intestinal mucosal injury in CD patients[206]. After six weeks period, biopsies proved lower small intestinal mucosal injury in patients treated with ALV003 than placebo-treated patients despite of persistent intestinal inflammation in many patients on a strict GFD. Placebo-treated patients were found to have suffered more adverse events, most commonly including abdominal distention, flatulence, eructation, abdominal pain and diarrhea[206].

Lactobacilli: Lactobacilli added to sourdough for fermentation are able to lyse the proline-/glutamine-rich gluten peptides and thus decrease immunotoxicity[207-210]. A mixture of fermented wheat flour with oat, millet and buckwheat allows sourdough bread to retain its baking characteristics. A pilot study in patients with CD suggested that this bread was well tolerated[209]. However, these patients were challenged for only 2 d, which is clearly not sufficient to draw any firm conclusions. Hence, another 60-d diet of fully hydrolyzed wheat flour with sourdough lactobacilli and fungal proteases (8 ppm residual gluten; n = 5) was further studied. The pretreated flour was rendered non-toxic by serological, morphometrical, and immunohistochemical analysis[211]. A larger group of subjects in the trial and palatability of digested flour baked products needs to be taken into consideration.

VSL#3: VSL#3 is a probiotic containing lyophilised bacteria, including bifidobacteria (*Bifidobacterium longum, Bifidobacterium infantis* and *Bifidobacterium breve*), lactobacilli (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp., Lactobacillus bulgaricus* and *Lactobacillus plantarum*) and *Streptococcus salivarius subsp., Thermophiles*. It is used to hydrolyse gliadin peptides in pre-treated flour and tested for efficacy in rat intestinal cell line and celiac jejunal biopsies[207]. VSL#3 pre-digested gliadins did not show an increase of the infiltration of CD3+ intraepithelial lymphocytes and caused a less pronounced effect on intestinal mucosa permeability (determined by lower F-actin rearrangement and zonulin release). Hence, VSL#3 may have importance during food processing to produce pre-digested gluten-free products.

Prevention of toxic gliadin peptide absorption

Larazotide: Larazotide (AT-1001, Alba Therapeutics, Baltimore, MA), is a synthetic hexapeptide derived from Zonula Occludens toxin of Vibrio cholera[212]. It is used to inhibit the opening of tight junctions of the small intestine epithelial cells. Clinical trial phase I in CD patients suggested that Larazotide therapy is well tolerated by patients and reduces intestinal barrier dysfunction, pro-inflammatory cytokine production, and gastrointestinal symptoms in CD individuals after gluten exposure[213]. Encouraging results were obtained from a 6-wk phase Ⅱb trial in terms of symptoms and antibody titers[214], showing larazotide acetate a promising drug candidate. This drug inhibits the paracellular route of gliadin absorption through tight junctions, which is not the only mechanism of gliadin absorption. Indeed, gliadin may gain access to the mucosa through transcellular pathways in addition to paracellular route[135,137]. Hence, this strategy might be best exploited in combination with other treatments.

Synthetic polymer poly (hydroxyethylmethacrylate-co-styrene sulfonate): Poly (hydroxyethylmethacrylate-co-styrene sulfonate) [P (HEMA-co-SS)] forms supramolecular particles upon gliadin complexation in gastric and intestinal conditions[215,216], and deteriorates gliadin's effect on epithelial cells[217]. This complexation decreases the effect of gastrointestinal (GI) digestive enzymes on gliadin absorption, and thus the formation of immunogenic peptides is reduced. Gluten-sensitive HLA-HCD4/DQ8 mice co-administered with P (HEMA-co-SS) showed attenuated gliadin-induced changes in permeability and inflammation[217]. Low side effect, cost and possibility to be taken, occasionally with gluten-containing food, makes it an attractive option. Further investigation of the mechanisms of action and its interaction with human tissues is required before its efficacy is investigated in human trials[218].

Anti-gliadin egg yolk antibody: Oral antibody passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the gastrointestinal tract[219]. Among antibodies, chicken egg yolk immunoglobulin (IgY), is ideal for passive immunotherapy, as it may be readily obtained in large quantities from egg yolk, presenting a more cost-effective, convenient, and hygienic alternative to mammalian antibodies. Oral immunotherapeutic IgY is a promising alternative to neutralize gliadin in the GI tract and prevention it from absorption[220]. Mannitol contained antibody preparation is highly resistant against GI enzymes and proved to effectively

neutralized gliadin under simulated GI conditions in the presence of food. *In vivo* study; BALB/c mice fed with IgY formulation and gliadin ratio of 1:5 (w/w), demonstrated that gliadin absorption in the gastrointestinal tract was minimal at < 1%[221]. Further investigations in CD patients is requires to prove its efficacy and determine dosing regimen of antibody relative to the amount of gliadin ingestion.

Blockage of selective deamidation of specific glutamine residues by tissue transglutaminase 2 inhibitor

Transglutaminases (a family of eight enzymes) have diverse functions in human and are involved in several biological and pathological processes[222]. tTG2 is an enzyme that has a pro-inflammatory effect and increases the immunostimulatory epitopes present in the lamina propria of the small intestine. Blockage of tTG2 may be a promising approach to inhibit the inflammatory process upon gluten ingestion. There are two essential classes of tTG2 inhibitors; irreversible and reversible inhibitors[223]. Irreversible inhibitors form a stable covalent bond with this enzyme, and thus prevent deamidation of gliadin peptides[223,224]. Reversible inhibitors are more desirable to minimize possible side effects. These include aldehyde-bearing tTG modulators[225], cinnamoyl triazole derivatives[226], and the highly specific modified peptide targeting the active cysteine site of tTG2[227]. Since a few gluten T-cell epitopes can be recognized without being deamidated by tTG2[228,229], this approach will not inhibit the innate response[101], or the immune response induced by non-deamidated peptides[82]. To be able to use tTG2 inhibitors clinically, it is critical to design highly specific inhibitors, since all human tTG share high sequence homology.

Vaccine application to restore immune tolerance towards gluten

Autoimmune enteropathy in CD has been proposed to be due to impairment of immunoregulatory mechanisms that controls oral tolerance. Systematic peptide mapping of T-cell was performed to determine gliadin reactive epitopes recognized by approximately 90% of CD patients. A clinical trial phase I study has been initiated as Nexvax2® (Nexpep Pty, Ltd., Australia) peptide vaccine-containing mixture of immunotoxic α- and ω-gliadins and B-hordein[230].

Engineered Lactococcus lactis secreting a DQ8-restricted gliadin peptide administered orally[231], or recombinant α-gliadin in HLA-DQ8 administered intranasally in transgenic mouse model[232], have been studied to modulate immune response to gluten. However, it is difficult to appreciate how the vaccine or the intranasal peptide administration can modulate the Tr1 response. More work is needed to assess the effect of these therapies on the spectrum of gluten peptides presented to the gut.

Dermal inoculation of human hookworm (*Necator americanus*) has also been used to modulate the immune response to gluten[233]. A phase Ⅱ trial with CD patients suggested that hookworm infection on its own may not obviate the necessity for a restricted diet in CD, but appears to be safe and might impact on immune pathology[234]. Here in, hookworm infection is expected to reduce gluten sensitivity and immune reactivity.

Modulation of immune response to dietary gliadin

HLA-DQ blocker: HLA-DQ blocker is used to block the binding sites of HLA-DQ2 or DQ8 for it to be unrecognized by T cells as well to block the presentation of the antigen. This is not a new concept that was developed without much success to treat type 1 diabetes mellitus and rheumatoid arthritis, due to difficulties in effective drug delivery[235,236]. By amino-acid substitution of gliadin T-cell stimulatory sequence, the epitope can be converted to an agonist or antagonist, abolishing the inflammatory cascade[237]. IFN-γ production by peripheral blood lymphocytes was prevented when either an alanine or lysine amino acid was substituted through the immunodominant α-gliadin peptide, corresponding to the peptide's anchor to the HLA-DQ cleft[238]. To develop this as a new therapeutic agent, more studies need to be performed, looking at the mass T-cell action of the gut towards these modified peptides.

Interleukin blocker: Modulation of cytokine production has been evaluated for the treatment of several autoimmune diseases, although their side effects may be severe. Modulation of proinflammatory IL-15 and anti-inflammatory IL-10 cytokines has been suggested to influence the immune balance between tolerance and autoimmunity[127,239-241]. Blocking IL-15 may promote maintenance of epithelial integrity, limit epithelial destruction, leading to decreased passage of dietary gliadin.

NKG2D antagonists: MICA molecules, strongly expressed on active CD epithelial cell surface upon gliadin challenge[132], interact with the NKG2D-activating receptor on human natural killer cells and CD8 T cells, leading to villous atrophy due to an IEL-mediated damage to enterocytes[131,132]. Thus, NKG2D antagonists[131] and anti-NKG2D antibodies[242], have been proposed as therapeutics in CD.

Restoration of intestinal architecture by R-spondin-1

R-spondin-1 is an intestinal mitogen, shown to stimulate crypt cell growth, accelerate mucosal regeneration and restore intestinal architecture in mouse models of colitis[243]. This agent has yet to be tested in human to be considered as a therapeutic agent in CD.

CONCLUSION

CD has been kept in the dark for decades with very little known about what is a relatively common medical condition. It is only recently that we have greater understanding of its prevalence, diagnosis and pathogenesis, which has supported the development of new therapeutic approaches to treat CD. There are several future

Table 9 Key points from recent findings

Cause
Environmental (gluten) and genetic factors (HLA and non-HLA genes)
Prevalence
0.5%-1% worldwide in normal at-risk population
Higher risk in the population with diabetes, autoimmune disorder or relatives of CD individuals
Pathogenesis
Gliadin gains access *via* trans- and para-cellular routes to the basal surface of the epithelium, and interact directly with the immune system
Types of CD symptoms: "typical" or "atypical"
Diagnosis
Positive serological (TGA or EMA) screening results suggestive of CD, should lead to small bowel biopsy followed by a favourable clinical and serological response to the GFD to confirm the diagnosis
Current treatment
Strict life-long GFD
Alternative future CD treatments strategies
Hydrolysis of toxic gliadin peptide
Prevention of toxic gliadin peptide absorption
Blockage of deamidation of specific glutamine residues by tissue
Restoration of immune tolerance towards gluten
Modulation of immune response to dietary gliadin
Restoration of intestinal architecture

HLA: Human leukocyte antigen; CD: Celiac disease; EMA: Endomysial; TGA: Transglutaminase; GFD: Gluten-free diet.

directions to follow to treat CD, which if successful will supplement or even replace the current only effective treatment, the use of a GFD. A greater understanding of the pathogenesis of CD allows alternative future CD treatments to hydrolyse toxic gliadin peptide, prevent toxic gliadin peptide absorption, blockage of selective deamidation of specific glutamine residues by tissue, restore immune tolerance towards gluten, modulation of immune response to dietary gliadin, and restoration of intestinal architecture (Table 9).

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REVIEW

Current progress in the treatment of chronic hepatitis C

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Abstract

Over the last decade, the standard of care for the treatment of chronic hepatitis C has been the combination of pegylated-interferon-alfa (PEG-IFN) and ribavirin (RBV) which results in sustained virological response (SVR) rates of 75%-85% in patients with genotypes 2 or 3 but only of 40%-50% in patients with genotype 1. Currently, there are rapid and continuous developments of numerous new agents against hepatitis C virus (HCV), which are the focus of this review. Boceprevir and telaprevir, two first-generation NS3/4A HCV protease inhibitors, have been recently licensed in several countries around the world to be used in combination with PEG-IFN and RBV for the treatment of genotype 1 patients. Boceprevir or telaprevir based triple regimens, compared with the PEG-IFN/RBV combination, improve the SVR rates by 25%-31% in treatment-naïve genotype 1 patients, by 40%-64% in prior relapsers, by 33%-45% in prior partial responders and by 24%-28% in prior null responders. At the same time, the application of response-guided treatment algorithms according to the on-treatment virological response results in shortening of the total therapy duration to only 24 wk in 45%-55% of treatment-naïve patients. There are, however, several challenges with the use of the new triple combinations in genotype 1 patients, such as the need for immediate results of HCV RNA testing using sensitive quantitative assays, new and more frequent adverse events (anemia and dysgeusia for boceprevir; pruritus, rash and anemia for telaprevir), new drug interactions and increasing difficulties in compliance. Moreover, the SVR rates are still poor in very difficult to treat subgroups of genotype 1 patients, such as null responders with cirrhosis, while there is no benefit for patients who cannot tolerate PEG-IFN/RBV or who are infected with non-1 HCV genotype. Many newer anti-HCV agents of different classes and numerous combinations are currently under evaluation with encouraging results. Preliminary data suggest that the treatment of chronic HCV patients with well tolerated combinations of oral agents without PEG-IFN is feasible and may lead to a universal HCV cure over the next 5-10 years.

Key words: Chronic hepatitis C; Pegylated interferon; Ribavirin; Protease inhibitors; Nucleos(t)ide analogue inhibitors; Non-nucleos(t)ide analogue inhibitors; Hepatitis C virus polymerase; NS5A inhibitors; Cyclophilin inhibitors

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects approximately 170 million people worldwide[1]. Chronic hepatitis C may lead to the development of cirrhosis, liver decompensation and hepatocellular carcinoma and is a major indication for liver transplantation, particularly in Western countries[2]. HCV is classified into 6 major genotypes. Some genotypes have a restricted geographical distribution (genotypes 4-6), while others (genotypes 1-3) are more broadly disseminated. Genotype 1 (subtypes 1a and 1b) is the most prevalent genotype in the world. Genotype 2 is found in clusters in the Mediterranean region, genotype 3 is most prevalent among intravenous drug users and genotype 4 is found mostly in Egypt, while genotypes 5 and 6 are less frequent[3,4]. The HCV genotypes strongly affect the likelihood of the response to treatment.

During the last decade, the standard of care (SOC) for chronic HCV patients consisted of pegylated interferon-alfa (PEG-IFN)-2a or -2b combined with ribavirin (RBV). The treatment duration has been based on the on-treatment virological responses, mainly estimated at 4 wk [rapid virological response (RVR)] and 12 wk of therapy (early virological response)[3,5]. Recently, polymorphisms of the interleukin 28b (*IL28B*, interferon lambda 3) gene were strongly associated with the rates of sustained virological response (SVR) to PEG-IFN/RBV therapy and therefore their determination may be useful to identify a patient's likelihood of response to treatment, but the predictive value is low[5]. In patients with HCV genotypes 2 or 3, the combination of PEG-IFN/RBV is usually given for 24 wk, achieving rates of SVR, i.e., absence of HCV RNA at 6 mo or more after cessation of therapy, of about 75%-85%. In patients with HCV genotypes 1 and 4, the combination of PEG-IFN and RBV is usually given for 48 wk, resulting in SVR rates of 40%-50% for genotype 1 and 55%-65% for genotype 4 patients[5,6]. The SVR rates are substantially lower in previous non-responders to PEG-IFN and RBV, in whom the proportion of genotype 1 patients is higher due to the lower initial SVR rates. It has been reasonable, therefore, that new treatments with improved efficacy were mostly needed for patients with genotype 1. In addition and regardless of HCV genotype, there are chronic HCV patients who cannot be treated with PEG-IFN and RBV for several reasons. First and of most clinical relevance, PEG-IFN therapy is contraindicated in patients with decompensated liver disease. Second, patients may not tolerate and/or may have other contraindication(s) to PEG-IFN or RBV. Thus, there has definitely been a need for new antiviral drugs with better efficacy, improved tolerance and good safety profiles for chronic HCV patients.

The current review focuses on the recent rapid and continuous developments in the management of chronic HCV infection, which have been based on a better understanding of the structure of the HCV genome and the key viral enzymes.

HCV GENOME ORGANIZATION AND NEW ANTIVIRALS

HCV has a positive-sense, single-stranded RNA genome of some 9.6 kilobases that encodes a polyprotein of about 3000 amino acids[7,8]. The open reading frame for the polyprotein is flanked by 5' and 3' untranslated regions, which contain elements that regulate translation and replication. The polyprotein is generated by the host cell translation machinery and cleaved co- and post-translationally by viral and host proteases to yield the mature viral proteins. The N-terminal segment of the polyprotein encodes the structural components of the virus (Core, E1, E2 and p7). Core protein forms the capsid shell into which the virus genome is packaged, while the glycoproteins are considered to locate to the lipid envelope surrounding the capsid. P7 is required for the virus assembly[9,10].

The C-terminal component of the polyprotein contains non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). NS2 and NS3 are viral proteases required for the processing of the HCV polyprotein[11]. NS3 is a multifunctional enzyme, which provides a serine protease and helicase/nucleotide triphosphatase activity and forms a stable eterodimeric complex with its cofactor NS4A, which is essential for protein folding. The NS3/NS4A complex cleaves the junction between NS3/4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. NS3 has also a helicase activity and is necessary for HCV replication supporting the unwinding of HCV[12]. NS4B is the presumed central organizer of the HCV replicase complex and a main inducer of intracellular membrane rearrangements[13]. NS5A is a RNA-binding phosphoprotein required for RNA replication and assembly of infectious virus particles[14]. NS5B RNA-dependent RNA-polymerase is required for viral replication.

The nonstructural proteins mentioned above have been the major targets for the evolving directly acting antivirals (DAAs) or specifically targeted antiviral therapy for hepatitis C. In particular, DAAs under development mainly include NS3/4A protease inhibitors, NS5B polymerase inhibitors and NS5A inhibitors. In addition, inhibitors of cyclophilin, which is a host protein with an important role in the HCV lifecycle, are also under development (Table 1).

NS3/4A PROTEASE INHIBITORS

Two first-generation, linear NS3/4A protease inhibitors, boceprevir and telaprevir, have recently been approved in several countries around the world for clinical use in patients with genotype 1, while many new NS3/4 protease inhibitors are currently under evaluation in clinical trials (Table 1). Boceprevir and telaprevir have high antiviral potency only against genotypes 1 and 2[15], but a low barrier to resistance. In particular, resistant HCV strains develop within a few days of monotherapy with one of these two agents[16,17], while most mutations confer

Table 1 New agents against hepatitis C virus currently evaluated in phase Ⅱ or Ⅲ trials

Drug	Company
NS3/4A protease inhibitors	
First generation, linear	
Boceprevir (Approved)	MERCK
Telaprevir (Approved)	JANSSEN
Narlaprevir	MERCK
First generation, macrocyclic	
BI201335	BOEHRINGER Ingelheim
TMC435	TIBOTEC/JANSSEN
Danoprevir	ROCHE
Vaniprevir	MERCK
Asunaprevir	BRISTOL-MYERS SQUIBB
ABT-450	ABBOTT
GS-9451	GILEAD
GS-9256	GILEAD
ACH-1625	ACHILLION
Second generation, macrocyclic	
MK-5172	MERCK
Nucleos(t)ide analogue inhibitors of HCV polymerase	
PSI/GS-7977	PHARMASSET/GILEAD
Mericitabine	ROCHE
IDX-184	IDENIX
Non-nucleoside analogue inhibitors of HCV polymerase	
Tegobuvir	GILEAD
Filibuvir	PFIZER
ANA-598/Setrobuvir	ANADYS/ROCHE
BI207127	BOEHRINGER Ingelheim
ABT-333	ABBOTT
ABT-072	ABBOTT
VX-222	VERTEX
NS5A inhibitors	
Daclatasvir	BRISTOL-MYERS SQUIBB
GS-5885	GILEAD
GSK2336805	GSK
Cyclophilin inhibitors	
Alisporivir[1]	NOVARTIS
SCY-635	Scynexis

[1]On hold by the United States Food and Drug Administration because of toxicity (pancreatitis cases with one death). HCV: Hepatitis C virus.

cross-resistance to both drugs (V36A/M, T54S/A, V55A, R155K/T/Q, A156S, A156T/V)[18]. HCV subtype 1a develops resistance more frequently and more rapidly than subtype 1b[19], as just one (instead of two in subtype 1b) nucleotide change (R155K) is enough for an amino acid replacement and emergence of a resistant strain. Because of the low barrier to resistance, boceprevir and telaprevir should always be used in triple combinations together with PEG-IFN/RBV. Since viral resistance may develop even in triple combinations with PEG-IFN/RBV, strict stopping rules are recommended to be applied with the use of boceprevir- or telaprevir-based regimens. Newer, first-generation NS3/4A inhibitors under development are mainly macrocyclic (danoprevir, vaniprevir, asunaprevir, *etc.*), and are expected to have better pharmacokinetics and tolerability compared with boceprevir and telaprevir. Second-generation NS3/4A inhibitors (MK-5172) are expected to have pan-genotype antiviral activity and improved resistance profiles.

Efficacy data from phase III clinical trials with boceprevir- or telaprevir-based regimens in treatment-naïve patients with genotype 1

SPRINT-2 was a randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of boceprevir-based regimens compared with the previous SOC, as well as the efficacy of boceprevir-based response-guided therapy (RGT) compared with a fixed 48-wk boceprevir regimen[20]. In total, 1097 (938 nonblack and 159 black) treatment-naïve genotype 1 patients were randomly assigned to one of three treatment arms (1:1:1). They all received PEG-IFN-2b and RBV for the first 4 wk (lead-in period) followed by (1) placebo plus PEG-IFN-2b/RBV for 44 wk (control arm); (2) boceprevir plus PEG-IFN-2b/RBV for 24 wk with or without an additional 20-wk course of placebo plus PEG-IFN-2b/RBV (boceprevir RGT arm); or (3) boceprevir plus PEG-IFN-2b/RBV for a standard period of 44 wk (boceprevir fixed arm). PEG-IFN-2b was administered subcutaneously at a dose of 1.5 μg/kg per week, RBV orally at a total daily dose of 600-1400 mg according to body weight and boceprevir orally with food at a dose of 800 mg every 7-9 h. In the boceprevir RGT arm, treatment was stopped at 28 wk in patients who achieved an extended RVR (eRVR) defined as undetectable HCV RNA between 8 and 24 wk, or continued until week 48 in patients who did not achieved such an eRVR.

The SVR rate was significantly lower in the control arm (38%) compared with both boceprevir arms (RGT: 63%, fixed: 66%; $P < 0.001$) (Table 2). In all arms, black patients achieved lower SVR rates compared with nonblack patients (control arm: 23% *vs* 40%, boceprevir RGT arm: 42% *vs* 67%, boceprevir fixed arm: 53% *vs* 69%). In the RGT arm, a total of 44% of patients were eligible to receive only 28 wk of treatment having an excellent SVR rate of 96% (97% for nonblacks and 87% for blacks). The relapse rates were 22% in the control arm and 9% in the two boceprevir arms. In conclusion, boceprevir-based regimens, compared with the PEG-IFN/RBV combination, offer a 25%-28% benefit in the likelihood of SVR in treatment-naïve genotype 1 patients, while a RGT-based duration of boceprevir-based therapy has excellent results in patients with an eRVR and is not overall inferior than a fixed 48-wk boceprevir-based regimen.

ADVANCE was a randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of telaprevir-based regimens compared with previous SOC as well as the optimal duration of telaprevir triple combination[21]. In total, 1088 treatment-naïve genotype 1 patients were randomized to one of three treatment arms (1:1:1): (1) telaprevir plus PEG-IFN-2a/RBV for the first 12 wk followed by 12 or 36 wk of PEG-IFN-2a/RBV (T12PR arm); (2) telaprevir plus PEG-IFN-2a/RBV for the first 8 wk and placebo plus PEG-IFN-2a/RBV for another 4 wk followed by 12 or 36 wk of PEG-IFN-2a/RBV (T8PR arm); or (3) PEG-IFN-2a/RBV for 48 wk together with placebo

Table 2 Sustained virological response rates in phase Ⅲ clinical trials with hepatitis C virus protease inhibitor-based regimens in treatment-naïve and treatment-experienced patients

Name of trial		SVR
Naïve patients - Treatment group		
SPRINT-2		
BOC/RGT		63%
BOC44/PR48		66%
PR48		38%
ADVANCE		
T12PR		75%
T8PR		69%
PR		44%
ILLUMINATE		
T12PR24 (eRVR+)		92%
T12PR48 (eRVR+)		87%
T12PR48 (eRVR-)		64%
< 20 wk		23%
Treatment-experienced patients - Treatment group		
RESPOND-2		
Prior relapsers	PR48	29%
	BOC/RGT	69%
	BOC44/PR48	75%
Prior partial responders	PR48	7%
	BOC/RGT	40%
	BOC44/PR48	52%
REALIZE		
Prior relapsers	PR48	24%
	LIT12PR48	88%
	T12PR48	83%
Prior partial responders	PR48	15%
	LIT12PR48	54%
	T12PR48	59%
Prior null responders	PR48	5%
	LIT12PR48	33%
	T12PR48	29%

BOC: Boceprevir; RGT: Response-guided therapy; P: Pegylated interferon-alfa; R: Ribavirin; T: Telaprevir; eRVR: Extended rapid virological response; LI: Lead-in; SVR: Sustained virological response.

for the first 12 wk (PR arm). PEG-IFN-2a was administered subcutaneously at a standard weekly dose of 180 μg, RBV orally at a total daily dose of 1000-1200 mg according to body weight and telaprevir orally with food at a dose of 750 mg every 8 h. In both telaprevir arms, treatment was stopped at 24 wk in patients who achieved an eRVR defined as undetectable HCV RNA at weeks 4 and 12, and continued up to 48 wk in patients who did not achieve such an eRVR.

Significantly more patients in the telaprevir arms achieved an SVR compared with controls (75% and 69% *vs* 44%, $P < 0.001$), while patients of the T12PR arm showed a trend for a higher SVR rate compared with patients of the T8PR arm, but this did not reach statistical significance (T12PR: 75% *vs* T8PR: 69%, $P = 0.088$). The rates of eRVR were 57% and 58% in the T12PR and T8PR arms, respectively, compared with 8% in the control arm. Among those patients with an eRVR who received only 24 wk of therapy, an SVR was achieved in 89% and 83% of cases in the T12PR and T8PR arms, respectively. Among patients who did not achieve an eRVR and continued therapy up to 48 wk, the SVR rates were 54% and 50% in the T12PR and T8PR arms, respectively (Table 2). The relapse rate was 9% in both telaprevir arms compared with 28% in the control arm.

Based on these data, we can conclude that telaprevir-based regimens, compared with the PEG-IFN/RBV combination, offer a 25%-31% benefit in the likelihood of SVR in treatment-naïve genotype 1 patients despite more than 50% of patients in the telaprevir arms receiving only 24 wk instead of 48 wk of therapy. Because of the numerically higher response rates in the T12PR than in the T8PR arm, the 12-wk telaprevir triple combination regimens were considered to be optimal for the treatment of genotype 1 patients.

ILLUMINATE was another phase Ⅲ trial which included 440 treatment-naïve genotype 1 patients to assess whether 24 wk of a telaprevir-based regimen was sufficient for patients with an eRVR[22]. All patients received telaprevir plus PEG-IFN-2a/RBV in the same doses used in the ADVANCE trial for the first 12 wk followed by PEG-IFN-2a/RBV for 12 or 36 wk[3]. In particular, patients with an eRVR (undetectable HCV RNA at weeks 4 and 12) were randomized at week 20 to continue PEG-IFN-2a and RBV until 24 or 48 wk, while all patients without an eRVR were maintained on PEG-IFN-2a/RBV until 48 wk. Among the 60% of patients who achieved an eRVR and continued treatment after 20 wk, SVR rates were comparable between those treated for a total duration of 24 or 48 wk (92% *vs* 88%, respectively) (Table 2). In the 22% of patients who did not achieve an eRVR but continued treatment after 20 wk, the SVR rate was 64%, while treatment was discontinued prematurely before the randomization at week 20 in 18% of cases. The conclusion of the ILLUMINATE trial was that 24 wk of a telaprevir-based regimen is enough for the treatment-naïve genotype 1 patients who achieve an eRVR.

Efficacy data from phase Ⅲ clinical trials with boceprevir- or telaprevir-based regimens in treatment-experienced patients with genotype 1

RESPOND-2 was a randomized, placebo-controlled trial designed to evaluate the efficacy and safety of boceprevir-based regimens compared with previous SOC for the retreatment of treatment-experienced genotype 1 patients. In total, 403 patients (259 relapsers: HCV RNA undetectable at the end but detectable at 6 mo after the end of previous therapy; 144 partial responders: HCV RNA decline > 2 $\log_{10}$ IU/mL at 12 wk but detectable during previous therapy) were randomly assigned to one of three treatment arms (1:2:2) similar to those used in the SPRINT-2 trial (control, boceprevir RGT and boceprevir fixed 48-wk arm)[23]. The only difference was in the RGT arm, which included the initial 4-wk lead-in phase with only PEG-IFN-2b and RBV followed by boceprevir plus PEG-IFN-2b/RBV for 32 wk (week 4 to 36) with or without the addition of 12 wk of PEG-IFN-

2b/RBV in patients with or without detectable HCV RNA at 8 wk of therapy.

SVR rates were higher in the two boceprevir arms (RGT: 59%; fixed: 66%) than in the control arm (21%, $P < 0.001$) (Table 2). SVR rates were also higher in the boceprevir arms in both relapsers (RGT: 69%, fixed: 75%, control: 29%) and partial responders (RGT: 40%, fixed: 52%, control: 7%), Among patients with undetectable HCV RNA at week 8, SVR was 86% after 36 wk of therapy in the boceprevir RGT arm and 88% after 48 wk of therapy in the boceprevir fixed arm. The overall SVR rates were found to be lower in the boceprevir RGT than the fixed arm in patients with advanced fibrosis (metavir F3-F4) (44% *vs* 68%) or mostly in patients with cirrhosis (35% *vs* 77%), but similar between these two arms in patients with milder fibrosis. The negative effect of cirrhosis on SVR was observed in both prior relapsers and prior partial responders. The probability of an SVR was also significantly higher in patients with than without a > 1 $\log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase (76% *vs* 33%). These data showed that boceprevir-based regimens compared with the PEG-IFN/RBV combination can improve the SVR rates by 40%-46% in previous relapsers and by 33%-45% in previous partial responders with genotype 1. Moreover, it was shown that a boceprevir-based RGT might be applied in treatment-experienced non-cirrhotic patients who achieve early (at 8 wk) HCV RNA undetectability. It should be noted, however, that the probability of an SVR is not very high in patients without a > 1 $\log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase of a boceprevir-based regimen.

Since null responders (< 2 $\log_{10}$ IU/mL decline in HCV RNA at 12 wk) were not included in the RESPOND-2 trial, a fixed 48-wk boceprevir-based regimen (4-wk lead-in with PEG-IFN-2b/RBV followed by 44 wk of triple combination with boceprevir plus PEG-IFN-2b/RBV) was subsequently evaluated in a rollover, single arm, prospective study[24]. Preliminary results reported an SVR rate of 38% in 42 previous null responders.

REALIZE was a randomized, placebo-controlled trial designed to evaluate the efficacy and safety of telaprevir-based regimens compared with the previous SOC in treatment-experienced genotype 1 patients as well as to determine whether a 4-wk lead-in therapy with only PEG-IFN/RBV can affect the probability of SVR in telaprevir-based regimens[1]. In total, 663 patients (354 relapsers, 124 partial responders, 184 null responders) were randomly assigned to one of three arms (1:2:2): (1) telaprevir plus PEG-IFN-2a/RBV for the first 12 wk followed by PEG-IFN-2a/RBV for another 36 wk (T12PR48 arm); (2) 4-wk lead-in phase with PEG-IFN-2a/RBV and then telaprevir plus PEG-IFN-2a/RBV for 12 wk followed by PEG-IFN-2a/RBV for another 32 wk (lead-in T12PR48 arm); or (3) PEG-IFN-2a/RBV for 48 wk (control PR48 arm).

SVR rates were similar in the two telaprevir arms (64% and 66%) and significantly higher compared with the control arm (17%, $P < 0.001$). In particular, SVR rates were higher in the telaprevir arms in prior relapsers (83% and 88% *vs* 24%, $P < 0.001$), prior partial responders (59% and 54% *vs* 15%, $P < 0.001$) and prior null responders (29% and 33% *vs* 5%, $P < 0.001$) (Table 2). The presence of cirrhosis was found to negatively affect the SVR rates in the telaprevir arms in prior partial responders (mild-moderate fibrosis: 72%, bridging fibrosis: 56%, cirrhosis: 34%) and mostly in prior null responders (mild-moderate fibrosis: 41%, bridging fibrosis: 39% cirrhosis: 14%), but not in prior relapsers (mild-moderate fibrosis: 86%, bridging fibrosis: 85% cirrhosis: 84%). Among the patients of the lead-in telaprevir arm, the probability of an SVR was significantly higher in patients with than without a > 1 $\log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase (82% *vs* 33%), but this effect was more clinically relevant in prior null responders (54% *vs* 15%) than in prior partial responders (59% *vs* 56%) or in prior relapsers (94% *vs* 62%). Thus, according to these data, telaprevir-based regimens compared with PEG-IFN/RBV improve the SVR rates by 59%-64% in prior relapsers, by 39%-45% in prior partial responders and by 24%-28% in prior null responders, while a 4-wk lead-in phase with only PEG-IFN/RBV does not offer any advantage to telaprevir-based regimens.

Safety issues with boceprevir- or telaprevir-based regimens

The most common and clinically important adverse event in the boceprevir trials was anemia, which developed in approximately 50% of patients treated with boseprevir-based regimens, compared with 30% of patients treated with only PEG-IFN/RBV[20,23]. Erythropoietin was administered by the investigators in 41%-46% of boceprevir-treated patients and in 21%-24% of the controls, while discontinuation due to anemia was necessary in 2% of patients in the boceprevir arms and in 1% of patients in the control arms. It should be noted that the SVR rates in the boceprevir arms were similar in patients with or without anemia, with or without erythropoietin use and with or without RBV dose reduction. Dysgeusia was another clinically important adverse event that was reported more frequently in the boceprevir than in the control arms (37%-43% *vs* 18%)[20,23].

The most clinically important adverse events in the telaprevir trials were pruritus, rash and anemia. In particular, pruritus was reported by 45%-50% of telaprevir-treated patients compared with 36% of controls, and rash developed in 35%-56% and 19%-37% of cases, respectively[21,22,25]. The rash during telaprevir therapy was typically eczematous and mild-to-moderate in > 90% of patients, while it was severe (involving > 50% of the body surface area) in 6%, leading to discontinuation of telaprevir in 5%-7% of patients (1% of controls) and of all drugs in 0.5%-1.4% of patients treated with telaprevir-based regimens (0% in controls)[21,22,25].

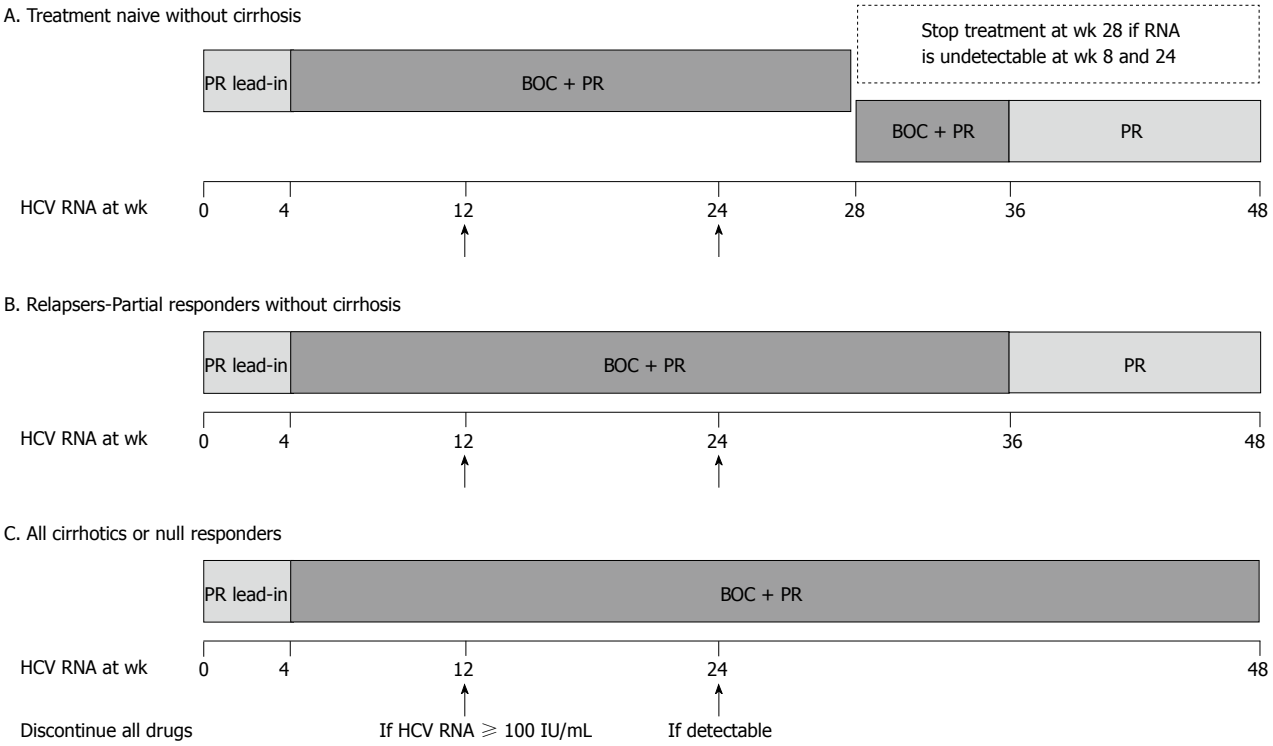

Figure 1 Treatment algorithms of boceprevir-based regimens for genotype 1 chronic hepatitis C virus patients recommended by the European Medicines Agency. All patients should start with a 4-wk lead-in phase with only pegylated interferon-alfa and ribavirin (PR). After 4 wk, boceprevir (BOC) is added. In treatment-naïve patients without cirrhosis who achieve an extended rapid virological response [eRVR: undetectable hepatitis C virus (HCV) RNA (< 10 IU/mL) at 8 and 24 wk], the triple therapy should last 24 wk and treatment end at 28 wk. In non-cirrhotic treatment-naïve patients who do not achieve such an eRVR and in all previous relapsers or partial responders without cirrhosis, the triple therapy should last 32 wk (until 36 wk of therapy) and should be followed by an additional 12 wk of PR. The United States Food and Drug Administration (FDA) recommendations suggest that prior relapsers or partial responders without cirrhosis who achieve an eRVR under BOC triple therapy can stop therapy at 36 wk without an additional 12-wk course of PR that is suggested by the European Medicines Agency. Finally, in all cirrhotics (treatment-naïve and experienced) and null responders, the triple therapy should last 44 wk (up to 48 wk of treatment). All patients should be tested for HCV RNA levels at 12 and 24 wk of total therapy and treatment should be discontinued for inefficacy if HCV RNA levels are > 100 IU/mL at 12 wk or HCV RNA is still detectable at 24 wk of therapy.

The mean time for the occurrence of rash was 22 d and the majority of rashes occurred during the first 4 wk of therapy. Anemia also developed more commonly in telaprevir-treated patients than in controls (37%-39% *vs* 19%)[21,22,25]. Anemia was managed with RBV dose reduction, which did not affect the SVR rate[21,22,25].

Another problem that may arise with the use of the protease inhibitors is the interactions with concomitant medications. Both boceprevir and telaprevir use hepatic drug metabolizing enzymes such as cytochrome P450 2C (CYP2C), CYP3A4, or CYP1A[15]. Therefore, caution is definitely required for the use of these agents in patients taking other dugs metabolized by the same pathways, such as statins, calcineurin inhibitors, antiretroviral agents, methadone, *etc*.[15]. Updated information on the possible drug-drug interactions with boceprevir and telaprevir can be found at the Food and Drug Administration (FDA) website (www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm) or other relevant sites (e.g., www.hep-druginteractions.org).

Use of boceprevir or telaprevir in current clinical practice

Both boceprevir and telaprevir have now been approved for the treatment of genotype 1 patients in several countries around the world. However, the approved treatment algorithms differ between the two drugs and according to the type of patients (Figures 1 and 2). In addition, there are some differences between the treatment algorithms of boceprevir-based regimens recommended by the United States and European regulatory authorities (Figure 1).

All patients who receive boceprevir should start with a 4-wk lead-in phase with only PEG-IFN/RBV. PEG-IFN-2b or PEG-IFN-2a may be used, while RBV should be administered at a weight-based dosage. After 4 wk, boceprevir is added, given with food at a dose of 800 mg (4 capsules of 200 mg each) every 8 h. In treatment-naïve patients without cirrhosis who achieve an eRVR [undetectable HCV RNA by a sensitive polymerase chain reaction (PCR) assay (HCV RNA < 10 IU/mL) at 8 and 24 wk], the triple therapy should last 24 wk and treatment finish at 28 wk. In non-cirrhotic treatment-naïve patients who do not achieve an eRVR and in all previous relapsers or partial responders without cirrhosis, triple therapy should last 32 wk (until 36 wk of therapy) and should be followed by an additional 12 wk of PEG-IFN/RBV. Finally, in all cirrhotics (treatment-naïve and experienced)

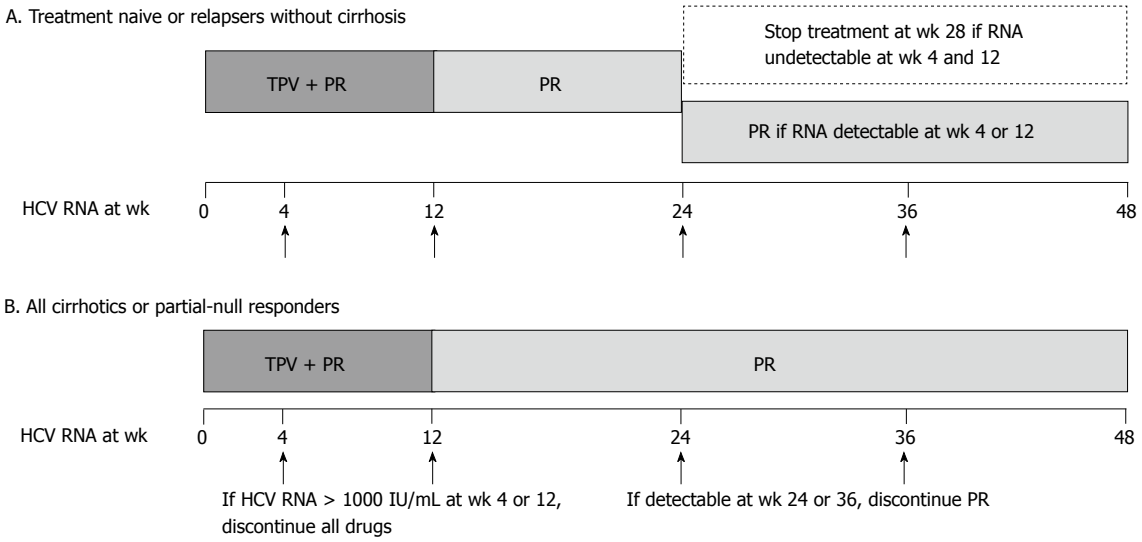

Figure 2 Treatment algorithms of telaprevir-based regimens for genotype 1 chronic hepatitis C virus patients recommended by both the European Medicines Agency and the Food and Drug Administration. All patients should start directly with the triple combination of telaprevir (TPV) plus pegylated interferon-alfa and ribavirin (PR). The triple combination should always last 12 wk. In treatment-naïve or previous relapser patients without cirrhosis who achieve an extended rapid virological response (eRVR) [undetectable hepatitis C virus (HCV) RNA (< 10 IU/mL) at 4 and 12 wk], triple therapy is followed by 12 wk of PR. In contrast, in non-cirrhotic treatment-naïve or relapser patients without an eRVR as well as in all cirrhotics or previous partial and null responders, triple therapy is followed by 36 wk of PR. Treatment should be discontinued for inefficacy if HCV RNA levels are > 1000 IU/mL at 4 or 12 wk or if HCV RNA is detectable at 24 or 36 wk of therapy.

and null responders, triple therapy should last 44 wk (up to 48 wk of treatment). All patients should be tested for HCV RNA levels at 12 and 24 wk of total therapy and treatment should be discontinued for inefficacy if HCV RNA levels are > 100 IU/mL at 12 wk or HCV RNA is still detectable at 24 wk of therapy.

All patients who receive telaprevir should start directly with the triple combination of telaprevir plus PEG-IFN/RBV. Theoretically, PEG-IFN-2a or PEG-IFN2b may be used, although PEG-IFN-2a has been used in all telaprevir trials. RBV is administered at a weight-based dosage, while telaprevir should be administered orally with a fatty meal at a dose of 750 mg (2 capsules of 375 mg each) every 8 h. The triple combination should always last 12 wk. In treatment-naïve or previous relapser patients without cirrhosis who achieve an eRVR [undetectable HCV RNA by a sensitive PCR assay (HCV RNA < 10 IU/mL) at 4 and 12 wk], triple therapy is followed by 12 wk of PEG-IFN and RBV. In contrast, in non-cirrhotic treatment-naïve or relapser patients without an eRVR as well as in all cirrhotics or previous partial and null responders, triple therapy is followed by 36 wk of PEG-IFN/RBV. Treatment should be discontinued for inefficacy if HCV RNA levels are > 1000 IU/mL at 4 or 12 wk or if HCV RNA is detectable at 24 or 36 wk of therapy.

OTHER DIRECT-ACTING ANTIVIRALS

NS5B polymerase inhibitors

There are 2 categories of NS5B polymerase inhibitors: nucleos(t)ide inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (Table 1). NIs mimic the naturally occurring nucleotides and thus are incorporated into the nascent RNA chain causing chain termination[26]. NIs are considered to have a high genetic barrier to resistance, although single amino acid substitutions are able to confer drug resistance *in vitro*. Nevertheless, because the active site of NS5 is highly conserved and amino acid substitutions in any position of the active site can result in loss of function, such resistant variants fit poorly, requiring weeks or months to grow to detectable levels in the presence of the drug. NIs have antiviral activity against all HCV genotypes (pan-genotype activity) as the active site of NS5B is well conserved across genotypes[27]. GS-7977 seems to be a promising representative of the NIs, as it appears to be rather safe and effective, achieving very high SVR rates (100%) in genotype 2 and 3 patients even when the drug is given for 12 wk only in combination with RBV[28].

NNIs bind to a distant site of the HCV polymerase and cause a conformational change rendering the enzyme ineffective. In particular, NNIs bind to one of 4 allosteric sites at the surface of HCV polymerase ("thumb" domain Ⅰ, "thumb" domain Ⅱ, "palm" domain Ⅰ, "palm" domain Ⅱ). NNIs have a more limited spectrum of activity being specifically against genotype 1. Because NNIs bind more distantly from the active site, resistant variants can fit in the presence of the drug, and therefore NNIs have a low barrier to resistance.

NS5A inhibitors

NS5A protein is a regulator of replication. NS5A inhibitors have high antiviral activity against different genotypes, but they have a low genetic barrier to resistance. Daclatasvir, a representative of this group (Table 1), is under evaluation in several combinations with promising results [29].

Cyclophilin A inhibitors

Cyclophilins are host proteins involved in protein folding. They play an important role in the HCV life cycle as a regulator of replication. The cyclophilin inhibitor, alisporivir (DEB-025) (Table 1), is a cyclosporine analogue without immunosuppressive properties that has shown pan-genotype antiviral activity and has been used either alone or in combination with PEG-IFN/RBV with promising results[30-32]. Phase Ⅲ trials with alisporivir were ongoing, but very recently the development of this drug was put on hold by the FDA due to safety concerns (a few cases of pancreatitis, one of which was fatal). SCY-635 (Scynexis) is another cyclophilin inhibitor under development.

NEW COMBINATIONS

Numerous trials of many combinations of the above drugs from different classes are currently ongoing. Much effort and interest has been given to the development of PEG-IFN-free regimens. Protease inhibitors have been combined with NNIs (telaprevir plus VX-222[33], BI201335 plus BI207127[34,35], GS-9256 plus tegobuvir[36]), NIs (danoprevir plus mericitabine)[37] or NS5A inhibitors as double or triple combinations including RBV[28]. Double combinations of a NI with RBV (GS-7977 and RBV) or with NS5 inhibitors are also being evaluated. Promising examples of PEG-IFN-free trials include the combination of the NI GS-7977 with RBV which has been shown to achieve an SVR in 10 out of 10 genotype 2 or 3 treatment-naïve chronic HCV patients treated for 12 wk[28,38] or the combination of the NS5A inhibitor daclatasvir and the NS3 protease inhibitor asunaprevir, which has shown interesting results in difficult to treat, genotype 1, prior null responders treated for 24 wk. The latter combination showed that an SVR can be achieved in 36% of 11 genotype 1 (mostly 1a) prior null responders from the United States[29] and in ⩾ 90% of 21 genotype 1b prior null responders coming from Japan[39]. Similarly, encouraging preliminary results have been reported by a 12-wk course of the NS3 protease inhibitor ABT-450 given with ritonavir boosting the combination of NNI ABT-072 and RBV, which achieved an SVR in > 90% of treatment-naïve genotype 1, IL28B rs12979860 genotype CC, non-cirrhotic chronic HCV patients[40]. Thus, it is clear that an SVR can be achieved with interferon-free regimens even in difficult to treat chronic HCV patients.

CONCLUSION

The recently approved boceprevir and telaprevir used in combination with PEG-IFN/RBV substantially improves the SVR rates in both treatment-naïve and treatment-experienced genotype 1 patients, while the treatment duration can be reduced to only 24 wk in a large proportion of mainly treatment-naïve patients. There are, however, several challenges with the use of the new triple combinations. There is a need for immediate results of HCV RNA testing using sensitive quantitative assays, there are new and more frequent adverse events and drug-drug interactions, and there will be increasing difficulties in compliance. In addition, the SVR rates are still poor in very difficult to treat subgroups of genotype 1 patients, such as null responders with cirrhosis, while there is no benefit in patients who cannot tolerate PEG-IFN/RBV and in patients infected with a non-1 HCV genotype. Many new drugs and combinations are currently under evaluation with encouraging results. Although it is yet early, preliminary data suggest that the treatment of chronic HCV patients with well tolerated combinations of oral agents without PEG-IFN is feasible and may lead to a universal HCV cure over the next 5-10 years.

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ORIGINAL ARTICLE

High densities of serotonin and peptide YY cells in the colon of patients with lymphocytic colitis

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Abstract

AIM: To investigate colonic endocrine cells in lymphocytic colitis (LC) patients.

METHODS: Fifty-seven patients with LC were included. These patients were 41 females and 16 males, with an average age of 49 years (range 19-84 years). Twenty-seven subjects that underwent colonoscopy with biopsies were used as controls. These subjects underwent colonoscopy because of gastrointestinal bleeding or health worries, where the source of bleeding was identified as haemorrhoids or angiodysplasia. They were 19 females and 8 males with an average age of 49 years (range 18-67 years). Biopsies from the right and left colon were obtained from both patients and controls during colonoscopy. Biopsies were fixed in 4% buffered paraformaldehyde, embedded in paraffin and cut into 5 μm-thick sections. The sections immunostained by the avidin-biotin-complex method for serotonin, peptide YY (PYY), pancreatic polypeptide (PP) enteroglucagon and somatostatin cells. The cell densities were quantified by computerised image analysis using Olympus software.

RESULTS: The colon of both the patient and the control subjects were macroscopically normal. Histopathological examination of colon biopsies from controls revealed normal histology. All patients fulfilled the diagnosis criteria required for of LC: an increase in intraepithelial lymphocytes (> 20 lymphocytes/100 epithelial cells) and surface epithelial damage with increased lamina propria plasma cells and absent or minimal crypt architectural distribution. In the colon of both patients and control subjects, serotonin-, PYY-, PP-, enteroglucagon- and somatostatin-immunoreactive cells were primarily located in the upper part of the crypts of Lieberkühn. These cells were basket- or flask-shaped. There was no statistically significant difference between the right and left colon in controls with regards to the densities of serotonin- and PYY-immunoreactive cells (P = 0.9 and 0.1, respectively). Serotonin cell density in the right colon in controls was 28.9 ± 1.8 and in LC patients 41.6 ± 2.6 (P = 0.008). In the left colon, the corresponding figures were 28.5 ± 1.9 and 42.4 ± 2.9, respectively (P = 0.009). PYY cell density in the right colon of the controls was 10.1 ± 1 and of LC patients 41 ± 4 (P = 0.00006). In the left colon, PYY cell density in controls was 6.6 ± 1.2 and in LC patients 53.3 ± 4.6 (P = 0.00007).

CONCLUSION: The change in serotonin cells could be caused by an interaction between immune cells and serotonin cells, and that of PYY density might be secondary.

Key words: Colon; Computer image analysis; Immunohistochemistry; Lymphocytic colitis; Peptide YY; Serotonin

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INTRODUCTION

Lymphocytic colitis (LC), which was first described in 1989[1], belongs to a group of conditions known as microscopic colitides (MC). This disorder is characterised mainly by chronic or intermittent watery diarrhoea[2], which can be severe. Up to 25% of patients present with more than 10 bowel movements per day in addition to nocturnal diarrhoea[3]. Other symptoms such as cramping, abdominal pain and weight loss may occur[4]. The aetiology of LC is not yet clear, although several hypotheses have been suggested, such as association to various autoimmune conditions and drug induction[2]. LC has an incidence of 3.1 to 9.8 and prevalence of 14.2 per 100 000 people[5-12].

Sequential treatment for LC is recommended, in which a "therapeutic ladder" is followed. The suggested drugs in this ladder are: loperamide, bismuth salicylate, budesonide, cholestyramine 5-aminosalicylic acid preparations, prednisolone, azathioprine, 6-mercaptopurine methotrexate or octreotide[2].

In a previous study, a high density of colonic chromogranin A immunoreactive cells were reported in patients with LC[13]. Chromogranin A is a general marker for all endocrine cells[14-16], but it is not clear which types of colonic endocrine cells are responsible for the increase in the density of chromogranin A cells in patients with LC. Therefore, the current study was performed to identify the endocrine cell types involved.

MATERIALS AND METHODS

Patients and controls

Fifty-seven patients with a diagnosis of lymphocytic colitis during the period 2007-2010 were included in this study. The patients were diagnosed in all 3 hospitals of Helse-Fonna region in Western Norway, namely Stord, Haugesund and Odda. They were 41 females and 16 males, with an average age of 49 years (range 19-84 years). These patients did not show any clinical signs of other autoimmune disorders. They did not had coeliac disease tested either by serology or duodenal biopsies. Twenty-seven subjects that underwent colonoscopy with biopsies were used as controls. Twenty of these subjects underwent colonoscopy because of gastrointestinal bleeding, where the source of bleeding was identified as haemorrhoids (18), or angiodysplasia (2), and seven were examined because of health worries caused by a relative being diagnosed with colon carcinoma. They were 19 females and 8 males with an average age of 49 years (range 18-67 years). All these subjects had no other gastrointestinal complaints than those mentioned above.

The study was performed in accordance with the Declaration of Helsinki and was approved by the local Committee for Medical Research Ethics. All subjects gave oral and written consent.

Colonoscopy

Colonoscopies were performed for all patients and controls, and two biopsies were taken from the caecum, from the ascending colon and from the right half of the transverse colon. These biopsies were pooled together and were labelled as right colon. In addition, two biopsies were taken from the left half of the transverse colon, from the descending colon and from the sigmoid colon. These 6 biopsies were pooled together and labelled as left colon.

Histopathology and immunohistochemistry

Biopsies were fixed in 4% buffered paraformaldehyde overnight, embedded in paraffin and cut into 5 μm-thick sections. The sections were stained with haematoxylin-eosin and immunostained by the avidin-biotin-complex (ABC) method using the Vectastain ABC-kit (Vector laboratories) as described earlier in detail[17]. The primary antibodies used were: monoclonal anti-human leucocytes common antigen (Dako, CD 45, clone 2B11), monoclonal anti-human CD8 lymphocytes (Dako, CD 57, clone 2B01), monoclonal mouse anti-serotonin (Dako, code no. M869), polyclonal anti-porcine peptide YY (PYY) (Eurodiagnostica, code B52-1), polyclonal rabbit anti-synthetic human pancreatic polypeptide (PP) (Dako, code no. A619), polyclonal rabbit anti-synthetic human somatostatin, and polyclonal rabbit anti-porcine glucagon (Eurodiagnostica, code B31). The antibodies were used at dilutions of 1:100, 1:200, 1:1500, 1:1500, 1:1000, 1:1600 and 1:200, respectively. The anti-PYY cross reacts with PYY in all vertebrates including humans. It does not cross react with PP or neuropetide Y in immunohistochemical system. Anti-glucagon is directed to N-Terminus of glicentin (enteroglucagon) and does not cross react with glucagon, vasoactive intestinal polypeptide or gastric inhibitory polypeptide. The second layer biotinylated mouse anti-immunoglobulin G (IgG) and rabbit anti-IgG were obtained from Dako. Negative and positive controls were the same as those described previously[17].

Computerised image analysis

The number of immunoreactive cells and the area of the epithelial cells were measured using Olympus software:

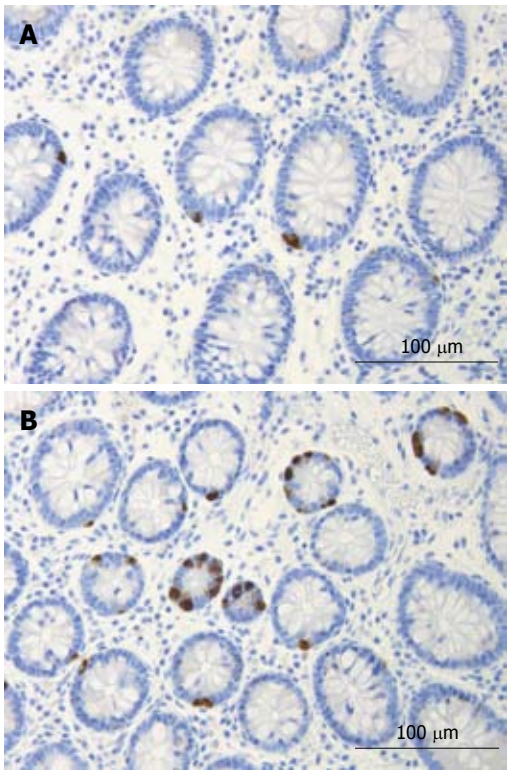

Figure 1 Serotonin-immunoreactive cells in the colon of a control (A) and of a patient with lymphocytic colitis (B).

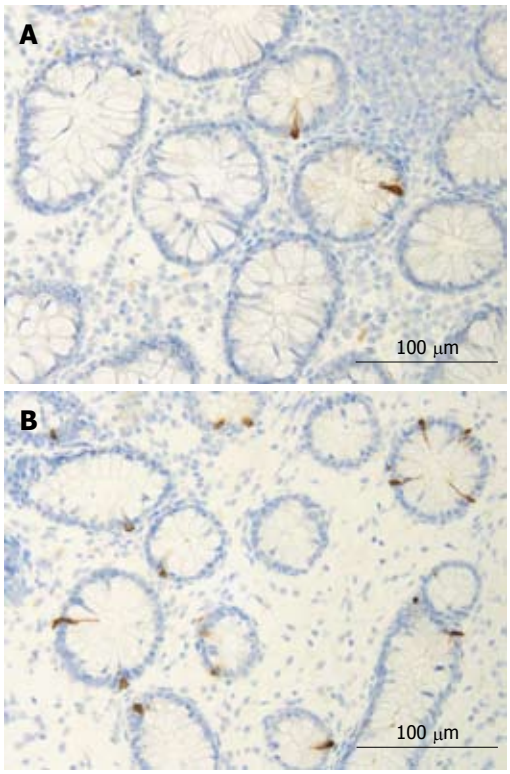

Figure 2 Colonic peptide YY -immunoreactive cells in a control (A) and in a patient with lymphocytic colitis (B).

Cell ^D. When using ×40 objectives, the frame (field) on the monitor represents an area of 0.14 mm^2 of the tissue. Measurements were performed in 10 randomly chosen fields for each individual and hormone. The X40 objective was used. The data from fields were tabulated, and the number of cells/mm^2 of the epithelium was computed and statistically analysed. The immunostained sections of IBS patients and controls were coded and mixed, and measurements were made without the knowledge of sections identity.

Statistical analysis

The non-parametric Mann-Whitney test was performed. $P < 0.05$ was considered to be statistically significant.

RESULTS

Endoscopy, histopathology and immunohistochemistry

The colon of both the patient and the control subjects were macroscopically normal. Histopathological examination of colon biopsies from controls revealed normal histology. All patients fulfilled the diagnosis criteria required for of LC: an increase in intraepithelial lymphocytes (> 20 lymphocytes/100 epithelial cells) and surface epithelial damage with increased lamina propria plasma cells and absent or minimal crypt architectural distribution. In the colon of both patients and control subjects, serotonin-, PYY-, PP-, enteroglucagon- and somatostatin-immunoreactive cells were primarily located in the upper part of the crypts of Lieberkühn. These cells were basket- or flask-shaped (Figures 1 and 2).

Computerised image analysis

PP-, enteroglucagon- and somatostatin-immunoreactive cells were sparse in the biopsy material examined. This made it difficult to perform a reliable quantification of these cell types.

There was no statistically significant difference between the right and left colon in controls with regards to the densities of serotonin- and PYY-immunoreactive cells ($P = 0.9$ and 0.1, respectively).

Serotonin cell density in the right colon in controls was 28.9 ± 1.8 (mean ± SE) and in LC patients 41.6 ± 2.6 ($P = 0.008$). In the left colon, the corresponding figures were 28.5 ± 1.9 and 42.4 ± 2.9, respectively ($P = 0.009$) (Figures 1 and 3). PYY cell density in the right colon of the controls was 10.1 ± 1 and of LC patients 41 ± 4 ($P = 0.00006$). In the left colon, PYY cell density in controls was 6.6 ± 1.2 and in LC patients 53.3 ± 4.6 ($P = 0.00007$) (Figures 2 and 4).

DISCUSSION

MC is a common cause of diarrhoea and 10% to 30% of older adults investigated for chronic diarrhoea have MC[18]. LC seems to be associated with several autoimmune diseases[19,20]. Furthermore, the prevalence of coeliac disease is high in patients with LC[21]. The information available on the gut endocrine cells in coeliac disease is restricted to the duodenum[22]. It is therefore difficult

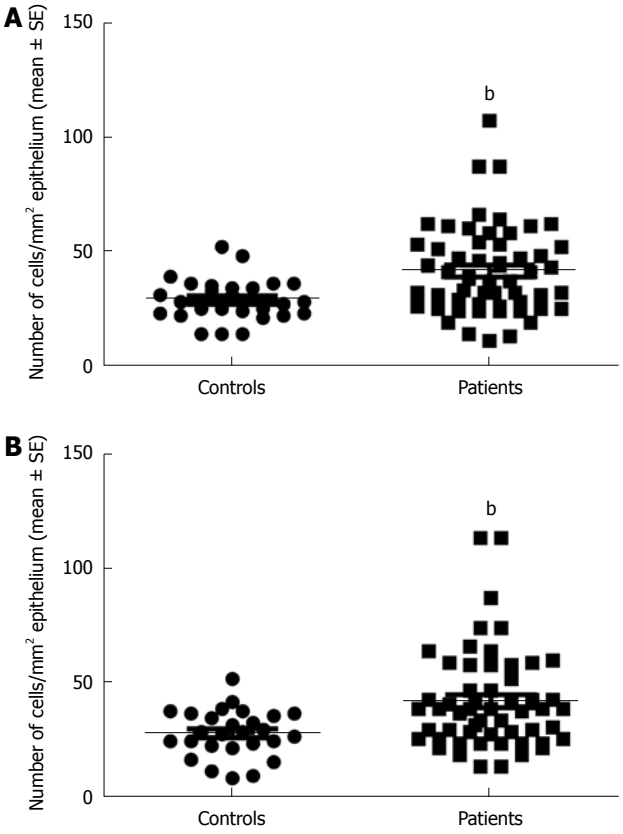

Figure 3 Serotonin cell density in the controls and patients with lymphocytic colitis in the right (A) and left colon (B). $^{b}P < 0.01$ *vs* controls.

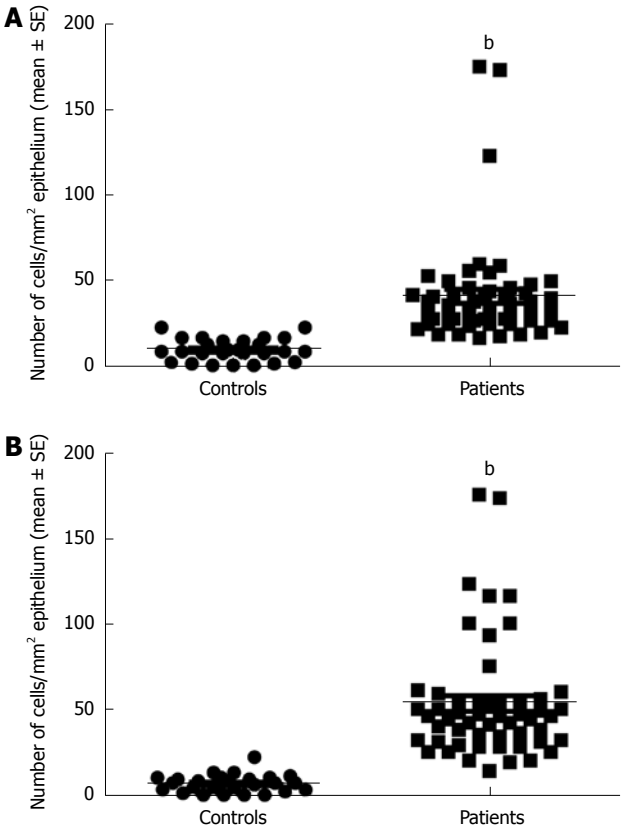

Figure 4 Peptide YY cell density in controls and patients with lymphocytic colitis in the right (A) and left colon (B). $^{b}P < 0.001$ *vs* controls.

to compare the outcome of the present study with findings in coeliac disease.

The current study showed that serotonin and PYY cell densities were increased in both the right and left colon of patients with LC. Serotonin activates the submucosal sensory branch of the enteric nervous system, and controls gastrointestinal motility and chloride secretion *via* inter-neurons and motor neurons[23,24]. PYY stimulates the absorption of water and electrolytes and is a major regulator of the "ileal brake"[24]. There are several studies showing that inflammation and immune cells affect the neuroendocrine system of the gut[25]. Thus, serotonin secreted by enterochromaffin (EC) cells can be enhanced or attenuated by secretory products of immune cells, such as CD4+T[26]. Furthermore, serotonin modulates the immune response[26]. The EC are in contact with or very close to CD3+ and CD20+ lymphocytes, and several serotonergic receptors have been characterised in lymphocytes, monocytes, macrophages and dendritic cells[27].

One may speculate that the high density of serotonin cells in LC patients is caused by the interaction between the immune cells and serotonin cells in the epithelium and submucosa of LC patients. The increase in serotonin would cause accelerated colonic motility and visceral hypersensitivity. Accelerated colonic motility and visceral hypersensitivity can cause diarrhoea and abdominal pain, symptoms that occur in LC. It is probable that the increase in PYY cell density is secondary to the increase in serotonin cell density in order to compensate for accelerated motility by activating the ileal brake and by increasing the absorption of water. In support for this assumption are the findings that large intestinal serotonin and PYY cells as well plasma levels are affected in patients with ulcerative colitis and Crohn's disease[28-32]. Similarly, these endocrine cells have been found to be affected in experimental animal model of colitis[33,34].

LC and post-infectious IBS (PI-IBS) show a striking similarity. They have the same clinical presentation and both can regress spontaneously[35,36]. Both LC and PI-IBS show intra-epithelial and submucosal infiltration of lymphocytes and mast cells, and exhibit a high density of colonic serotonin and PYY cells[36-41]. This raises the question as to whether LC and PI-IBS are actually the same disorder. If this is proven to be true, it would have an important clinical implication. Thus, PI-IBS can be treated by the same "therapeutic ladder" which is proven to be effective in LC.

COMMENTS

Background

Microscopic colitis is a chronic condition with watery diarrhoea as the cardinal symptom, but other symptoms such as cramping, abdominal pain and weight loss may occur Radiologic and endoscopic findings in these patients are normal. However, histopathological examinations of the colon reveal abnormal histology, which is of two distinctive types: lymphocytic colitis (LC) and collagenous

colitis. LC exhibits an increased number of colonic intra-epithelial lymphocytes (> 20/100 epithelial cells), increased inflammatory cells within the lamina propria and preserved crypt architecture. LC and irritable bowel syndrome (IBS) have similar symptoms and both are without radiologic or endoscopic abnormalities. Thus, LC can be mistakenly diagnosed as IBS. In a study on the colonic chromogranin A cell density in IBS patients, nine patients out of 50 showed extremely high colonic chromogranin A cell density. This high density was in contrast to the low density of chromogranin cells in the rest of the IBS patients studied. Re-examination of these nine patients revealed that they suffered from LC. This unexpected finding was confirmed on a larger patient's material. As chromogranin A is a common marker for endocrine cells, the present investigation was performed to identify the colonic endocrine cell-types that are affected.

Research frontiers

The current study is a further investigation of the unexpected observation that LC patients have high colonic chromogranin A cell density, which has been shown to be an excellent biomarker for the diagnosis of LC. This unexpected observation led to a novel approach toward LC, where the colonic hormones role in the symptom development in patients and their role in the pathogenesis of the disease come under focus. The current study showed that the endocrine cell-types that are affected in the colon of LC patients were peptide YY (PYY) and serotonin cells. This underlines the importance of theses two hormones in LC.

Innovations and breakthroughs

The present findings underline the importance of the interaction between the gut hormones and the local immune system in the gut (the endocrine/immune axis) and its role in the pathogenesis and symptom development in disease. Such interactions should be put under the spotlight in several gastrointestinal diseases, especially those with known inflammation such as inflammatory bowel disease and coeliac disease. The similarity in the endocrine changes between LC and post-infectious irritable bowel syndrome (PI-IBS) has drawn attention to other histopathological and clinical similarities such as: both LC and PI-IBS show intra-epithelial and submucosal infiltration of lymphocytes and mast cells; both have the same clinical presentation; and both can regress spontaneously. This lead to the notion that LC and PI-IBS may be the same disorder.

Applications

Understanding the interaction between gut hormones and the local immune system of the gut would result in better understanding of the pathogenesis of gut inflammatory diseases and possibly open a new avenue for treatment. If LC and PI-IBS are proven to be the same disorder, PI-IBS can be treated by the same "therapeutic ladder" that has been proven to be effective in LC. PI-IBS constitutes a large subset of IBS without any effective treatment.

Terminology

Chromogranin A: Chromogranin A is a 68-kDa protein comprising 439 amino-acid residues. Chromogranin is co-stored and co-released with monoamines and peptide hormones of the adrenal medulla, pituitary gland, parathyroid, thyroid C-cells, pancreatic islets, endocrine cells of the gastrointestinal tract and sympathetic nerves; PYY: PYY is localised in endocrine cells in the ileum and large intestine; Serotonin: Serotonin is a monoamine that is localised in the enterochromaffin cells throughout the entire gastrointestinal tract. It also occurs also in the enteric nervous system and acts as a hormone and a neurotransmitter.

Peer review

This is an excellent paper which shows new light on lymphocytic colitis. The study was performed to identify the endocrine cell types in the colonic epithelium of LC by immunostaining using representative 5 antibodies against serotonin, PYY, pancreatic polypeptide, somatostatin, and glucagon. The results were that serotonin and PYY cell densities were increased in both the right and left colon of patients with LC, when compared with controls. They concluded that the high density of serotonin cells in LC patients were caused by the interaction between immune cells and serotonin cells, which occurs in the epithelium and submucosa of LC patients, and that the increase in PYY density is secondary to the increase in serotonin cell density.

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ORIGINAL ARTICLE

Galactosylated chitosan/5-fluorouracil nanoparticles inhibit mouse hepatic cancer growth and its side effects

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Abstract

AIM: To observe the curative effect of galactosylated chitosan (GC)/5-fluorouracil (5-FU) nanoparticles in liver caner mice and its side effects.

METHODS: The GC/5-FU nanoparticle is a nanomaterial made by coupling GC and 5-FU. The release experiment was performed *in vitro*. The orthotropic liver cancer mouse models were established and divided into control, GC, 5-FU and GC/5-FU groups. Mice in the control and GC group received an intravenous injection of 200 μL saline and GC, respectively. Mice in the 5-FU and GC/5-FU groups received 200 μL (containing 0.371 mg 5-FU) 5-FU and GC/5-FU, respectively. The tumor weight and survival time were observed. The cell cycle and apoptosis in tumor tissues were monitored by flow cytometry. The expression of p53, Bax, Bcl-2, caspase-3 and poly adenosine 50-diphosphate-ribose polymerase 1 (PARP-1) was detected by immunohistochemistry, reverse transcription-polymerase chain reaction and Western blot. The serum blood biochemical parameters and cytotoxic activity of natural killer (NK) cell and cytotoxicity T lymphocyte (CTL) were measured.

RESULTS: The GC/5-FU nanoparticle is a sustained release system. The drug loading was 6.12% ± 1.36%, the encapsulation efficiency was 81.82% ± 5.32%, and the Zeta potential was 10.34 ± 1.43 mV. The tumor weight in the GC/5-FU group (0.4361 ± 0.1153 g *vs* 1.5801 ± 0.2821 g, $P < 0.001$) and the 5-FU (0.7932 ± 0.1283 g *vs* 1.5801 ± 0.2821 g, $P < 0.001$) was significantly lower than that in the control group; GC/5-FU treatment can significantly lower the tumor weight (0.4361 ± 0.1153 g *vs* 0.7932 ± 0.1283 g, $P < 0.001$), and the longest median survival time was seen in the GC/5-FU group, compared with the control (12 d *vs* 30 d, $P < 0.001$), GC (13 d *vs* 30 d, $P < 0.001$) and 5-FU groups (17 d *vs* 30 d, $P < 0.001$). Flow cytometry revealed that compared with the control, GC/5-FU caused a higher rate of G0-G1 arrest (52.79% ± 13.42% *vs* 23.92% ± 9.09%, $P = 0.014$) and apoptosis (2.55% ± 1.10% *vs* 11.13% ± 11.73%, $P < 0.001$) in hepatic cancer cells. Analysis of the apoptosis pathways showed that GC/5-FU upregulated the expression of p53 at both the protein and the mRNA levels, which in turn lowered the ratio of Bcl-2/Bax expression; this led to the release of cytochrome C into the cytosol

from the mitochondria and the subsequent activation of caspase-3. Upregulation of caspase-3 expression decreased the PARP-1 at both the mRNA and the protein levels, which contributed to apoptosis. 5-FU increased the levels of aspartate aminotransferase and alanine aminotransferase, and decreased the numbers of platelet, white blood cell and lymphocyte and cytotoxic activities of CTL and NK cells, however, there were no such side effects in the GC/5-FU group.

CONCLUSION: GC/5-FU nanoparticles can significantly inhibit the growth of liver cancer in mice *via* the p53 apoptosis pathway, and relieve the side effects and immunosuppression of 5-FU.

Key words: Galactosylated chitosan; Nanoparticles; 5-fluorouracil; Hepatocellular cancer; Targeted therapy; Apoptosis

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INTRODUCTION

Hepatocellular cancer (HCC) is one the most prevalent malignancies[1,2]. Liver transplantation remains the most effective therapeutic option for HCC; however, due to the lack of donors and the relatively high cost, a substantial number of patients die while waiting for a donor liver[3,4]. The disadvantages of most anti-cancer drugs that are currently available include low bioavailability, poor selectivity because they can act on both tumor cells and healthy cells, and immunosuppression that can cause complications and even patient death[5]. However, targeted therapy for HCC may be useful because it is relatively less expensive compared with the current therapies and it also produces fewer side effects[6,7]. 5-fluorouracil (5-FU) is a pyrimidine anticancer drug. Since its development by Heidelberg in 1957, 5-FU has occupied an important position in the cancer chemotherapy field. Because 5-FU is highly effective against a broad spectrum of malignancies, it is widely used in chemotherapy regimens against cancers such as hepatocellular, gastric, pancreatic and breast cancers; 5-FU is very important in the management of liver cancer. 5-FU belongs to the cell cycle specific drugs and can be converted into fluorouracil deoxynucleotide to bind with thymidine synthase, which leads to the disruption of RNA, DNA and protein biosynthesis. However, because of the similarity of the nucleic acid metabolism pathways between tumor and normal tissues, 5-FU can also target normally proliferating tissues, leading to bone marrow suppression and gastrointestinal reactions. Other disadvantages of 5-FU include irregular absorption, a short half-life, and rapid turnover, which require lengthy, high-dose intravenous administration to maintain its effective *in vivo* concentration for a suitable period[8,9]; these disadvantages can significantly restrict the clinical application of 5-FU. The emergence of a novel, sustained-release formulation of 5-FU is of clinical significance because it has fewer side effects compared to the regular 5-FU formulation[10-12]. Galactosylated chitosan (GC) is a galactose ligand, with chitosan modifications on the molecular structure[13-15]. Asialoglycoprotein receptor (ASGPR) is a receptor found on the membrane of hepatocytes facing the sinusoids, with specificity for glycoproteins with galactose or acetyl galactosamine at the end. Each hepatocyte contains approximately two million binding sites for ASGPR[16]. The binding of the galactose ligand with ASGPR induces liver-targeted gene transfer. Our lab previously synthesized a GC nanoparticle as a gene carrier and showed that the GC nanoparticle can successfully transfer genes into the liver *in vitro* and *in vivo*. We also confirmed that this nanoparticle material has a high selectivity to the liver and a low cytotoxicity[17]. In the present study, we synthesized GC/5-FU nanoparticles by combining the GC material with 5-FU, and tested its effect on liver cancer *in vitro* and *in vivo*. We found that the GC/5-FU nanoparticles can specifically target the liver and that the addition of GC increases the cytotoxicity of 5-FU and apoptosis mediated by the p53 pathway. GC/5-FU nanoparticles can ameliorate the side effects and immunosuppressive action of 5-FU.

MATERIALS AND METHODS

Reagents

Chitosan (deacetylation degree > 85%) GC was synthesized and stored by our group. HCl was from Shanghai Medpep, AR. LC-10A HPLC (Shimadzu, Japan), flow cytometry (FACS Calibunr, United States). The immunohistochemistry kit was from GBI, United States. Caspase-3 and poly ADP-ribose polymerase 1 (PARP-1) antibodies were from Santa Cruz, CA, United States; Bax and Bcl-2 antibodies were from Temecula, CA, United States; and p53 antibody was from Beverly, MA, United States.

Mice and cell lines

The mouse liver cancer cell lines (H22) were purchased from the Cancer Institute of Fudan University, China. Female BALB/c mice, 7 wk of age and weighing 25 g, were

obtained from the Department of Experimental Animals of Fudan University, China. All mice were housed in specific pathogen-free level B animal facility and animal experiments were conducted following the guidelines of the Animal Research Ethics Board of Fudan University.

Synthesis of GC/5-FU

The 5-FU/GC was mixed at a mass ratio of 10:1 in solution, using vortex oscillator (2500 r/min) for 30 s; the final concentration of 5-FU was 1.857 g/L. The product was kept at room temperature for 30 min to assess for further particle formation. The final product was kept at 4 ℃. The drug loading and encapsulation efficiency were calculated according to the following equations: drug loading = the amount of 5-FU within nanoparticle/nanoparticle mass × 100%; encapsulation efficiency = the amount of 5-FU within nanoparticle/total amount of 5-FU added × 100%.

In vitro release experiment

GC/5-FU nanoparticles (20 mg) were mixed with 30 mL of simulated body fluid (pH 7.4) in dialysis bags and incubated at 37 ℃ using a shaker with a fixed speed of 60 r/min. Samples were taken at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 d after mixing. The optical density (*A*) was measured at 265 nm by an automated microplate reader (Bio-Rad Inc, California, United States). The amount of 5-FU released at different time points was calculated according to a standard absorbance curve. The concentration and cumulative release rate were calculated according to the standard curve equation. Each experiment was performed in triplicate.

Animal model

The subcutaneous liver cancer mouse model was established using the mouse HCC cell line H22. After euthanasia and dissection, fresh fast-growing tumor tissues were minced and made into a tumor cell suspension at a density of 6×10^4/L. Recipient mice were anesthetized by 20% urethane, followed by an injection of 50 μL of tumor cell suspension into the left liver lobe capsule. Approximately two min after completion of the procedure, when there was no leaking, the abdomen was sutured and the orthotropic liver cancer mouse model was established successfully[18].

Curative effect of GC/5-FU in the orthotropic liver cancer mouse model

Five days after the establishment of the orthotropic liver cancer mouse model, the tumor reached a size of about 4-6 mm in diameter (Figure 1). The mouse models were randomly assigned into 4 groups labeled as control, GC, 5-FU and GC/5-FU. Mice in the control group received an intravenous injection of 200 μL saline. GC group received 200 μL GC nano-material. 5-FU and GC/5-FU groups received 200 μL (containing 0.371 mg 5-FU) 5-FU and GC/5-FU, respectively. The drugs were given continuously for 5 d starting from day 5 after the tumor was established. At day 15, 10 mice were sacrificed and the tumor growth was monitored. The remaining 15 mice in each group were kept for survival analysis.

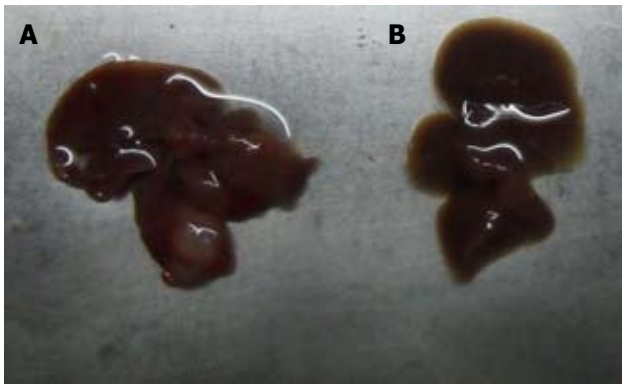

Figure 1 Establishment of the hepatic cancer mouse model. A: Liver cancer; B: Normal mouse liver.

Cell cycle and apoptosis analysis by flow cytometry

The cell suspension was made of 1-2 mL tumor tissues from each individual group. Cells were washed three times by 0.1 mol/L phosphate buffered solution (PBS) and fixed by 70% ethanol. Cells were then incubated with 50 mg/L propidium iodide (Zhengzhou Sigma Chemical, Zhengzhou, China), 1.0% Triton X-100 and 10 mg/L RNaseA for 30 min at 4 ℃ in dark. Cell cycle distribution was analyzed by flow cytometry. Proliferation index (PI) = (S + G2/M)/(G0/G1 + S + G2/M). Apoptosis was determined by staining cells with annexin V-FITC (Pharmingen, San Diego, CA, United States) and propidium iodide because annexin V can identify the externalization of phosphatidylserine during the progression of apoptosis and therefore can detect early apoptotic cells. To quantify apoptosis, cells were washed twice with cold PBS and resuspended in binding buffer at 1×10^3 cells/L. A quantity of 100 μL of this suspension was transferred to a 5 mL culture tube with 5 mL of annexin V-FITC and 10 mL of 20 mL/L propidium iodide, and analyzed using the flow cytometry.

Immunohistochemistry

The 4 μm sections were deparaffinized by incubation at 65 ℃. Sections were soaked in 3% H_2O_2 for 10 min at room temperature to deactivate endogenous peroxidases. Antigen retrieval was performed using a microwave. The primary antibody was incubated at 37 ℃ for 1 h in a humidified chamber; the secondary antibody was incubated at 37 ℃ for 30 min. After washing, the sections were developed using diaminobenzidine, and counter stained with hematoxylin. After dehydration, the sections were analyzed under a light microscope[19]. p53 staining was mainly observed in the nucleus, which appeared brown and granular with little background. Caspase-3 staining was mainly present in cytoplasm, showing a brown granular staining pattern. PBS was used instead of primary antibody for a negative control. The Image-pro plus 6.0

system was used to analyze five fields randomly chosen from each slide. The images were amplified 200-fold, converted into gray-scale so as to distinguish the positive staining area from background. The positive-stained area and the total area of the field were measured by the system and the area ratio was calculated using the following equation: staining area/total area × 100%. The stained area of each individual slide was determined by averaging the area ratio.

Reverse transcription-polymerase chain reaction

Primers were purchased from Shanghai R and S Biotechnology Co., Ltd. The oligonucleotide primers used were: Bcl-2: 5'-CGGGCTGGGGATGACTTCTCT-3' (sense), 5'-GCATCCCAGCCTCCGTTATCC-3' (antisense); Bax: 5'-AGACACCTGAGCTGACCTTGGAG-3' (sense), 5'-AGACACCTGAGCTGACCTTGGAG-3' (antisense); PARP-1: 5'-TCCCAAGGACTCCCTCCGCATGG-3' (sense), 5'-CTTTGCCTGCCACGCCTCCAGCC-3' (antisense); Caspase-3: 5'-TTGGAACAAATGGACCTG-3' (sense), 5'-ACAAAGCGACTGGATGAA-3' (antisense); P53: 5'-GTGGCCTCTGTCATCTTCCG-3' (sense), 5'-CCGTCACCATCAGAGCAACG-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control : 5'-ACCGCAAAGACTGTGGATGC-3' (sense), 5'-TGAGCTTGACAAAGTGG TCG-3' (antisense). Tissue total RNA was extracted by TRIZOL (Invitrogen, California, United States). Total RNA (1 μL) was used to reverse transcribe into cDNA using 0.5 μL AMV reverse transcriptase. Polymerase chain reaction (PCR) was performed using 2.5 μL cDNA, 0.1 μL Ex Taq HS, 0.1 μL forward primer and 0.1 μL reverse primer. The PCR reaction conditions were as follows: 94 ℃ for 2 min, 35 cycles of 94 ℃ for 40 s, 50 ℃ to 6 ℃ for 40 s and 72 ℃ for 1 min, followed by 72 ℃ for 5 min. PCR products were kept at -20 ℃[20]. GAPDH was used as internal control. The PCR product (6 μL) was resolved in 2% agarose gel for 30 min at 120 V, 100 mA, stained with ethidium bromide solution for 5 min, imaged by a ultraviolet gel imaging system, and analyzed by Quantity One software (Bio-Rad Inc, California, United States). The expression of target genes was presented as the ratio of target to internal control GAPDH.

Western blotting analysis

After the concentration was determined, the samples were loaded onto the 12% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) gel and resolved at 80 V followed by 120 V. Methanol-pretreated polyvinylidene difluoride (PVDF) membrane was then soaked in transfer buffer (pH 8.3, 25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol) for 10 min. Proteins on the SDS-PAGE gel were then transferred onto the PVDF membrane under 100 volts (V) for 70 min. The membrane was blocked by 5% FBS/PBS at 4 ℃ overnight. Primary antibodies were diluted at 1:2000 and incubated with a membrane for 3 min at room temperature. The membrane was then washed three times for 10 min each with PBS containing 0.05% Tween 20. Goat-anti-mouse immunoglobulin G secondary antibody (1:8000) was added to incubate with the membrane for 3 h at room temperature, followed by washing three times using the same washing solution. The membrane was then developed for 1 min using an enhanced chemiluminescence kit with equal volumes of A and B solution[21]. After imaging, Image J version 1.44 software (National Institutes of Health) was used to analyze the average density values.

Analysis of blood biochemical parameters

The animals were sacrificed by day 10 after treatment. Blood chemistry including the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen and creatinine was examined by a Fuji Drychem 3500 automated analyzer (Fuji Medical System Co. Ltd., Tokyo), the blood routine such as hemoglobin (Hbg), platelet (PLT), white blood cell (WBC), lymphocyte and neutrophil was detected by a Sysmex XS-800i automated analyzer (Sysmex Shanghai Ltd, Shanghai, China) .

Cytotoxic assay for natural killer cell and cytotoxicity T lymphocyte

Natural killer cell (NK) and cytotoxicity T lymphocyte (CTL) cytotoxic activity was measured by the 3-(4, 5-dimethylthiazd-2-yl)-2,5-diphenyltentrazolium bromide (MTT) colorimetry assay (Sigma, United States) as reported previously[17]. 1×10^4 YAC-1 as target cells were seeded in a 96-well plastic plates. Spleen cells used as effector cells were prepared from the mice and were simultaneously seeded in a 96-well plate at a 50:1 ratio of effector to target (E:T) in CTL assay. The effector cells from spleen cells were incubated with H22 target cells at a 50:1 ratio of E:T. All cytotoxic activity assays were performed in triplicate.

The activities of CTL and NK were calculated using the following formulas: CTL activity (%) = [1 - (A_{E+T} - A_E)/A_T] × 100%; NK activity (%) = [1 - (A_{E+T} - A_E)/A_T] × 100%, where A_E indicates the mean A value of effector cells, A_T indicates the mean A value of target cells, and A_{E+T} indicates the mean A value of effector cells + target cells.

Statistical analysis

All data was collected and expressed as mean ± SD. Analysis of variance (ANOVA) was used to analyze data within the same group, one-way ANOVA was used to analyze data between groups, while the least significant digit method was used for pairwise comparison between groups. A value of $a = 0.05$ and $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and characterization of GC/5-FU nanoparticles

5-FU/GC nanoparticles were successfully synthesized,

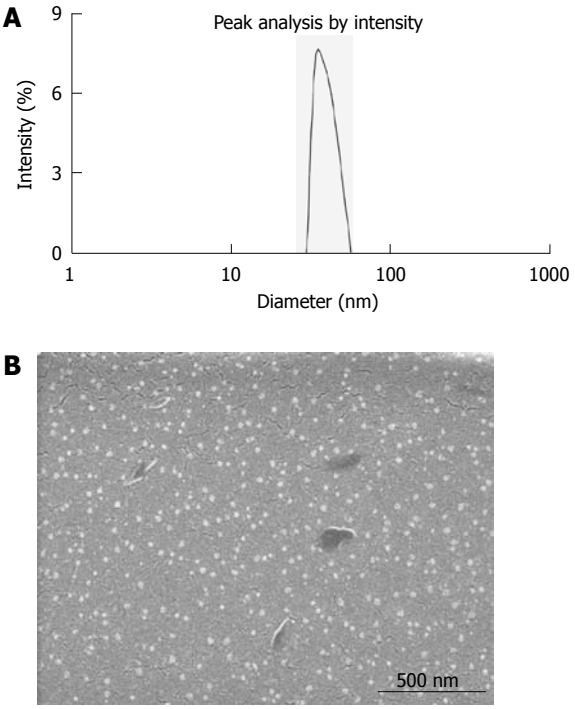

Figure 2 Particle size and scanning electron microscope image of galactosylated chitosan/5-fluorouracil. A: Particle size graph showing the diameter of galactosylated chitosan/5-fluorouracil (GC/5-FU) (35.19 ± 9.50 nm); B: Scanning electron microscope image of GC/5-FU. The particles show spherical structure with a smooth surface and no adhesion between nanoparticles.

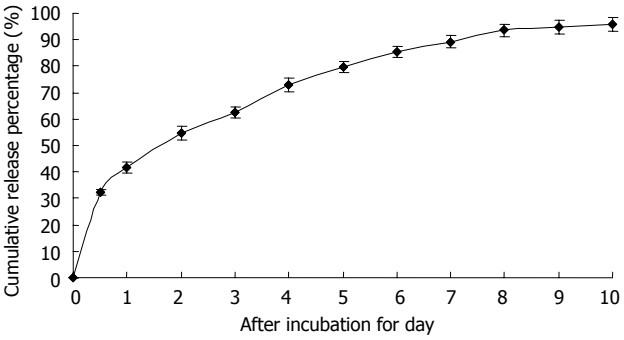

Figure 3 The *in vitro* release curve of nanoparticles in simulated body fluid (37 ℃, pH 7.4, *n* = 3). A rapid release was observed from time 0 h to 12 h, with a cumulative release percentage of 32.4%; a smooth slow-release occurred between day 1 and day 8, with a cumulative release percentage of 93.50%. During days 8 to 10, the release reached a plateau, with a cumulative release percentage of 95.70% at day 10.

and the radius of the nanoparticles was 35.19 ± 9.50 nm, which had a normal distribution (Figure 2A). Electron microscopy showed that the particles were in regular spherical shape, with a smooth surface, a uniform size, and no adhesion between nanoparticles (Figure 2B). The drug loading was 6.12% ± 1.36%, the encapsulation efficiency was 81.82% ± 5.32%, and the Zeta potential was 10.34 ± 1.43 mV. Figure 3 shows the *in vitro* release curve of nanoparticles in simulated body fluid (37 ℃, pH 7.4). A rapid release was observed from 0 h to 12 h, with a cumulative release percentage of 32.4%, presumably due to the diffusion of surface 5-FU into the solution; a smooth slow-release occurred between day 1 and day 8, with a cumulative release percentage of 93.50%, indicating that the GC/5-FU nanoparticles have a sustained release effect from days 1 to 8. From days 8 to 10, the release reached a plateau, with a cumulative release percentage of 95.70% at day 10.

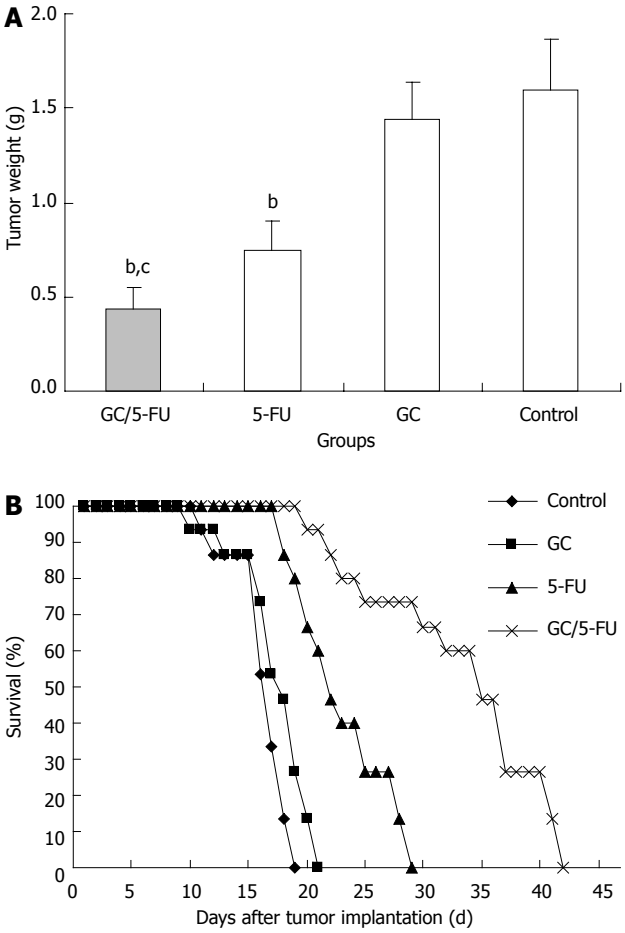

Figure 4 The curative effect of galactosylated chitosan/5-fluorouracil on liver cancer in the orthotopic transplant model of hepatocellular carcinoma. A: Five days after the tumor was established, galactosylated chitosan/5-fluorouracil (GC/5-FU), 5-FU, GC or phosphate buffered solution was given to the mice. Tumor weight was measured at day 15; B: Treatment was given as described previously and the survival was monitored. The median survival for control, GC, 5-FU and GC/5-FU groups were 12, 13, 17 and 30 d, respectively. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* 5-FU group.

Effect of GC/5-FU on tumor mass and survival in the mouse model

The tumor samples were harvested and weighted 15 d after treatment (Figure 4A). The weight of the tumor was 0.4361 ± 0.1153 g in GC/5-FU group, 0.7932 ± 0.1283 g in 5-FU group, 1.3989 ± 0.2125 g in GC group and 1.5801 ± 0.2821 g in control group. The differences between the groups was statistically significant ($P < 0.01$). The tumor weight in the GC/5-FU and the 5-FU groups were significantly lower than in the GC group and control group ($P < 0.01$) while tumor weight in the GC/5-FU group was significantly lower than in the 5-FU group ($P < 0.01$);

however, the tumor weight in the GC group and the control group was not different ($P > 0.05$). After the model was developed, the mice were randomly assigned to four groups with 15 mice each, and treated as described above. The survival of the mice was monitored, and there was a 100% mortality in all groups. The Kaplan-Meier survival curve (Figure 4B) showed that mice all the mice in the control group died between day 6 and day 14, with a median survival time of 12 d. In the GC group, all mice died between day 5 and day 16, the median survival time being 13 d. There was no statistical difference in the survival time between the control and the GC groups ($P > 0.05$). Mice treated with 5-FU also all died between day 13 and day 24, with a median survival of 17 d. All mice in the GC/5-FU group died between day 15 and day 37, with a median survival time of 30 d. The median survival time of mice treated with either 5-FU or GC/5-FU was significantly longer than that of mice in the GC or control groups; the longest median survival time was seen in the GC/5-FU group ($P < 0.01$ compared with the control, GC and 5-FU groups).

Effect of GC/5-FU on cell cycle, proliferation and apoptosis of H22 cells

Flow cytometry was used to analyze the liver cancer samples harvested 15 d after beginning the treatment. As shown in Figure 5A and B, the percentage of cells in the G0-G1 phases was significantly higher in the GC/5-FU- and 5-FU-treated tumors ($P < 0.01$), while the PI was lower than that in the GC and control groups ($P < 0.01$), suggesting that GC/5-FU and 5-FU had an overt anti-proliferative effect and arrested the tumor cells in the G0-G1 phases. The percentage of apoptotic cells in the GC/5-FU and 5-FU groups was significantly increased when compared with that in the control and GC groups ($P < 0.01$). Also, the percentage of apoptotic cells of GC/5-FU group was higher than that in the 5-FU group ($P < 0.01$), suggesting that GC is able to enhance the cellular influx of 5-FU, thereby improving the pro-apoptotic effect of 5-FU (Figure 5C).

GC/5-FU induced hepatic cancer cell apoptosis via activating the p53 pathway

To understand which pathway(s) mediated the GC/5-FU-induced apoptosis, we examined the expression of p53 at both protein and mRNA levels. Compared with the control and GC groups, the expression of p53 was increased in the 5-FU and GC/5-FU groups, with the highest increase seen in the GC/5-FU group ($P < 0.01$, Figures 6, 7A and B). The ratio of Bcl-2/Bax showed a decreasing tendency from control to GC to 5-FU to GC/5-FU groups ($P < 0.01$, Figure 7D); specifically, the ratio in 5-FU and GC/5-FU was significantly lower than that in the control and GC groups, with a lowest ratio observed in the GC/5-FU group ($P < 0.01$). GC/5-FU can also significantly induce the expression of caspase-3 in the tumor tissues ($P < 0.01$, Figures 6, 7A and B). The expression of PARP-1 also displayed a decreasing tendency from control to GC to 5-FU to GC/5-FU groups ($P < 0.01$, Figure 7C), with the most significant reduction seen in the GC/5-FU group. Therefore, it is likely that GC/5-FU was involved in upregulating the genes in the p53 pathway.

Side effects of GC/5-FU

In order to understand the side effect of liver and kidney function and blood cells, we examined the blood of the mouse model by day 10 after treatment. The levels of AST and ALT in 5-FU group were obviously higher than those in control group ($P < 0.01$), while those in GC/5-FU group were lower compared with 5-FU group ($P < 0.01$), there were no differences among control, GC and GC/5-FU groups ($P > 0.05$). The numbers of PLT, WBC and lymphocyte in 5-FU group were decreased more obviously as compared with the control, GC and GC/5-FU groups ($P < 0.05$ or < 0.01), however, there were no differences among these three groups ($P > 0.05$). The levels of blood urea nitrogen, creatinine, Hbg and neutrophil were approximate in different groups ($P > 0.05$), as shown in Table 1.

Cytotoxic activities of CTL and NK in mice

We evaluated whether the GC/5-FU could affect the activity of CTL and NK *in vivo* in mouse model. The harvested splenocytes were washed with PBS. The activity of CTL and NK was detected by MTT colorimetry. Figure 8 shows that the cytotoxic activities of CTL and NK cells were significantly decreased in 5-FU group compared with other three groups ($P < 0.01$), while the crosscurrent was found in GC group compared with 5-FU group ($P < 0.05$). There were no differences between control and GC/5-FU groups. These results thus demonstrate that the GC/5-FU nanoparticles could ameliorate the decreased cytotoxic activities of CTL and NK in 5-FU group.

DISCUSSION

The utilization of nanotechnology and nano-materials in the pharmaceutical field gave rise to the drug-nanoparticle carrier-release system, which is a drug delivery system using nanoparticles as the drug carriers. A particle ranging from 0.1 nm to 100 nm is considered to be a nanoparticle[22]. The size of a nanoparticle is very important for drug delivery, as the spaces between the cells in various tissues are different: it is now known that the aperture of vascular endothelial within most normal tissues is 2 nm, the aperture of the postcapillary venule is 6 nm, while that of non-continuous tumor blood vessels ranges from 100 nm to 780 nm[23,24]. The size of the nanoparticles used in this study was approximately 35.19 nm (Figure 2A), which is smaller than most nanoparticles reported[25], allowing them to enter the space within tumor cells but restricting them from penetrating the normal tissues. Scanning electron microscopy revealed a spherical structure with a smooth surface and no adhe-

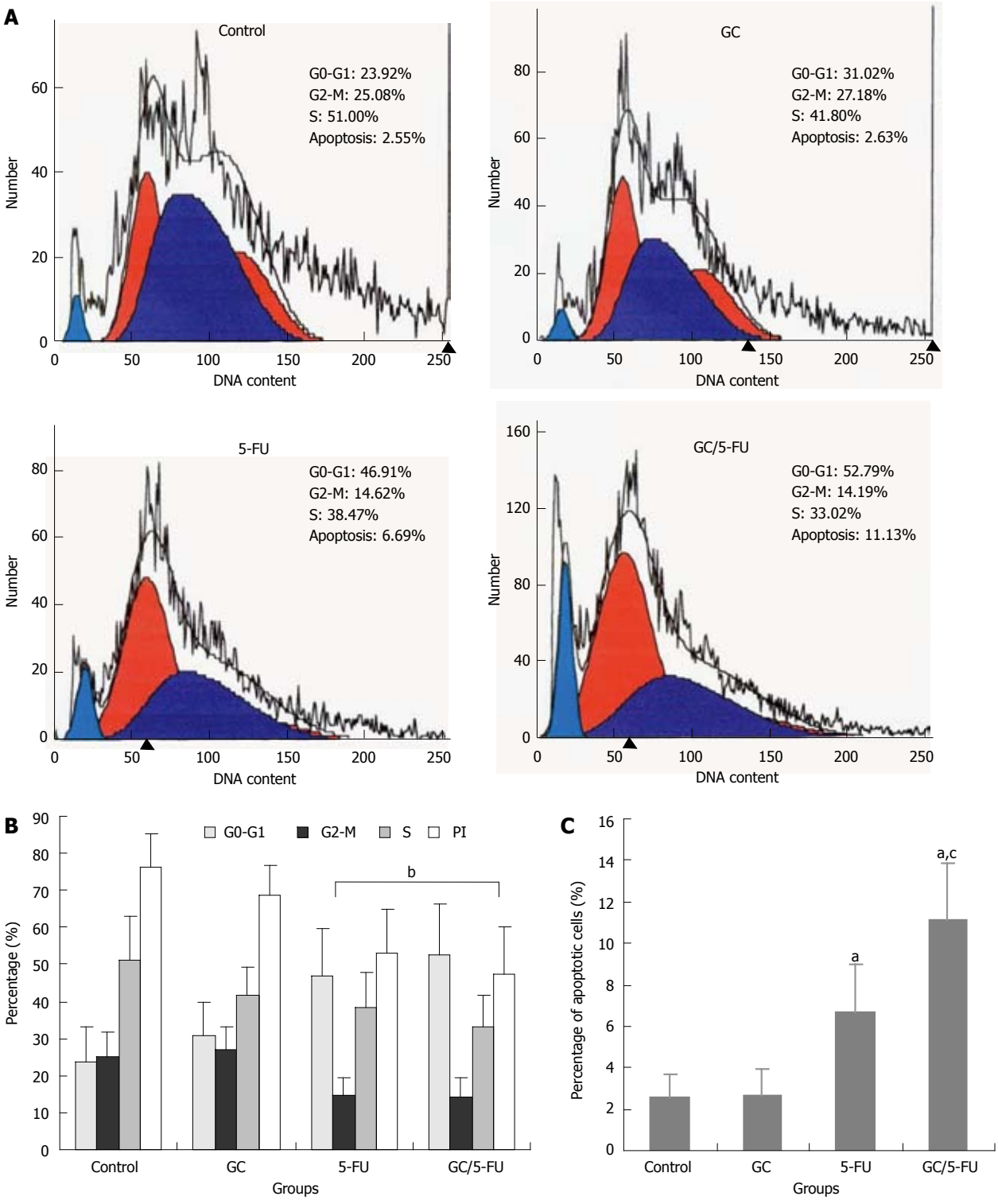

Figure 5 The effects of different treatments on cell cycle, proliferation index and apoptosis index. A: Flow cytometry analysis of cell cycle distribution of mouse liver cancer cell line (H22) cells; B: Quantification of cell cycle distribution and proliferation index of H22 cells. Percentage of cells in G0-G1 in the galactosylated chitosan/5-fluorouracil (GC/5-FU) and 5-FU groups was higher than that in control and GC groups, while the proliferation index (PI) decreased significantly ($P < 0.01$); C: Quantification of apoptosis of H22 in different treatment groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* 5-FU group.

sion between nanoparticles (Figure 2B), which is consistent with previous reports[25,26]. In order to confirm the sustained release effect, we performed an experiment on GC/5-FU. The *in vitro* release curve of GC/5-FU in simulated body fluid showed that the sustained release of the nanoparticle lasted 1-8 d. Such sustained release effect makes the drug evenly distribute in the body, thereby increasing the half-life of GC/5-FU in the circulation system and decreasing the toxic effects of 5-FU on normal tissues[27].

In order to evaluate the curative efficiency of intravenously injected GC/5-FU in a liver cancer mouse model,

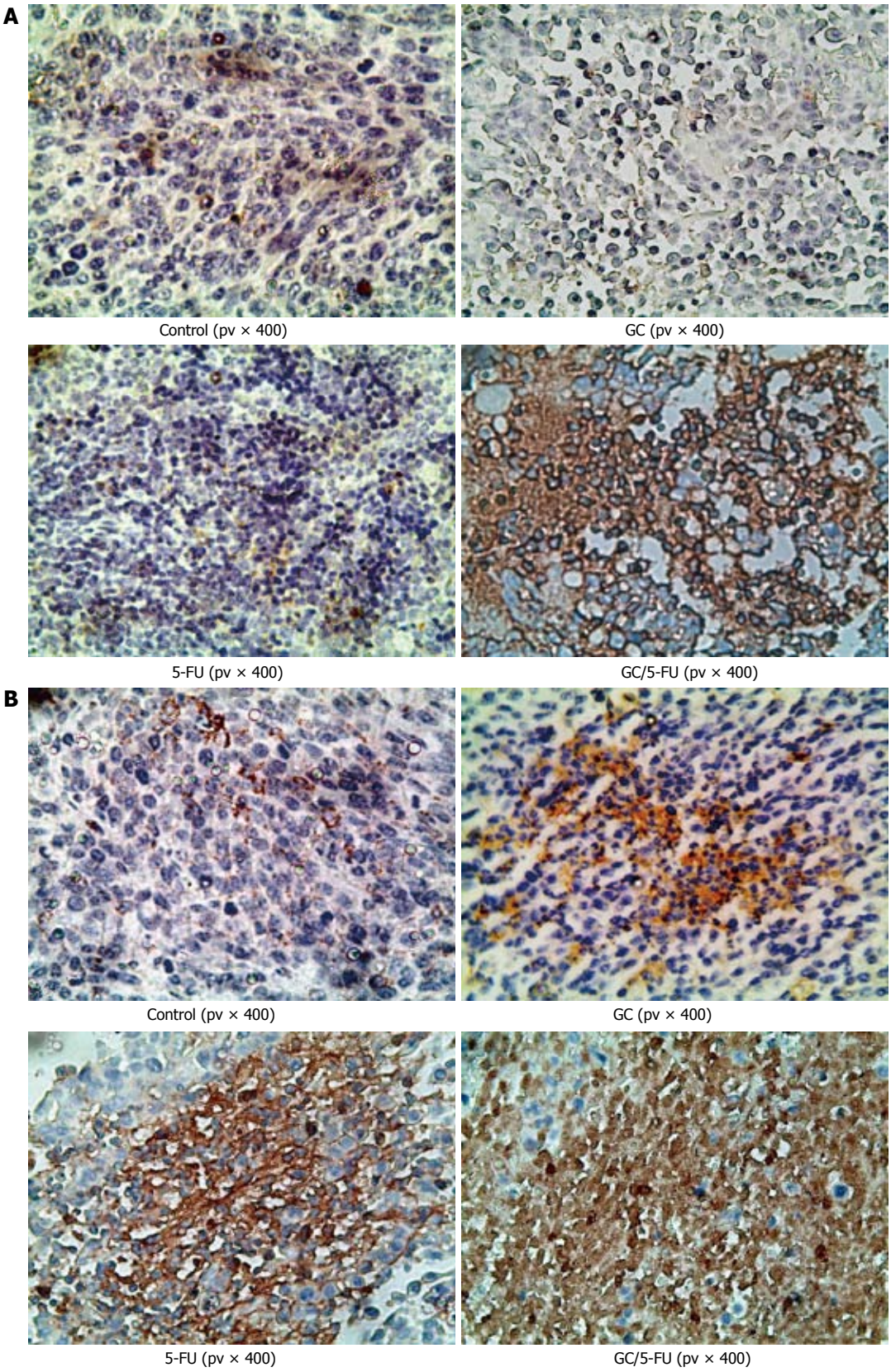

Figure 6 Immunohistochemistry of p53 and caspase-3 in the tumor sections from mice with different treatments. A: p53 staining in the control and galactosylated chitosan (GC) groups showed a scattered nuclear distribution pattern, in dark yellow or dark brown; while in 5-fluorouracil (5-FU) and GC/5-FU groups, p53 showed a sheet staining pattern, which was more dramatic; B: Caspase-3 staining in the control and GC groups showed a scattered cytoplasmic distribution pattern, in dark yellow or dark brown; while in 5-FU and GC/5-FU groups, caspase-3 showed a sheet staining pattern, which was more dramatic in the GC/5-FU group.

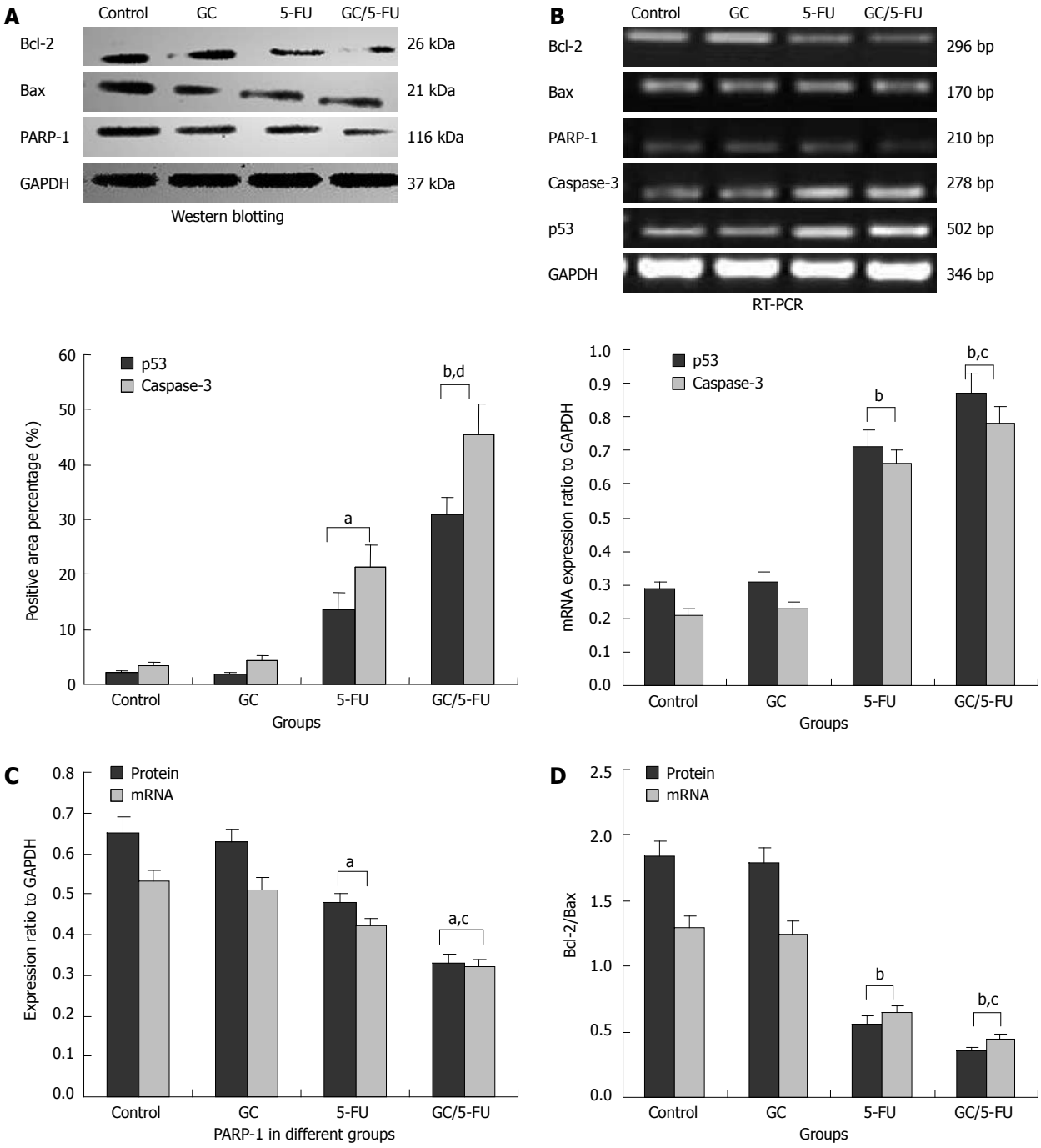

Figure 7 Expression of p53, caspase-3, Bax, Bcl-2 and poly adenosine 50-diphosphate-ribose polymerase 1 in the tumor tissues from mice with different treatments. A: Quantification of p53 and caspase-3 expression as detected by immunohistochemistry and shown pictorially in Figures 6 and 7; B: mRNA levels of p53 and caspase-3 in individual tissues was measured by reverse transcription-polymerase chain reaction (RT-PCR), and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); C: Poly adenosine 50-diphosphate-ribose polymerase 1 (PARP-1) expression in individual tumor samples was determined by both RT-PCR and western blot analysis; results were normalized to GAPDH; D: Expression of Bcl-2 and Bax was quantified by both RT-PCR and Western blotting; the ratio of Bcl-2/Bax was shown. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* 5-fluorouracil (5-FU) group. GC: Galactosylated chitosan.

some of the mice were sacrificed and analyzed at day 15. As shown in Figure 4A, the tumor weight in mice treated with GC/5-FU and 5-FU was significantly less than that in the mice treated with GC or in the control; the weight of GC/5-FU-treated tumors was even lower than the 5-FU-treated tumors, while the GC- or control-treated tumors did not show any statistically significant difference. All mice died after treatment. In the control group, mice died between day 6 and day 14, with a median survival of 12 d; in the GC group, mice died between day 5 and day 16, with a median survival of 13 d, showing no difference from the control group (Figure 4B, $P > 0.05$). Mice in the 5-FU group succumbed to a tumor-associated death between day 13 and day 24, with a median survival time of 17 d, while mice in the GC/5-FU group died between day 15 and day 37, with a median survival

Table 1 Serum levels of blood biochemical parameters in different groups by day 10

Groups	AST (U/L)	ALT (U/L)	BUN (mmol/L)	Creatinine (μmol/L)	Hbg (g/L)	PLT ($\times 10^9$/L)	WBC ($\times 10^9$/L)	Lymphocyte ($\times 10^9$/L)	Neutrophil ($\times 10^9$/L)
Control	92.79 ± 8.74	49.73 ± 4.83	23.74 ± 5.84	0.23 ± 0.09	117.32 ± 9.87	69.43 ± 8.94	6.32 ± 1.24	3.86 ± 1.34	2.18 ± 0.73
GC	92.34 ± 7.65[d]	49.89 ± 5.13[d]	23.25 ± 6.54	0.24 ± 0.08	118.823 ± 10.85	71.43 ± 6.54[d]	6.53 ± 1.32[d]	3.95 ± 1.35[d]	2.17 ± 0.68
5-FU	113.25 ± 7.65[b]	81.48 ± 6.81[b]	23.64 ± 5.45	0.25 ± 0.07	109.41 ± 10.73	55.63 ± 7.43[a]	3.83 ± 1.18[b]	1.57 ± 1.20[a]	2.75 ± 0.87
GC/5-FU	93.42 ± 8.32[d]	48.97 ± 4.93[d]	22.94 ± 5.26	0.24 ± 0.05	116.38 ± 8.53	68.64 ± 7.38[c]	6.21 ± 1.04[d]	3.81 ± 1.17[c]	2.17 ± 0.71
F value	8.227	33.222	0.020	0.058	0.868	4.349	5.498	4.249	0.712
P value	0.002	0.000	1.012	0.941	0.482	0.018	0.008	0.024	0.526

Data were expressed as mean ± SD. $n = 5$ in each group. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* 5-fluorouracil group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BUN: Blood urea nitrogen; Hbg: Hemoglobin; PLT: Platelet; WBC: White blood cell; GC: Galactosylated chitosan; 5-FU: 5-fluorouracil.

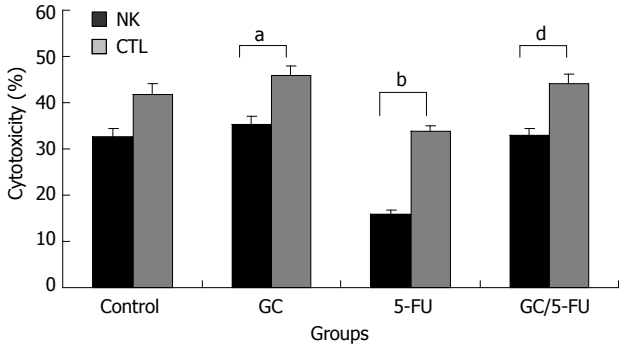

Figure 8 The activity of cytotoxicity T lymphocyte and natural killer cell was detected by 3-(4, 5-dimethylthiazd-2-yl)-2,5-diphenyltentrazolium bromide in the orthotopic transplant model of hepatocellular carcinoma. The cytotoxic activities of cytotoxicity T lymphocyte (CTL) and natural killer (NK) cells were significantly decreased in 5-fluorouracil (5-FU) group compared with other three groups ($P < 0.01$), while, the crosscurrent was found in galactosylated chitosan (GC) group compared with 5-FU group ($P < 0.05$). [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* 5-FU group.

time of 30 d. The survival time of mice treated with either 5-FU or GC/5-FU was significantly longer than that of mice in the control and GC groups, with the longest survival time seen in the GC/5-FU group. This result suggested that although GC alone cannot affect tumor growth, the conjugation of GC to 5-FU improved the tumor suppressive effect of 5-FU. To determine the mechanism of effect of GC/5-FU nanoparticles on the hepatic cancer, we used flow cytometry to examine tumor cell apoptosis. The results revealed that both 5-FU and GC/5-FU enhanced apoptosis when compared with either control or GC, and compared with 5-FU alone, GC/5-FU further increased the apoptosis index, suggesting that GC improves the pro-apoptotic effect of 5-FU by promoting its entry into the cells. In addition, as shown in Figure 5A and B, compared with control and GC treatment, 5-FU and GC/5-FU can increase the percentage of cells in the G0-G1 phases, but lower the PI, suggesting 5-FU and GC/5-FU are cytotoxic to the proliferating cells by arresting them in the G0-G1 phases; this is consistent with previously reported research[28,29]. Therefore, GC facilitates intracellular transport of 5-FU, improving the effects of 5-FU on tumor cell apoptosis and on inhibition of the cell cycle.

To further study whether the apoptosis induced by the GC/5-FU nanoparticles was mediated by the p53 pathway, we used immunohistochemistry, reverse transcription-PCR and Western blotting analysis to examine the expression of p53, Bax, Bcl-2, caspase-3 and PARP-1. We found that the addition of GC/5-FU and 5-FU induced p53 expression at both the protein and the RNA levels; the strongest induction of p53 was noted in the GC/5-FU group, and a moderate to strong induction seen in the 5-FU group (Figure 7A and B). The change of Bcl-2/Bax ratio also showed a similar pattern. Administration of GC/5-FU and 5-FU decreased the Bcl-2/Bax ratio, with the most dramatic reduction observed in the GC/5-FU group (Figure 7D). It is now known that Bax, belonging to the Bcl-2 family, is able to promote apoptosis. Although both Bax and Bcl-2 coexist in cells as dimers, each suppresses the function of the other. Physiologically, both Bax and Bcl-2 are present in cells in the same amounts, ensuring the normal growth of the cells. If Bcl-2 is overexpressed, the heterodimer Bcl-2/Bax is induced to suppress apoptosis, while if the level of Bax increases, the formation of Bax/Bax homodimer promotes apoptosis by antagonizing the anti-apoptotic effect of Bcl-2[30]. Wild-type p53 induces Bax synthesis to mediate apoptosis, while mutant p53 can inhibit apoptosis leading to uncontrolled proliferation[31]. We also found that GC/5-FU was able to significantly enhance the expression of caspase-3 ($P < 0.01$), which is known to be an important promoter of apoptosis. Caspase-3 can be activated by cytochrome c in the cytosol, which is released from mitochondria under the control of Bax and Bcl-2. Therefore, the ratio of Bax and Bcl-2 determines the activation of caspase-3[32,33]. Figure 7C shows a tendency toward a decrease in PARP-1 expression from the control to the GC to the 5-FU to the GC/5-FU groups, with a most significant reduction in the GC/5-FU group. It has been reported that caspase-3 is a pivotal effecter in apoptosis. Activation of PARP-1 after severe DNA damages results in depletion of cellular energy. In order to prevent the consumption of NAD+ and adenosine triphosphate, activated caspase-3 cleaves and inactivates PARP-1, leading to apoptosis[34]. Taking into consideration all the above results, GC improved the apoptotic effect of 5-FU in hepatic cancer cells. The mechanism underlying GC/5-FU nanoparticle-induced apoptosis was

inducing the expression of p53 at the protein and mRNA levels. The elevated p53 level can significantly lower the Bcl-2/Bax ratio which in turn promotes the release of cytochrome c from the mitochondria into the cytosol, leading to the activation of caspase-3. Upregulation of the caspase-3 gene and protein contributed to the reduction of PARP-1 at both the protein and mRNA levels, thus triggering apoptosis. Therefore, GC/5-FU-induced apoptosis is p53 dependent.

5-FU is a common chemotherapy drug, and its common side effects are the suppression of bone marrow[35], dysfunction of liver and kidney and suppression of immune function[36-38], leading to a decreased efficacy and survival time of the patients with cancer. In this experiment, 5-FU increased significantly the levels of AST and ALT, decreased obviously the numbers of PLT, WBC and lymphocyte in tumor-bearing mice compared with the control group. The levels of ALT and AST, the numbers of PLT, WBC and lymphocyte, remained stable in GC/5-FU group compared with control group. It is indicated that GC nanoparticles can improve the damage of liver function caused by 5-FU and the suppression state of bone marrow. We found that the cytotoxic activities of CTL and NK cells by 5-FU were significantly inhibited, and the GC nanoparticles could relieve the suppression state of NK and CTL cells by 5-FU, which is consistent with our previously reported experiments which verified that GC nanoparticles can stimulate the cytotoxic activities of CTL and NK cells in tumor-bearing mice[17]. So the GC/5-FU nanoparticles can alleviate the inhibition of 5-FU on the body's immunity.

In conclusion, we demonstrated that GC is a good carrier for nano-material, especially 5-FU. GC/5-FU nanoparticles had a sustained release effect. GC/5-FU nanoparticles can also significantly inhibit the tumor growth in the orthotropic liver cancer mouse model, and this *in vivo* effect was stronger than that of 5-FU alone. The mechanism underlying GC/5-FU nanoparticles may be the elevated G0-G1 arrest and apoptosis mediated by the p53 pathway. GC/5-FU nanoparticles can ameliorate the side effects and immunosuppressive action of 5-FU.

COMMENTS

Background

Biodegradable polymer nanoparticle drug delivery systems are characterized by targeted drug delivery, improved pharmacokinetic and biodistribution, enhanced drug stability and reduced side effects. These drug delivery systems are widely used for delivery of cytotoxic agents. The galactosylated chitosan (GC)/5-fluorouracil (5-FU) nanoparticle is a nanomaterial made by coupling GC, a polymer known to have the advantages described above, and 5-FU.

Research frontiers

5-FU can target normal, proliferating tissues, but with side effects of bone marrow suppression and gastrointestinal reactions. Targeted therapy for hepatocellular cancer may be useful because it is relatively less expensive compared with the current therapies and it also has fewer side effects. The emergence of a novel, sustained-release formulation of 5-FU is of clinical significance because it has fewer side effects compared with the regular 5-FU formulation.

Innovations and breakthroughs

In this paper, the authors examined the effects of GC/5-FU nanoparticle on liver cancer mouse model. As a result, GC/5-FU treatment could significantly lower the tumor weight and increase the survival time of mice when compared with 5-FU treatment alone. In addition, it was suggested that the abovementioned effects of GC/5-FU was associated with G0-G1 arrest and apoptosis of tumor cells mediated by p53 pathway. GC/5-FU nanoparticles can relieve the side effects and immune- suppressive action of 5-FU.

Terminology

The effects of GC/5-FU nanoparticle on liver cancer mouse model. GC is a galactose ligand, with chitosan modifications on the molecular structure. Asialoglycoprotein receptor (ASGPR) is a receptor found on the membrane of hepatocytes facing the sinusoids, with specificity for glycoproteins with galactose or acetyl galactosamine at the end. The galactose ligand with ASGPR in GC/5-FU nanoparticle induces liver-targeted 5-FU transfer.

Peer review

The significance of this study is evident because the improvement of chemotherapy for liver cancer is an important subject in the clinical setting. In addition, the animal experiments were generally performed appropriately.

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BRIEF ARTICLE

Small intestine contrast ultrasonography *vs* computed tomography enteroclysis for assessing ileal Crohn's disease

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Abstract

AIM: To compare computed tomography enteroclysis (CTE) *vs* small intestine contrast ultrasonography (SICUS) for assessing small bowel lesions in Crohn's disease (CD), when using surgical pathology as gold standard.

METHODS: From January 2007 to July 2008, 15 eligible patients undergoing elective resection of the distal ileum and coecum (or right colon) were prospectively enrolled. All patients were under follow-up. The study population included 6 males and 9 females, with a median age of 44 years (range: 18-80 years). Inclusion criteria: (1) certain diagnosis of small bowel requiring elective ileo-colonic resection; (2) age between 18-80 years; (3) elective surgery in our Surgical Unit; and (4) written informed consent. SICUS and CTE were performed ⩽ 3 mo before surgery, followed by surgical pathology. The following small bowel lesions were blindly reported by one sonologist, radiologist, surgeon and histolopathologist: disease site, extent, strictures, abscesses, fistulae, small bowel dilation. Comparison between findings at SICUS, CTE, surgical specimens and histological examination was made by assessing the specificity, sensitivity and accuracy of each technique, when using surgical findings as gold standard.

RESULTS: Among the 15 patients enrolled, CTE was not feasible in 2 patients, due to urgent surgery in one patients and to low compliance in the second patient, refusing to perform CTE due to the discomfort related to the naso-jejunal tube. The analysis for comparing CTE *vs* SICUS findings was therefore performed in 13 out of the 15 CD patients enrolled. Differently from CTE, SICUS was feasible in all the 15 patients enrolled. No complications were observed when using SICUS or CTE. Surgical pathology findings in the tested population included: small bowel stricture in 13 patients, small bowel dilation above ileal stricture in 10 patients, abdominal abscesses in 2 patients, enteric fistulae in 5 patients, lymphnodes enlargement (> 1 cm) in 7 patients and mesenteric enlargement in 9 patients. In order to compare findings by using SICUS, CTE, histology and surgery, characteristics of the small bowel lesions observed in CD each patient were blindly reported in the same form by one gastroenterologist-sonologist, radiologist, surgeon and anatomopathologist. At surgery, lesions related to CD were detected

in the distal ileum in all 13 patients, also visualized by both SICUS and CTE in all 13 patients. Ileal lesions > 10 cm length were detected at surgery in all the 13 CD patients, confirmed by SICUS and CTE in the same 12 out of the 13 patients. When using surgical findings as a gold standard, SICUS and CTE showed the exactly same sensitivity, specificity and accuracy for detecting the presence of small bowel fistulae (accuracy 77% for both) and abscesses (accuracy 85% for both). In the tested CD population, SICUS and CTE were also quite comparable in terms of accuracy for detecting the presence of small bowel strictures (92% *vs* 100%), small bowel fistulae (77% for both) and small bowel dilation (85% *vs* 82%).

CONCLUSION: In our study population, CTE and the non-invasive and radiation-free SICUS showed a comparable high accuracy for assessing small bowel lesions in CD.

Key words: Crohn's disease; Ileal lesions; Computed tomography enteroclysis; Small intestine contrast ultrasonography; Surgical findings

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INTRODUCTION

Accurate assessment of the lesions is mandatory for a proper pre-operative evaluation in Crohn's disease (CD). Diagnostic imaging of the small bowel traditionally included the small bowel follow through (SBFT) or small bowel enteroclysis[1]. A comparable accuracy for assessing small bowel lesions in CD has been shown in referral centres[2]. More recently, magnetic resonance enterography (MRE) and computed tomography enterography or enteroclysis (CTE) showed to accurately assess the presence, site and extent of small bowel lesions in CD, including stenosis, bowel dilation above stenosis and accurate measurement of the lumen diameter[3-6]. These techniques also provide detailed extraluminal findings, including the bowel wall thickness (BWT), fistulas, abscesses and phlegmons not detected by barium studies[7-9]. For these reasons, MRE and CTE currently represent the standard techniques for assessing small bowel lesions in CD[10]. The major limit of CTE is represented by the high radiation exposure for the patient[5-11]. However, CTE has a greater availability and is less time-consuming than MRE[10]. Therefore, as CTE and MRE show a comparable sensitivity for assessing small bowel lesions in CD, their use is also related to the local feasibility and availability of an experienced radiologist[10].

Transabdominal ultrasonography also has been proposed for detecting small bowel lesions in patients with suspected or known CD, showing a sensitivity and specificity of 67%-84% and 81%-95%, respectively[12-14]. The use of oral contrast significantly increases the sensitivity of ultrasonography for assessing small bowel lesions in CD (by more than 95%)[10,15-19]. In particular, small intestine contrast ultrasonography (SICUS) performed by an experienced sonographer may visualize both established CD lesions (i.e., stenosis with possible pre-stenotic dilation) and minor changes of the small bowel[10,15-20]. In experienced hands, SICUS may detect lesions in suspected small bowel diseases with a high (> 95%) sensitivity and specificity, when compared with SBFT and enema[10,15-19]. The use of SICUS has also been proposed in the follow-up of CD patients after ileo-colonic resection, in order to avoid radiation exposure or the more invasive ileocolonoscopy[19].

To our knowledge, only one retrospective study compared CTE and surgical pathology findings in patients with small bowel CD[21]. A detailed information of the small bowel lesions is mandatory before elective surgery in CD[10]. Moreover, surgical indication in subgroup of patients with small bowel CD may also be related to characteristics of the lesions, including abscesses, marked lumen narrowing and/or strictures with prestenotic dilation. On the basis of these observations, in a prospective longitudinal study, we aimed to compare the sensitivity, specificity and accuracy of SICUS *vs* CTE for assessing the presence of small bowel lesions in patients with CD undergoing elective ileo-colonic resection, when using surgical pathology findings as a gold standard.

MATERIALS AND METHODS

Patients

From January 2007 to July 2008, 18 eligible patients undergoing elective resection of the distal ileum and coecum (or right colon) with ileo-colonic anastomosis were enrolled. Among these 18 patients, there were 15 patients with ileal CD (8 males, median age 44 years, range: 19-73 years) and, as a control group, 3 patients (2 males, mean age 69 years, range: 60-77 years), requiring ileal resection due to small bowel duplication, carcinoid or ischemic enteritis. All patients were under regular follow-up in our unit.

Inclusion criteria included: (1) Patients with a certain

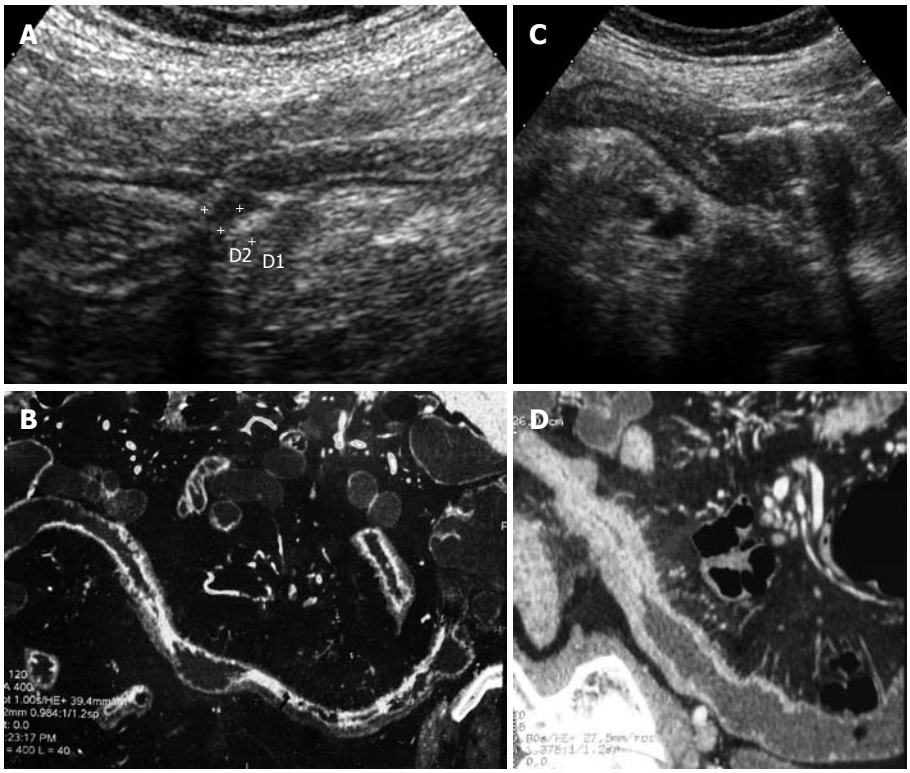

Figure 1 The figure shows images from the distal ileum from one patient with Crohn's disease of the distal and neo-terminal ileum undergoing ileo-colonic resection, as assessed by small intestine contrast ultrasonography and computed tomography-enteroclysis. A: Small intestine contrast ultrasonography (SICUS) showed a stenosis of the terminal ileum, with a thickened bowel wall, with lumen narrowing. The lumen diameter did not change after the ingestion of polyethylen glycole; B: Computed tomography-enteroclysis (CTE) showed findings comparable with SICUS, including a marked narrowing of the distal ileum, associated with an increased bowel wall thickness; C: SICUS showed a stricture of the neo-terminal ileum presenting as a thickened bowel wall, with lumen narrowing associated with pre-stenotic dilation; D: CTE showed findings comparable with SICUS, including a marked narrowing of the distal ileum, associated with an increased bowel wall thickness, but no bowel dilation).

diagnosis of small bowel diseases including CD or other non-inflammatory bowel disease (IBD) related conditions, requiring elective ileo-colonic resection; (2) age between 18-80 years; (3) elective surgery in our Referral Surgical Unit; and (4) written informed consent.

Exclusion criteria included*:* (1) Low compliance to perform both SICUS and CTE, including the introduction of a naso-gastric tube; (2) patients requiring urgent surgery; (3) obesity (body mass index > 30) not allowing a proper assessment by SICUS; and (4) allergy to contrast agents. In patients with CD, the diagnosis was made according to standard clinical, endoscopic and radiological criteria[10]. Clinical characteristics of each of the 16 patients studied are summarized in Table 1.

Study protocol

From January 2007 to July 2008, all patients fulfilling the inclusion criteria and requiring elective ileo-colonic resection in our Unit due to small bowel CD were prospectively enrolled. In all patients, ileal lesions were assessed by using both SICUS and CTE within 3 mo before surgery, followed by surgical pathology findings used as a gold standard. Histological assessment of the surgical specimen was performed. In order to compare findings by using SICUS, CTE, histology and surgery, characteristics of the small bowel lesions were blindly reported in the same form by one gastroenterologist-sonologist (Calabrese E), radiologist (Fiori R), surgeon (Simonetti G) and anatomopathologist (Palmieri G). The following parameters detailing the characteristics of the small bowel lesions were blindly reported by each specialist: site of the lesions (ileum, ileum-colon, colon, others), extent of the lesions (< 10 cm *vs* > 10 cm), strictures (yes/no, number), fistulae (yes/no, number), abscesses (yes/no, number), bowel dilation above strictures (yes/no), lymphnodes enlargement > 1 cm (yes/no, number), mesenteric enlargement (yes/no) (Figure 1).

CTE

CTE was performed by one experienced radiologist unaware of SICUS findings, from the Department of Diagnostic Imaging from our university, as previously described[22]. Colonic cleaning was performed the day before CTE by using polyethylen glycole (PEG) 4000 solution. A 20G needle was placed in the antecubital vein and an 8-F naso-jejunal catheter with a Teflon-covered guide wire was positioned under fluoroscopic guidance (Guerbetm Guerbet GmbH D65838, Sulzbach/Ts) and the distal tip was located in the distal duodenum. The patient was then taken into CT room and contrast material (1500 mL of PEG) was administered manually with

Table 1 Clinical characteristics of the 16 patients considered in the analysis

Disease	Sex	Age (yr)	Surgical indication	Lesions extent (cm)	CD pattern
CD	M	39	Sub-obstructions	30	Fibrostricturing
CD	F	19	Abscess	20	Fistulizing
CD	F	49	Sub-obstructions	30	Fibrostricturing
CD	M	38	Abscess	21	Fibrostricturing
CD	F	73	Sub-obstructions	40	Fibrostricturing
CD	F	33	Sub-obstructions	25	Fibrostricturing
CD	F	57	Sub-obstructions	30	Fibrostricturing
CD	M	31	Abscess	15	Fistulizing
CD	F	49	Sub-obstructions	35	Fibrostricturing
CD	M	41	Sub-obstructions	20	Fibrostricturing
CD	M	45	Sub-obstructions	15	Fibrostricturing
CD	M	30	Sub-obstructions	40	Fibrostricturing
CD	M	30	Sub-obstructions	35	Fibrostricturing
Small bowel duplication	M	60	Sub-obstructions	20	NA
Ischemic enteritis	M	69	Abdominal pain	10	NA
Small bowel carcinoid	F	77	Diarrhoea, weight loss	35	NA

CD: Crohn's disease; M: Male; F: Female; NA: Not applicable (non-CD).

60-mL syringes, with a continuous injection rate of 150 mL/min followed by a flow rate of 200 mL/min until the maximum tolerance of the patient. Before the examination, a smooth muscle relaxant (N butyl-scopolamine) was administered. CTE was performed by a 64-slice multidetector (LightSpeed VCT, General Electric Medical System, Milwaukee, WI, United States). After PEG infusion, CT scan was performed before and after the administration of *i.v.*, iodinated contrast material. The contrast-enhanced study was acquired 70 s after the administration of contrast material (Ultravist 370, Schering AG, Berlin, Germany) with a "double-bolus" technique (a first bolus of 60 cc at a flow rate of 1.5 mL/s and a second bolus of 80 cc at a flow rate of 2.0 mL/s).

SICUS

SICUS was performed as previously described[15,18,19]. In particular, SICUS was performed after the ingestion of 375 mL (range: 250-500 mL) of oral contrast solution consisting of PEG (Promefarm, Milano, Italy), by using a convex transducer (frequency 3.5-5 MHz) and then with a high frequency linear-array transducer (5-12 MHz) (Hitachi, EUB 6500, Japan). All procedures were performed by the same expert EC (> 2000 examinations).

The following findings were considered compatible with CD[16,18,19]: (1) increased BWT (> 3 mm); (2) "stiff loop", identified by the presence of small bowel loop, with increased BWT not distended by contrast solution; (3) small bowel dilation, defined as a lumen diameter > 2.5 cm; (4) bowel stricture defined as lumen diameter < 1 cm, measured at the level of maximally distended loop, independently of the presence of pre-stenotic dilation; (5) fistulae defined as hypoechoic tract with or without hyperechoic content; (6) mesenteric enlargement and/or masses; (7) lymphnodes enlargement (> 1 cm); and (8) abscesses identified as roundish anechoic lesions, with an irregular wall, often presenting internal echoes and posterior echo enhancement.

Surgical assessment

At time of intestinal resection, one single GS filled up the above reported form in order to assess the small bowel lesions. Findings at surgery were considered as the gold standard for assessing the small bowel lesions described by SICUS and CTE. The surgical specimen was fixed in formalin for histological examination.

Histological assessment

The surgical specimen was examined by the one single GP unaware of previous findings at SICUS, CTE and surgery. At this purpose, routine hematoxylin and eosin staining was performed and the GP filled up the same form used by the sonographist, radiologist and surgeon.

Statistical analysis

All results were expressed as median and range. Comparison between findings at SICUS, CTE, surgical specimens and histological examination was made by assessing the specificity, sensitivity and accuracy of each technique, when using surgical findings as a gold standard.

RESULTS

From January 2007 to July 2008, 15 CD patients undergoing elective ileo-colonic resection and fulfilling the inclusion criteria were prospectively enrolled. Among these 15 patients, 2 CD patients performed SICUS but were not able to perform CTE, as one patient required "urgent surgery", and the second patient refused to perform CTE due to the discomfort related to the nasojejunal tube. Therefore, among the initial 15 patients eligible for the study, only 13 patients were studied by both SICUS and CTE and were therefore considered for the analysis. No side effects were reported after SICUS and CTE procedures.

Among the 13 CD patients considered in the analysis, surgical pathology findings included: small bowel stricture in 13, small bowel dilation above stricture in 10, abdominal abscesses in 2, fistulae in 5 (associated with abscess in 2), lymphnodes enlargement (> 1 cm) in 7 and mesenteric enlargement in 9 patients (Table 2).

Small bowel assessment by CTE and SICUS vs findings at surgery

Site of the lesions: At surgery, lesions were detected in the distal ileum in all 13 patients, and also in the right colon in 5. Both SICUS and CTE also visualized ileal lesions in all 13 patients, while concomitant lesions in the right colon were detected in 3 out of the 5 patients by both SICUS and CTE (including the same patients in 2 cases) (Table 2). Histological findings were comparable to surgery in all CD patients.

Table 2 Characteristics of the lesions in the 13 Crohn's disease patients, as assessed by surgical pathology (considered as a gold standard), small intestine contrast ultrasonography, computed tomography-enterography and histology

Characteristics	Surgery	SICUS	CTE	Histology
CD site				
Ileum	8/13	10/13[1]	10/13	8/13
Ileum-colon	5/13	3/13	3/13	5/13
CD extent (cm)				
< 10	0/13	12/13	12/13	9/13
⩾ 10	13/13	1/13	1/13	4/13
Strictures				
Yes	13/13	12/13	13/13	13/13
No	0/13	1/13	0/13	0/13
Dilation				
Yes	10/13	10/13	11/13	5/13
No	0/13	0/13	2/13	8/13
Fistulae				
Yes	5/13	6/13	4/13	6/13
No	8/13	7/13	9/13	7/13
Abscesses				
Yes	3/13	5/13	3/13	4/13
No	11/13	8/13	10/13	9/13

[1]Including the jejunum in 1 patient. CD: Crohn's disease; SICUS: Small intestine contrast ultrasonography; CTE: Computed tomography-enterography.

Extent of the small bowel lesions: In CD group (Table 2), histology detected ileal lesions of > 10 cm length in only 9 patients.

Small bowel strictures: Ileal strictures were detected at surgery in all 13 CD patients. Comparable findings were detected by using CTE and histology, while no strictures were detected in one CD patient by using SICUS (Table 2).

Bowel dilation above strictures: Dilation above ileal strictures was detected by both surgery and SICUS in 10/13 CD patients, by CTE in 11/13 patients and by histology in 5/13 patients (Table 2). However, discrepant findings *vs* surgery were observed by using SICUS in 4/13 CD (dilation not detected in 2; dilation detected only by SICUS in 2), by using CTE in 5/13 CD (dilation not detected in 2; dilation detected only by CTE in 3) and by histology in 5/13 patients (dilation not detected in 5, discrepant findings *vs* surgery but comparable with SICUS in 2 and with CTE in 1) (Table 2). In the same 2 CD patients, both SICUS and CTE concordantly reported dilation above strictures not detected at surgery. Findings at CTE and SICUS were comparable in only 8/13 patients, as dilation was detected only by SICUS in 2 (confirmed at surgery) and only by CTE in 3 CD patients (confirmed at surgery in 2).

Fistulae: The presence of enteric fistulae were detected in 5 out of the 13 CD patients at surgery, in 6 patients when using SICUS, in 4 patients by using CTE, while histology reported the presence of fistulae in 6 CD patients (Table 2). Findings different from surgery were detected by SICUS in 3/13 patients (fistulae detected by SICUS and not at surgery in 2 patients; fistulae detected at surgery and not by SICUS in 1 patient) by CTE in 4/13 patients (fistulae detected only by CTE in 2; detected only at surgery in 2); and by histology in 3/13 patients (fistulae detected by histology and not at surgery in 2, and detected at surgery and not by histology in 1). In 3 patients, SICUS and CTE concordantly reported enteric fistulae not confirmed at surgery (detected histologically in 2). When comparing SICUS *vs* CTE, the presence of fistulae was concordantly detected in 9/13 patients, while fistulae were detected only by SICUS in 1 CD patient.

Table 3 Sensitivity, specificity and accuracy of small intestine contrast ultrasonography and computed tomography-enterography for detecting the presence of small bowel abscesses, strictures, fistulae and bowel dilation in Crohn's disease

Parameter	SICUS			CTE		
	Sens.	Spec.	Accuracy	Sens.	Spec.	Accuracy
Strictures	92	0	92	100	0	100
	(TN 0; TP 12; FN 1; FP 0)			(TN 0; TP 13; FN 0; FP0)		
Dilation	100	50	85	78	0	82
	(TN2; TP 9; FN 0; FP2)			(TN 0; TP 7; FN 2; FP 4)		
Fistulae	60	88	77	60	88	77
	(TN 7; TP 3; FN 2; FP 1)			(TN 7; TP 3; FN 2; FP 1)		
Abscesses	100	80	85	67	100	85
	(TN 8; TP 3; FN 0; FP 2)			(TN 9; TP 2; FN 1; FP 1)		

SICUS: Small intestine contrast ultrasonography; CTE: Computed tomography-enterography; TN: True negative; TP: True positive; FN: False negative; FP: False positive; Sens.: Sensitivity; Spec.: Specificity.

Abscesses: Abdominal abscesses were detected at surgery in 3/13 CD patients (surgically drained in 1), by using SICUS in 5, by CTE in 3 and by histology in 4 patients (Table 2). Findings different from surgery were reported by SICUS in 2 patients (abscess detected only by SICUS in both), by CTE in 2 patients (abscess detected only by CTE in 1, only at surgery in 1), and by histology in 3 patients (abscess detected only by histology in 2 and only at surgery in 1 patient performing surgical drainage). In one patient, abdominal abscess was detected by both SICUS and CTE but not by surgical pathology and histology. When comparing SICUS and CTE, the presence of abscesses was concordantly detected in 11 out of the 13 patients, while in 2 patients SICUS only reported the presence of an intestinal abscess (confirmed at surgery in one of them).

Sensitivity and specificity of SICUS vs CTE

When using surgical findings as a gold standard, sensitivity, specificity and accuracy of SICUS and CTE for assessing the presence of stenosis, dilation above stenosis and fistulae are reported in Table 3. As indicated, the two techniques showed the same sensitivity, specificity and accuracy for detecting the presence of small bowel fistulae (accuracy 77% for both) and abscesses (accuracy 85% for both). SICUS and CTE were also quite comparable for detecting the presence of small bowel strictures, fistulae and abscesses. Nevertheless, there was a

not significant trend for a higher sensitivity and accuracy of CTE *vs* SICUS for assessing small bowel strictures (accuracy 100% *vs* 92%; the observed 0% specificity related to the absence of true negative findings), while SICUS showed a not significantly higher accuracy *vs* CTE for detecting small bowel dilation (85% *vs* 82%).

DISCUSSION

Appropriate surgical treatment of CD involves an accurate knowledge of the characteristics of the lesions, including the site, extent and possible presence of complications (strictures, dilation above strictures, fistulae, abscesses). The development of a marked bowel dilation above stricture or abscesses may represent indication for surgery[10]. Colonoscopy represents the gold standard technique for assessing colonic lesions, while small bowel lesions were previously assessed by SBFT or small bowel enteroclysis[1,2]. More recently, CTE or MRE represent the gold standard techniques at this purpose[10]. These techniques indeed provide not only an accurate assessment of the presence, site and extent of the lesions, but they also allow the visualization of extraluminal findings related to the disease (i.e., increased BWT, fistulae, abscesses, mesenteric enlargement)[3-9]. The preferential use of CTE *vs* MRE is related to the feasibility and easy access to these techniques in each IBD referral centre. At this purpose, both appropriate radiologic instruments and an experienced radiologist with specific competence in the field are required[10]. MRE shows the advantage of a radiation-free procedure.

SICUS also has also been recently suggested as a non-invasive technique able to assess, in experienced hands, the presence of small bowel lesions in CD, including the BWT, strictures, bowel dilation, fistulae and abscesses[16,18,21]. Indication for surgery in CD may also be related to the characteristics of the small bowel lesions (i.e., marked dilation above strictures, abscesses). Whether CTE and SICUS provide a comparable definition of the small bowel lesions in CD is currently unknown. On the basis of these observations, in the present study we aimed to compare these 2 techniques in terms of assessment of the small bowel lesions in patients with CD undergoing elective ileo-colonic resection. The use of small bowel capsule endoscopy has also been shown to accurately visualize small bowel lesions in CD[23-25]. However, the use of small bowel capsule endoscopy (SBCE) is limited by the impact risk in patients with intestinal stenosis[23-25]. In our study, according to the inclusion criteria, all CD patients were undergoing elective ileo-colonic resection. Therefore, all the enrolled CD patients were by definition at high risk of SBCE impact, related to severe lesions requiring surgical resection. For this reason, this useful technique able to visualize the entire small bowel was not feasible in the present study aimed to compare findings using CTE *vs* SICUS. A comparative estimate of the costs of the current techniques able to assess small bowel lesions, including not only CTE and SICUS, but also MRE and SBCE, could be of great interest. However, these cost may greatly differ in different hospitals, thus limiting the usefulness of this estimate. Nevertheless, among techniques tested in the present study, it appears conceivable to consider CTE more expensive than SICUS.

Limitations of the study include the limited number of tested patients (n = 15), related to difficulties to perform 2 consecutive small bowel examinations in patients with active CD undergoing surgical resection. Additional limitation include the absence of a control group, as the purpose of the study was to compare the accuracy of SICUS *vs* CTE for assessing small bowel lesions in patients with a certain diagnosis of CD. Results from our limited series suggest that SICUS and CTE are quite comparable techniques at this purpose. However, while the accuracy of these two procedures for assessing the presence of strictures was quite comparable, SICUS showed a slightly higher accuracy for detecting the presence of dilation above strictures. In our series, CTE and SICUS were absolutely comparable for assessing the presence of fistulae and abscesses. SICUS is not feasible in obese patients, due to inaccurate findings and may be less accurate than CTE for visualizing lesions in the deeper layer of the abdominal cavity[12]. Nevertheless, it seems relevant to note that CTE could not be performed in 2 out of the 17 enrolled CD patients (11.7%) already studied by SICUS. These 2 patients were therefore excluded from the analysis, as CTE could not be performed due to low compliance in one patient refusing the nasojejunal tube and to need of urgent surgery in the second patient. These observations therefore support that CTE may not be performed in a relatively high proportion of patients undergoing ileo-colonic resection for CD.

Nevertheless, differently form SICUS, CTE is an invasive procedure associated with a high radiation exposure for the patient[11]. This issue assumes particular relevance when considering that small bowel assessment before surgery for CD is most often required in young patients already performing other diagnostic radiological procedures and treated with immunomodulatory drugs[26-29]. These observations, together with findings from our study therefore suggest that in referral IBD centres with a feasible experienced ultrasonographer, SICUS may represent the procedure of choice when compared with CTE, for assessing small bowel lesions in patients undergoing elective ileo-colonic resection for CD.

COMMENTS

Background

Magnetic resonance enterography (MRE) and computed tomography enterography or enteroclysis (CTE) accurately assess small bowel lesions in Crohn's disease (CD), representing the standard techniques at this purpose. The major limit of CTE is represented by the high radiation exposure for the patient. Recently, small intestine contrast ultrasonography (SICUS) performed by an experienced sonographer has been shown to visualize CD lesions of the small bowel. These findings suggest that SICUS may be used for assessing CD lesions, although comparison with CTE when using surgical pathology as standard is unknown.

Research frontiers

Proper follow up of CD patients includes the assessment of the lesions in order to choice appropriate treatment strategies and the presence of complications. In this study, the authors compared the sensitivity, specificity and accuracy of SICUS *vs* CTE for assessing the presence of small bowel lesions in patients with CD undergoing elective ileo-colonic resection, when using surgical pathology findings as a gold standard.

Innovations and breakthroughs

Small bowel lesions in CD may be accurately detected by CTE or MRE. However, the use of CTE is associated with a high radiation exposure, while MRE shows a low availability. Moreover, the need of intestinal preparation, insertion of the naso-gastric tube may limit the use of both techniques. The authors performed a prospective longitudinal study in patients undergoing elective surgery, aimed to assess the accuracy of SICUS *vs* CTE for assessing small bowel lesions in CD, when using surgical pathology as gold standard.

Applications

This study provides the first evidence that SICUS and CTE show a comparable high accuracy for assessing small bowel lesions in CD. These results suggest that the radiation-free, non-invasive SICUS performed by an experienced sonographer may be used for assessing small bowel lesions in patients with CD.

Peer review

CTE and MRE may not be performed in patients with low compliance. Results from this study support that, differently from CTE, SICUS may be performed in all CD patients undergoing elective surgery. As SICUS and CTE showed a comparable high accuracy for assessing small bowel lesions in CD, the non-invasive SICUS should be used at this purpose in referral centres provided of an experienced and available sonologist.

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BRIEF ARTICLE

Quasispecies dynamics in main core epitopes of hepatitis B virus by ultra-deep-pyrosequencing

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Abstract

AIM: To investigate the variability of the main immunodominant motifs of hepatitis B virus (HBV) core gene by ultra-deep-pyrosequencing (UDPS).

METHODS: Four samples (2 genotype A and 2 genotype D) from 4 treatment-naïve patients were assessed for baseline variability. Two additional samples from one patient (patient 4, genotype D) were selected for analysis: one sample corresponded to a 36-mo treatment-free period from baseline and the other to the time of viral breakthrough after 18 mo of lamivudine treatment. The HBV region analyzed covered amino acids 40 to 95 of the core gene, and included the two main epitopic regions, Th50-69 and B74-84. UDPS was carried out in the Genome Sequencer FLX system (454 Life Sciences, Roche). After computer filtering of UDPS data based on a Poisson statistical model, 122 813 sequences were analyzed. The most conserved position detected by UDPS was analyzed by site-directed mutagenesis and evaluated in cell culture.

RESULTS: Positions with highest variability rates were mainly located in the main core epitopes, confirming their role as immune-stimulating regions. In addition, the distribution of variability showed a relationship with HBV genotype. Patient 1 (genotype A) presented the lowest variability rates and patient 2 (genotype A) had 3 codons with variability higher than 1%. Patient 3 and 4 (both genotype D) presented 5 and 8 codons with variability higher than 1%, respectively. The median baseline frequencies showed that genotype A samples had higher variability in epitopic positions than in the other positions analyzed, approaching significance (P = 0.07, sample 1 and P = 0.05, sample 2). In contrast, there were no significant differences in variability between the epitopic and other positions in genotype D cases. Interestingly, patient 1 presented a completely mutated motif from amino acid 64 to 67 ($E_{64}LMT_{67}$), which is commonly recognized by T helper cells. Additionally, the variability observed in all 4 patients was particularly associated with the $E_{64}LMT_{67}$ motif. Codons 78 and 79 were highly conserved in all samples, in keeping with their involvement in the interaction between the HBV virion capsid and the surface antigens (HBsAg). Of note, codon 76 was even more conserved than codons 78 and 79, suggesting a possible role in HBsAg interactions or even in hepatitis B e antigen

conformation. Sequential analysis of samples from patient 4 (genotype D) illustrated the dynamism of the HBV quasispecies, with strong selection of one minor baseline variant coinciding with a decrease in core variability during the treatment-free and lamivudine-treated period. The drop in variability seemed to result from a "steady state" situation of the HBV quasispecies after selection of the variant with greatest fitness.

CONCLUSION: Host immune pressure seems to be the main cause of HBV core evolution. UDPS analysis is a useful technique for studying viral quasispecies.

Key words: Hepatitis B virus; Ultra-deep-pyrosequencing; Epitopes; Quasispecies; Linkage analysis

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. Around 350 million people are chronically affected with this pathogen, which confers a higher risk of developing liver disease, liver cirrhosis, and hepatocellular carcinoma. The course of HBV infection is closely related to the host immune response and genetic factors[1], and disease progression is related to mutations in the HBV core gene[2-4].

HBV core gene codes for two partially collinear proteins, the hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). These proteins, together with the surface antigen (HBsAg) are important targets for antiviral immunity, but HBcAg seems to be the most immunogenic[5]. Several epitopes have been identified in the HBV core gene. Among them, two regions play a particularly important immunodominant role: the sequence from amino acid 50 to 69, which immunostimulates CD4+ T-helper lymphocytes (Th50-69)[6] and the sequence from amino acid 74 to 84, which stimulates B lymphocytes (B74-84)[7,8].

During chronic HBV infection, a large number of amino acid substitutions are seen in the core gene, mainly clustered in epitopic regions. These amino acid changes have been associated with viral persistence because of their impact on the host immune response and the natural course of HBV infection[7,9-14]. The largest number of core gene changes is associated with interferon (IFN) therapy[10-12]. The effect of nucleoside/nucleotide analogues on the core gene has been little investigated, although some variability in a minor epitope (Th28-47) was recently reported[11]. In another recent study, entecavir and adefovir were associated with an enhanced immune response[15]. Selection of core gene amino acid changes might result in evasion of HBV from the host immune system, thereby lengthening the life of infected hepatocytes. For this reason, core gene baseline variability in chronic hepatitis B patients might be crucial for understanding the evolution of the viral quasispecies in response to host immune pressure.

Next-generation sequencing technologies enable deep assessment of gene variability and are especially useful to study the dynamics of viral quasispecies[16-21]. Core gene variability can be studied with this technology, specifically, the 454 FLX platform, which analyzes fragments of 250- to 400-bp length. Although this length does not permit complete analysis of the gene, ultra-deep analysis of the main immunodominant regions of the core protein (Th50-69 and B74-84) is possible. The aim of this study was to analyze the variability of these main HBV core epitopes in chronic hepatitis B patients by ultra-deep pyrosequencing (UDPS).

MATERIALS AND METHODS

Patients and samples

Four chronic hepatitis B patients with complete clinical documentation were selected for the study; baseline characteristics are indicated in Table 1. All patients were diagnosed with active HBV replication and treated with lamivudine (LVD) 100 mg/d (Zeffix, Glaxo Welcome, United Kingdom). After 18 to 24 mo, they all presented mutations conferring resistance to treatment. Owing to their similarities in LVD non-response, they were selected for inclusion in the study. To evaluate baseline variability, a sample taken at the time of the diagnosis (antiviral treatment naïve) was selected for each patient. HBV DNA was retested in all samples by TaqMan real-time polymerase chain reaction (PCR) (Roche) technology, and all presented values higher than 5 $\log_{10}$ IU/mL.

Two sequential samples from patient 4 were additionally selected for UDPS analysis. HBV-DNA had been quantified using the branched-DNA (bDNA, limit of detection 5 logs IU/mL) technology available at that time, but the samples selected were retested with TaqMan technology for this study. At the time of the diagnosis (baseline sample), high HBV-DNA levels were detected by bDNA (retesting with TaqMan, 7 $\log_{10}$ IU/mL). However, HBV-DNA spontaneously dropped below the limit of detection of bDNA technology and the patient remained untreated, according to the guidelines at that time. After 36 mo (treatment-free sample), bDNA significantly increased (> 8 $\log_{10}$ IU/mL on TaqMan retesting) and LVD was started. After an initial suboptimal response (HBV DNA decrease to 4 logs), viral breakthrough (7

Table 1 Baseline characteristics of the four patients included in the study

Patient	Sex	Age (yr)	ALT (IU/mL)	HBV DNA (log10 IU/mL)	Genotype	HBeAg status
1	Male	46	167	7.4	A	-[1]
2	Male	39	95	7.8	A	+
3	Male	31	392	8.3	D	-
4	Female	55	117	7.5	D	-

Age at the time of sample collection. [1]Wild type in main precore mutation. ALT: Alanine transaminase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

$\log_{10}$ HBV-DNA) was observed after 18 mo, and the rtL180M and rtM204V HBV polymerase variants were selected. Ultimately, adefovir was added to LVD.

Epitopic region amplification and UDPS amplicon preparation

All the samples included in this study had HBV viral loads higher than 6 logs IU/mL. HBV-DNA was extracted from serum by QIAamp microspin columns (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To obtain optimal amplification of HBV DNA, the process was optimized with two PCRs. To minimize the error rate of the PCR process (false nucleotide substitutions), high fidelity polymerase (Pfu Ultra-Ⅱ, Stratagene, La Jolla, United States) was used. At the time the study was designed, the maximal amplicon length that could be analyzed by the FLX platform was 250 nucleotides; PCR primers were selected for amplification of a specific 210-bp HBV fragment, which included main epitopic regions (Th50-69 and B74-84). The first PCR primers were sense (position 1662-1681); 5'-^{C}/$_{T}$ATAAG AGGACTCTTGGACT-3' and anti-sense primers (position 2912-2931); 5'-TGTTCCCA^{A}/$_{G}$GAATA^{A}/$_{T}$GGTGA-3'. The nested primers included the recognition site for UDPS, in italics. The sequence of the sense primer (position 1997-2016) was 5'-*GCCTCCCTCGCGCCATCAG*ACCGCCTCAGCTCT ^{C}/$_{T}$TAT CG-3', and the anti-sense primer (position 2178-2206) was 5'-*GCCTTGC CAGCCCGCTCAG*CCACA^{A}/$_{G}$AGTTGCCTGA^{A}/$_{G}$CTT-3'. PCR products were isolated from 0.9% agarose gel and quantified using Quan-iT Picogreen sDNA reagent (Invitrogen). Before the sequencing reaction, each amplicon was pooled to obtain a concentration of 4×10^6 molecules of the HBV region. This working solution was enriched with the capture beads needed for sequencing. After optimal enrichment, clonal amplification in beads was performed in forward and reverse directions (emPCR kits Ⅱ and Ⅲ, 454 Life Sciences). UDPS was carried out in the Genome Sequencer FLX system (454 Life Sciences). The HBV region analyzed covered amino acids 40 to 95 of the core gene.

Bioinformatics filter

A total of 122 813 sequences was obtained. Reads were acquired with forward and reverse sequences and were aligned according to the primer sequence (designed by our group). Initial raw data filtering was performed as previously reported[16-18,20-22].

After applying the Poisson-based statistical filter validated in a study from our group[21], the empirical distribution of mismatch errors determined by UDPS analysis of an HBV DNA clone from the same region yielded an average of 0.006%; however in 8 positions, errors were higher than 0.02% but lower than 0.05%. Therefore, the sensitivity of UDPS to detect mutations was primarily limited by the highest mismatch error rate in the HBV DNA clone of 0.05%, which is similar to the value recently obtained in UDPS amplicons including an internal sequence as a control[20]. Thus, the measurements and biological conclusions in this study are based only on mutations present at a percentage above 0.05%.

Phenotyping, mutagenesis and cell culture

Cloning of a more than full-length HBV genome[20] in pTriEx-mod vector was performed as described by Durantel *et al*[23]. The influence on HBV viral replication of the most conserved position observed in UDPS analysis, codon 76, was analyzed by site-directed mutagenesis (Agilent Technologies, Stratagene, United States) according to the manufacturer's instructions. The wild-type clone had an L in codon 76, which was changed to V or P to test the effect of maintaining or deeply altering the physical-chemical properties of core codon 76. The introduction of mutations was confirmed by direct sequencing, as previously described[20].

Huh7 human hepatoma cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum. Transfection of plasmids was performed as previously described[20] using Fugene-HD (Roche, Germany). The supernatant was used to quantify HBsAg (Architect, Abbot), HBeAg (Vitros, Johnson and Johnson), and HBV DNA (CobasTaqman, Roche) production. The results were statistically analyzed with the Student *t* test. DNA was extracted from the supernatant (QiagenAMP DNA Mini Kit, Qiagen, Germany) following the manufacturer's instructions and used to evaluate HBV-DNA production. As has been previously described[20], to confirm that HBV-DNA detected after transfection was the result of HBV replication and not due to contamination from the HBV genome in the pTriEx-mod vector of the transfection experiments, the supernatant extractions were used in 1/10, $1/10^2$, $1/10^3$, and $1/10^4$ dilutions and PCR amplification of HBV-DNA and pTriEx-mod was performed.

Statistical analysis

To obtain the percentage of amino acid variability in each sample, the total number of amino acid substitutions was divided by the total number of amino acids analyzed. This value gave the theoretical variability for each position, and was used to estimate the expected variability for the regions studied (the theoretical variability was multiplied by the length of the epitope: 20

Table 2 Baseline variability of all the codons analyzed

Codon	Patient N sequences Master AA	Pt 1 33245 % var	Pt 2 28254 % var	Pt 3 27156 % var	Pt 4 19748 % var	Median baseline variability
40	E	0.17	0.00	0.27	36.27	9.18
41	A*134*/S2	0.04	7.54	1.58	0.03	2.30
42	L	0.20	0.08	0.14	0.09	0.13
43	E	0.06	0.07	0.08	0.12	0.08
44	S	0.03	0.01	0.01	0.05	0.03
45	S*1*/P*234*	0.24	0.07	0.10	0.09	0.12
46	E	0.06	0.05	0.06	0.08	0.06
47	H	0.06	0.06	0.04	0.03	0.05
48	C	0.09	0.18	0.07	0.27	0.15
49	S	0.09	0.07	0.03	0.01	0.05
50	P	0.07	0.04	0.08	0.02	0.05
51	H	0.14	0.11	0.06	0.01	0.08
52	H	0.03	0.04	0.02	0.02	0.03
53	T	0.11	0.14	0.07	0.04	0.09
54	A	0.14	0.18	0.17	0.19	0.17
55	L	0.03	0.05	0.06	18.58	4.68
56	R	0.21	0.22	0.14	0.10	0.17
57	Q	0.05	0.05	0.03	0.00	0.03
58	A	0.15	0.18	0.09	0.04	0.12
59	I*134*/V2	0.04	4.75	1.68	0.03	1.62
60	L	0.03	0.02	0.03	0.06	0.04
61	C	0.10	0.08	0.04	0.03	0.06
62	W	0.15	0.21	0.19	0.09	0.16
63	G	0.17	1.93	0.34	0.18	0.65
64	D*14*/E*23*	0.69	0.50	0.41	29.96	7.89
65	V*1*/L*234*	0.08	0.02	0.09	0.19	0.09
66	T*1*/M*234*	0.12	0.21	0.11	0.11	0.14
67	N*1*/T*234*	0.05	0.06	0.06	0.04	0.05
68	L	0.04	0.02	0.03	0.02	0.03
69	A	0.05	0.12	0.10	0.09	0.09
70	T	0.05	0.02	0.02	0.01	0.02
71	W	0.11	0.24	0.20	0.17	0.18
72	V	0.07	0.12	0.07	0.03	0.07
73	G	0.17	0.28	0.19	0.11	0.19
74	N*12*/A*3*/V*4*	0.46	0.04	2.06	13.72	4.07
75	N	0.01	0.02	0.02	0.02	0.02
76	L	0.00	0.01	0.02	0.02	0.01
77	G*134*/Q2	0.14	0.30	1.94	3.03	1.35
78	D	0.03	0.08	0.03	0.08	0.05
79	P	0.16	0.10	0.07	0.03	0.09
80	A*123*/T*4*	0.11	0.17	0.12	8.09	2.12
81	S	0.04	0.03	0.01	0.00	0.02
82	R	0.19	0.12	0.15	0.06	0.13
83	D	0.05	0.04	0.06	0.12	0.07
84	Q*1*/L*234*	0.21	0.52	0.12	0.05	0.23
85	V	0.02	0.01	0.03	0.01	0.02
86	V	0.02	0.03	0.01	0.04	0.03
87	N*1*/S*234*	0.06	0.03	1.93	0.02	0.51
88	Y	0.08	0.03	0.04	0.05	0.05
89	V	0.01	0.00	0.02	0.03	0.02
90	N	0.02	0.02	0.08	0.00	0.03
91	T	0.07	0.05	0.02	0.03	0.04
92	N	0.03	0.02	0.70	18.93	4.92
93	M*123*/V*4*	0.02	0.05	0.15	18.89	4.78
94	G	0.04	0.12	0.15	0.09	0.10
95	L	0.03	0.02	0.15	0.14	0.09

Italic numbers indicate the patient in whom the master amino acid (AA) was detected.

for Th50-69, 11 for B 74-84, and 25 for the remaining positions).

Fisher's exact test was used to evaluate possible relationships between the most variable codons (variability $\geqslant$ 1%) and their positions in the epitopic region or other regions. The Wilcoxon signed-rank test was used to compare the evolution of the codons in the sequential analysis.

RESULTS

Baseline variability of main epitopic regions of HBV core gene

The amplicon analyzed was limited to codons 40 to 95, which include the main Th50-69 and B74-84 epitopes. A total of 122 814 sequences corresponding to 4 baseline samples were analyzed, and 108 403 of them were validated by bioinformatics and Poisson filtering. A total of 61 499 sequences were from genotype A samples and the remaining from genotype D. Variability was analyzed attending to the percentage of changes in all codons of the amplicon, and the results obtained for each position are shown in Table 2.

In the two genotype A samples (patients 1 and 2), differences between the master sequences were found in ten codons (41, 45, 59, 64, 65, 66, 67, 77, 84 and 87, Table 2), seven of which were located in Th50-69 or B74-84. Of particular note, the master sequence of the motif delimited by codons 64 to 67, commonly defined by $E_{64}LMT_{67}$ and recognized by T-cells[13], differed in patient 1. The sequence found, $D_{64}VTN_{67}$, was completely different from the consensus sequence of genotypes A and D. The amino acid variability detected in patient 1 (average, 0.1%), which ranged from 0.69% to values under the cut-off (< 0.05%), was the lowest in all 4 samples. In this patient, the main epitopic regions contained 67.7% of the changes, a percentage 1.2 times higher than would be expected by the length of these regions, and the changes were equally distributed between the two epitopes. In contrast, patient 2 had higher variability (average, 0.35%), particularly in codons 41 (7.54%), 59 (4.75%) and 63 (1.93%). Only 53.1% of these changes were located in epitopic regions, a rate similar to the expected random percentage, but in the Th50-69 epitope the substitutions were 1.3 times higher than would be expected. Interestingly, two of the main substitutions detected in patient 2, S41 (A, 7.51%) and V59 (I, 4.21%), coincided with the master sequence of patient 1. The third main variant position was G63 (V, 1.62%).

The two genotype D baseline samples (patients 3 and 4) had the same master sequence, except in codons 64, 74, 80 and 93, which were also the most highly variable in patient 4. In patient 3, five codons with more than 1% variability were detected: A41 (1.58%), I59 (1.68%), A74 (2.1%), E77 (1.94%) and S87 (1.93%) (3 of them in epitopic regions). The average amino acid variability was 0.26%, and 57.8% of changes were located in the main epitopic regions. Overall, this percentage was not higher than expected; however, changes in B74-84 were 1.6 times higher than the expected random percentage (31.6% *vs* 19.7%). In patient 4, variability was higher

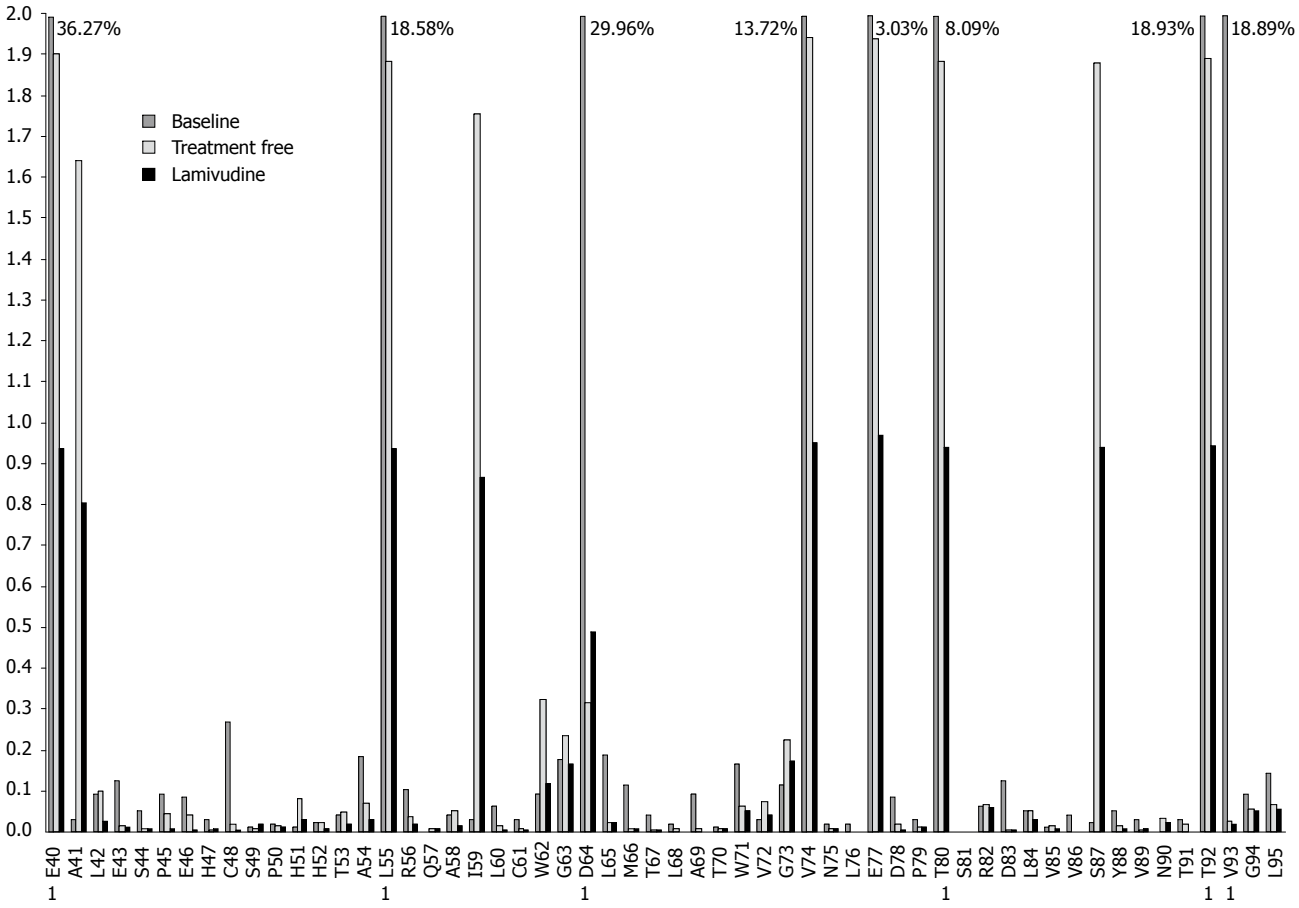


Figure 1 Variability of all codons analyzed from the 3 sequentially studied samples, corresponding to patient 4. Percentages higher than 2% are indicated at the top of the bars. 1: Master aminoacid different between treatment free and lamivudine samples.

than 1% in 8 codons - Q40 (36.27%), L55 (18.58%), D64 (29.96%), V74 (13.72%), E77 (3.03%), T80 (8.08%), T92 (18.93%) and V93 (18.89%) - 5 of which were in epitopic regions. Linkage analysis showed that some of the main variants seen in this patient (S at codon 41, V at 59, N at 74, E at 77 and N at codon 87) were located in the same viral strain (1.5% of quasispecies). This observation seems to indicate possible selection by the effect of immune pressure on the core gene. Surprisingly, despite the high total amino acid variability detected in patient 4 (2.69%), only 49.8% of changes affected positions located in main epitopic regions, a value lower than was expected in both Th50-69 and B74-84.

Median baseline frequencies were compared between the epitopic and other positions. Only genotype A samples showed high variability approaching significance ($P = 0.07$, sample 1 and $P = 0.05$, sample 2) in epitopic positions. Regarding the median baseline variability (Table 2), 6 of the highest values were located in positions within the main epitopic regions (codons 55, 59, 64, 74, 77 and 80), and 4 positions outside the main epitopes (codons 40, 41, 92 and 93) accumulated high percentages of changes. The variability in positions 40, 92 and 93 was due to changes in patient 4, whereas the variability of codon 41 (median 2.3% of changes) was due to changes in patients 2 and 3. Interestingly, positions 64 and 66, corresponding to the $E_{64}LMT_{67}$ motif of Th50-69, showed significant variability in all 4 samples (0.69%, 0.5%, 0.41% and 29.96% in position 64 and 0.12%, 0.21%, 0.11% and 0.11% in position 66).

Attending to the conserved positions, 12 codons showed variability lower than the system error rate (< 0.05%): positions 44, 52, 57, 68, 70, 75, 76, 78, 81, 85, 86 and 89. The most highly conserved was leucine in codon 76, with frequencies clearly below the system error rate (0.003-0.02%) and a median baseline error of 0.013% (Table 2). Based on this finding, codon 76 was analyzed by site-directed mutagenesis analysis.

HBV quasispecies dynamics: The sequentially studied patient

Patient 4 was selected for sequential analysis, and 3 samples were processed (Figure 1): a baseline sample (also included in the *Baseline Study*), a sample following a treatment-free period of 36 mo, and a sample following 18 mo of LVD non-response. After application of the bioinformatic filter, 34 320 sequences from the treatment-free sample and 43 257 sequences from the LVD sample were obtained. The average amino acid variability of the baseline sample was higher than that of the treatment-free one (2.69% *vs* 0.34%, $P = 0.001$) and the average amino acid variability of the treatment-free sample was

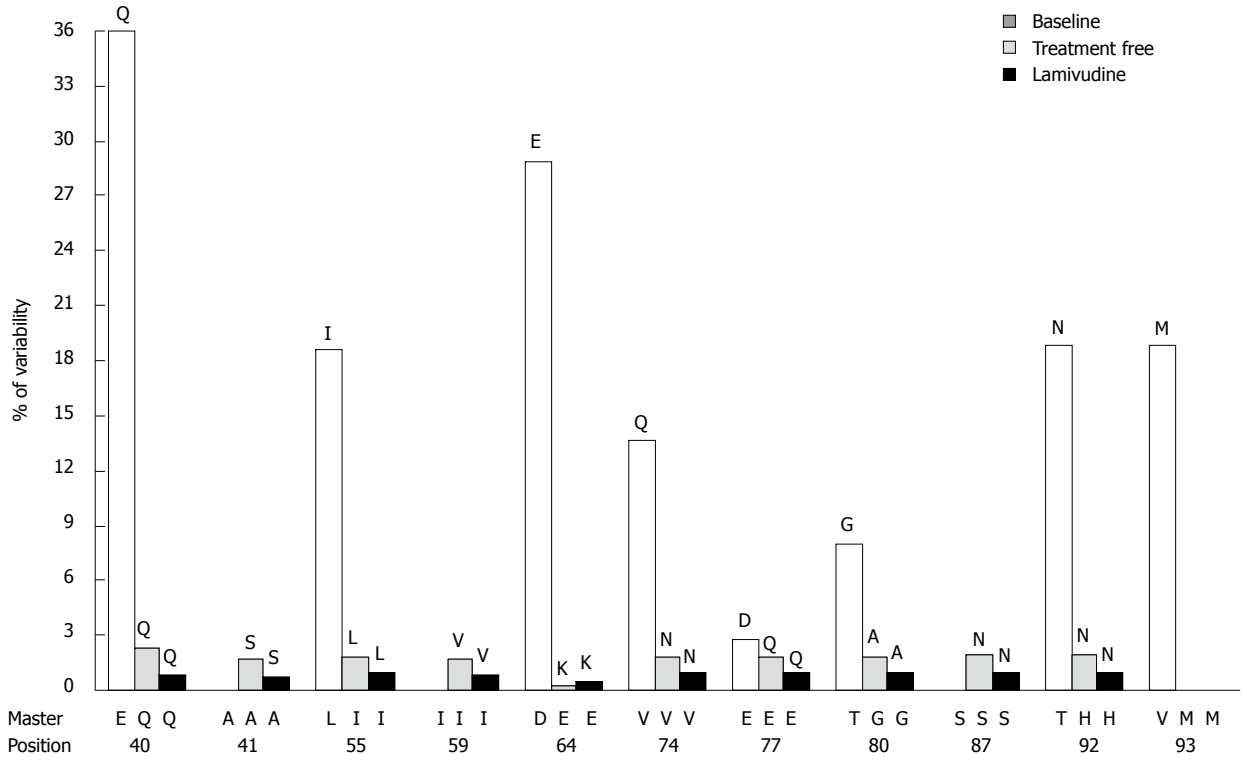


Figure 2 Evolution of the most variable codons of the core region analyzed in the sequentially studied patient. The variable position and its corresponding master amino acid are shown on the X-axis. The main mutated amino acids are indicated at the top of the bars.

higher than that of the LVD treatment sample (0.34% *vs* 0.18%, $P = 0.001$). The main variants detected by sequential analysis are represented in Figure 2. Of note, 6 of these 11 variable positions (codons 55, 57, 64, 74, 77, 80) were located in main core epitopic regions.

Linkage analysis was performed to determine whether the most frequent amino acid substitutions were simultaneously present in the same viral sequence (Figure 3A). At baseline, 50.15% of sequences in the positions studied were wild type, and 38.88% were mutated sequences in the most variable positions (15 different mutated variants, Figure 3A); 10.97% showed mutations in other positions. The mutated sequences in highly variable positions detected at baseline were found to be decreased in the treatment-free period (1.85%, Figure 3B) and after LVD breakthrough (1.24%, Figure 3B).

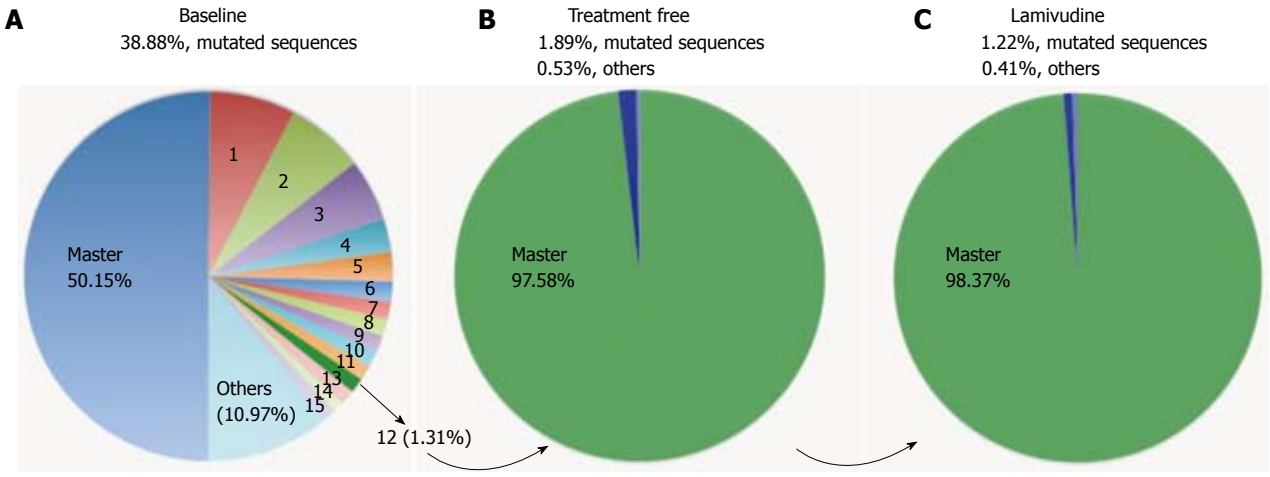
Attending to these variable positions, the most common strain at baseline (7.5%) had only one mutated codon (E40Q), followed by a strain (6.84%) with 5 mutated codons (E40Q, D64E, V74G, T92N and V93M) and another strain (5.45%) with 3 mutated codons (E40Q, L55I and D64E). Surprisingly, the baseline strain that had been selected as master after the treatment-free period and maintained during LVD was a low-frequency baseline mutant strain (1.31%) with the following substitutions: E40Q, L55I, D64E, T80G, T92H and V93M (variant 12, Figure 3A).

The time period with an absence of therapy (between baseline and treatment-free sampling) represented the complete time of HBV infection, and the HBV core quasispecies showed a tendency to decreased variability. This was reflected by a drop in the percentage of accumulated variability from baseline (38.88%) to the treatment-free sample (1.85%) and coincided with the change in the master sequence between the two samples, which could indicate a possible alternative immune escape mechanism. No significant differences were observed between samples from the treatment-free and LVD periods, which showed similar composition and percentages of mutated variant strains (Figure 3).

Evaluation of the conserved position, leucine 76, by site-directed mutagenesis

As is described above, leucine (L) at codon 76 was the most highly conserved position in all the samples analyzed (Table 2). Although there were other conserved positions (Q57, T70, D78 and S81), codon 76 focused our interest because it was a leucine (one amino acid coded by 6 different codons), because of its location in the core gene, and because it has never been described as essential. L76 was even more conserved than D78 (0.05%) or P79 (0.09%), both of which are reported to be involved in the core-HBsAg interaction[24]. Mutagenic studies of L76 were performed to evaluate a possible essential role of this amino acid. The experiments included a change to valine (V), whose hydrophobicity is similar to that of L, and a change to proline (P), whose physical-chemical properties differ from those of L.

After transfection, HBsAg, HBeAg and HBV DNA were quantified in cell culture supernatants. The presence



Baseline composition:

M: E40-A41-L55-I59-D64-V74-E77-T80-S87-T92-V93
1: Q40-A41-L55-I59-D64-V74-E77-T80-S87-T92-V93 7.5%
2: Q40-A41-L55-I59-E64-G74-E77-T80-S87-N92-M93 6.9%
3: Q40-A41-I55-I59-E64-V74-E77-T80-S87-T92-V93 5.5%
4: Q40-A41-I55-I59-D64-V74-E77-T80-S87-T92-V93 2.9%
5: Q40-A41-I55-I59-E64-V74-E77-G80-S87-T92-V93 2.6%
6: E40-A41-L55-I59-D64-V74-E77-T80-S87-N92-M93 1.8%
7: E40-A41-L55-I59-E64-G74-E77-T80-S87-N92-M93 1.5%
8: Q40-A41-E55-I59-D64-V74-E77-T80-S87-T92-V93 1.4%
9: Q40-A41-L55-I59-E64-G74-E77-T80-S87-T92-V93 1.4%
10: Q40-A41-L55-I59-D64-V74-D77-T80-S87-N92-M93 1.4%
11: E40-A41-L55-I59-E64-V74-E77-T80-S87-T92-V93 1.4%
12: Q40-A41-I55-I59-E64-V74-E77-G80-S87-H92-M93 1.3%
13: E40-A41-I55-I59-E64-V74-E77-T80-S87-T92-V93 1.3%
14: Q40-A41-I55-I59-E64-V74-E77-T80-S87-N92-M93 1%
15: E40-A41-L55-I59-D64-V74-E77-G80-S87-T92-V93 1%

Figure 3 Quasispecies composition of the hepatitis B virus core region in the 3 sequentially analyzed samples. A: Baseline variants (1-15) in percentages ⩾ 1%; B: Treatment-free variability; C: Lamivudine sample variability of the most frequent amino acid substitutions defined in Figure 2. Linkage analysis was also performed attending these most variable codons.

of P in position 76 significantly decreased production of HBsAg, HBeAg and HBV-DNA in comparison with the wild type (L76) (both, $P < 0.001$). However, when V was in position 76, a reduction was observed in HBsAg levels ($P < 0.001$), but not in HBV DNA. Surprisingly, the V substitution resulted in a four-fold increase in HBeAg production in comparison with L76 ($P < 0.001$).

DISCUSSION

Several epitopic regions have been described in HBV core gene. Core region variability under antiviral treatment has been extensively studied, particularly in relation to IFN therapy[10,12]. However, differing patterns of amino acid substitutions under the effect of nucleotide/nucleoside analogues and during periods without treatment have been recently described[11]. Some studies have used the classical clonal method to analyze the evolution and composition of the entire HBV core gene[25-27]. However, in all these reports, only a small number of clones were processed and minor populations could not be studied. Thus, high-resolution clonal studies are needed to deeply analyze the composition of the HBV core gene quasispecies. To this end, the recently developed UDPS technology provides an opportunity to bypass the restrictions of the classical clonal method to determine the composition of viral quasispecies[16,17,19-21].

When this study was designed, UDPS technology based on the 454 Life Science Platform (GS-FLX, Roche Applied Science) only allowed analysis of around 200 nucleotide sequences. Because of this limitation, the present work was focused on analysis of the main immunodominant core motifs, Th50-69 and B74-84[6,7]. The high economic cost and complex computing analysis of UDPS strongly restrict the number of samples to be processed. For this reason, we were only able to study six selected samples: four samples to evaluate the baseline variability and two more samples to sequentially analyze one patient.

To achieve the main aim of UDPS analysis, a parallel analysis was needed to define the cut-off for analyzing the UDPS data. In our previous report of UDPS analysis of the HBV quasispecies[20], inclusion of an internal control sequence within the analyzed amplicon enabled establishment of a cut-off percentage to define the limit of viral variability, which was set at 0.03%. In the present study, a Poisson computational model recently validated by our group was applied by processing an HBV-DNA clone[21]; the percentage established to differentiate variability from UDPS error was set at 0.05%[21].

In the study of baseline HBV quasispecies, the most variable codons (median baseline variability ⩾ 1%) were 40, 41, 55, 59, 64, 74, 77, 80, 92 and 93, all of which have been previously described in a study using clonal methodology to analyze acute exacerbations in HBeAg-negative patients[25]. Furthermore, some of these highly variable codons (codons 64, 74 and 77) were previously described as common changes in untreated chronic hepatitis B[11]. Genotype A sequences showed fewer codons with variability ⩾ 1% and, consequently, lower over-

all median variability than genotype D. The statistical analysis of the median baseline frequencies showed that genotype A samples had higher variability in epitopic positions, approaching significance (P = 0.07, sample 1 and P = 0.05, sample 2). In contrast, in genotype D cases, no significant differences in variability were observed between the epitopic and other positions. These findings could be related to the high frequencies of mutations in codon 40, 87, 92 and 93 in genotype D samples (patients 3 and 4), which might be involved in the minor epitopic regions Th28-47 and Th82-101[6,11]. These differences in HBV core gene variability seem to indicate an influence of genotype on immune activation.

Patient 1 showed the lowest variability, but interestingly, the codon 64 to 67 master sequence was defined by the sequence $D_{64}VTN_{67}$, which is completely different from the well-characterized $E_{64}LMT_{67}$ motif. This motif is commonly recognized by T-cells, and the simultaneous E64D and T67N change has been reported to reduce T-cell proliferation *in vitro*[13]. Hence, we suggest that the changes observed in patient 1 might be the result of an alternative mechanism to escape from immune pressure, over the $E_{64}LMT_{67}$ motif. In fact, the finding that positions 64 and 66 of this motif showed significant variability in all 4 samples suggests that the motif could be a central target for immune pressure in HBV infection[11]. The low variability observed in sample 1 (0.1% of total amino acid variability) could reflect attainment of a kind of "steady state" in the viral quasispecies, resulting from strong selection of an escape variant with the mutated motif $D_{64}VTN_{67}$, similar to that seen in the longitudinal study of patient 4.

Evolution of the HBV quasispecies was evaluated in a single patient and involved a baseline sample and two additional samples, one taken after a period without treatment and one taken after LVD treatment. During the treatment-free period, a decrease in quasispecies variability was observed, with a reduction in the number of viral strains. This change may have been a consequence of host immune pressure[25]. One of the baseline viral strains present in a small percentage (variant 12, 1.31%, Figure 3) was strongly selected and became the master in the treatment-free and LVD samples, a fact suggesting that this variant might be an escape mutant, whose selection could be related to better fitness, regardless of its initial frequency.

Based on these results, we postulated that the HBV quasispecies achieved a kind of "steady state" after the treatment-free period that did not change with LVD treatment, because immune pressure could also be decreased during treatment. The significant differences in average variability in the two periods suggest that the equilibrium is dynamic. The structure of the HBV quasispecies in the three samples represents a complex reservoir of different minor variants, resulting from natural HBV evolution and likely affected by antiviral treatment[28].

Several codons in our region were found to be highly conserved (positions 57, 70, 76, 78 and 81). Position 76, located at the tip of the spike was of special interest, being next to the major immunodominant region and part of the B74-84 main epitope. Indeed, this position has never been described as essential, in contrast to the nearby positions 78 and 79[24]. The HBV core sequence is involved in the process of capsid conformation by interacting with the surface antigens in viral particle assembly. However, the interaction between HBsAg and HBcAg in virions is still unclear[29-31]. The core region analyzed in the present study is part of the assembly domain (amino acids 1-149); thus, the changes in this region might potentially modify the shell conformation.

Electrostatic interactions between core and HBsAg take place at the tip of the spike of the core antigen (codons 74-84)[32,33]. Cryo-electron microscopy studies have shown that codon 78 and 79 are within the contact area of core with envelope proteins[24]. This essential role might explain the high level of conservation of these positions (especially codon 78, 0.05%) observed in our UDPS analysis. However, surprisingly, the most conserved codon of the HBV core region in baseline and sequential samples was leucine at position 76.

To our knowledge, few studies[30,31] have analyzed the effect of single core amino acid mutations on HBV replication *in vitro*. Only one such study conducted by Ponsel *et al*[31] evaluated the leucine 76 position (among others) by inducing a change to alanine; no significant reduction in nucleocapsid or virion production was observed. In the present study, we induced changes in the hydrophobic characteristics of position 76 and determined their effect on HBsAg, HBeAg and HBV DNA production. In contrast to the results of Ponsel *et al*[31], we found a significant reduction in HBsAg production with both the V and P changes, suggesting possible involvement of L76 of HBcAg in the HBsAg interaction. A significant decrease in HBV replication in the presence of P76 was detected, leading us to speculate that the hydrophobic characteristics of position 76, conferred by the presence of L or V, are needed for HBV replication. The increase in HBeAg production in the presence of the V mutation and the absence of increasing HBV DNA were surprising, particularly because some authors have reported a correlation between HBeAg and HBV DNA levels[34]. Nonetheless, this correlation was not found by other authors[35] and currently remains controversial. We suggest that this unexpected HBeAg increase may indicate alternative pathways between HBeAg and HBV replication, as has been indicated previously[36]. Based on our *in vitro* results, it can be postulated that the amino acid changes induced in the core sequence are not as important as the structure adopted by the capsid and HBeAg.

Our study is mainly limited by the high cost of the UDPS process, which restricted the number of samples studied and created a risk that some results could be due to random chance. A larger number of samples, additional sequential studies, and duplicate UDPS experiments would have given more information about the

HBV core sites involved in quasispecies evolution and potentially related to HBV chronic infection and to treatment non-response. In addition, at the time of the study, the available UDPS methodology only allowed analysis of sequences up to 250 bp. For this reason, we limited the study to the widely described main epitopic regions included in the HBV core, previously investigated by conventional methodologies[6,7,11,25].

In conclusion, this study validates application of UDPS to study the variability of the main core epitopes, substantiates the significant richness of the HBV baseline quasispecies, and suggests a relationship between core variability and HBV genotype. The highest variability was mainly detected in Th50-69 and B74-84, supporting their role as the main immune-stimulating core regions. The significant variability associated with well-characterized Th-cell motifs, such as $E_{64}LMT_{67}$, seems to indicate that the host immune system may be the main factor responsible for HBV core evolution. The relevant conservation of codon 76 may be related to possible interactions with the viral envelope. In the single longitudinally analyzed patient, a minor variant present in the baseline quasispecies was selected as the main variant in the absence of treatment and was maintained after lamivudine. These findings indicate the utility of UDPS to describe the dynamic behavior of the HBV quasispecies. More extended analyses with a larger number of samples must be performed to confirm the findings. The expected spread of this technology will probably allow a significant decrease in the cost, enabling processing a large number of samples. The UDPS application for diagnostic and routine analysis might serve for the quantitative estimation of the viral quasispecies, for defining mutant variants or for establishing the quasispecies complexity. All these parameters would be useful as prognostic factors for disease outcome or therapy efficacy.

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COMMENTS

Background

Hepatitis B virus (HBV) infection is a global health problem, with more than 300 million HBV carriers. Viral distribution in individual infection is defined as a quasispecies, which means that variability can be accumulated along the HBV genome. The core gene is the least overlapped gene of the HBV genome and core antigen is the most immunogenic of all the viral peptides. Therefore, the region encoding the main core epitopes is useful to study the effect of the host immune response and to analyze the variability of HBV quasispecies composition.

Research frontiers

The distribution of viral infection as a quasispecies (in HBV, but also hepatitis C virus and human immunodeficiency virus) is an important advantage for the virus. It enables fast, easy establishment of infection and enables adaptation against changes in the viral environment. The study of viral quasispecies has been limited by the available methods, with which analysis of a significant number of clonal sequences was extremely difficult. The possibility of obtaining thousands of sequences in a single sample, provided by next-generation sequencing methods such as ultra-deep-pyrosequencing (UDPS), will allow more in-depth study of the viral quasispecies. In the present study, the authors applied the UDPS to analyze HBV quasispecies variability and adaptability of the virus in single patient.

Innovations and breakthroughs

To date, few studies have used UDPS to study the HBV quasispecies, likely because of the current high cost of the technique for this purpose. However, the results presented in these recently published studies have shown clear advantages of UDPS for viral quasispecies analysis. Most of these studies have been mainly focused on the polymerase gene (treatment resistance variants). The present study is the first work analyzing the core gene by this method, to evaluate the effect of the host immune response and the variability of this little overlapped region of the HBV genome.

Applications

This study illustrates the value of deep quantitative analysis of the HBV quasispecies composition to investigate its clinical relevancy. The results provide an indication of the role of HBV core gene quasispecies structure in the natural host immune response and have prompted us to continue investigating HBV variability by UDPS, particularly the precore and core regions because of their relationship with the immune response.

Terminology

Viral quasispecies distribution in an infected patient refers to a group of viruses that are different, but highly related. This distribution results in competition between viruses from the same infection, but also confers plasticity and adaptability to environmental changes.

Peer review

The authors analyzed the quasispecies of HBV core epitopic regions by UDPS. They found that positions with highest variability rates, mainly clustered in the main core epitopes, showed some relationship with HBV genotype, and were particularly associated with the T-helper motif. The authors suggest that immune system pressure is the main cause of HBV core evolution.

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BRIEF ARTICLE

Cost of treating chronic hepatitis B: Comparison of current treatment guidelines

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Abstract

AIM: To compare program costs of chronic hepatitis B (CHB) screening and treatment using Australian and other published CHB treatment guidelines.

METHODS: Economic modeling demonstrated that in Australia a strategy of hepatocellular cancer (HCC) prevention in patients with CHB is more cost-effective than current standard care, or HCC screening. Based upon this model, we developed the B positive program to optimize CHB management of Australians born in countries of high CHB prevalence. We estimated CHB program costs using the B positive program algorithm and compared them to estimated costs of using the CHB treatment guidelines published by the Asian-Pacific, American and European Associations for the Study of Liver Disease (APASL, AASLD, EASL) and those suggested by an independent United States hepatology panel. We used a Markov model that factored in the costs of CHB screening and treatment, individualized by viral load and alanine aminotransferase levels, and calculated the relative costs of program components. Costs were discounted by 5% and calculated in Australian dollars (AUD).

RESULTS: Using the B positive algorithm, total program costs amount to 13 979 224 AUD, or 9634 AUD per patient. The least costly strategy is based upon using the AASLD guidelines, which would cost 34% less than our B positive algorithm. Using the EASL and the United States Expert Group guidelines would increase program costs by 46%. The largest expenditure relates to the cost of drug treatment (66.9% of total program costs). The contribution of CHB surveillance (20.2%) and HCC screening and surveillance (6.6%) is small - and together they represent only approximately a quarter of the total program costs.

CONCLUSION: The significant cost variations in CHB screening and treatment using different guidelines are relevant for clinicians and policy makers involved in designing population-based disease control programs.

Key words: Chronic hepatitis B; Markov model; Hepatocellular cancer; Treatment guidelines

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INTRODUCTION

Although hepatocellular cancer (HCC) remains relatively uncommon in Australia, its incidence has increased approximately fivefold since 1972, and based on current trends, a threefold increase is expected by 2020[1]. HCC rates are highest in Southwestern Sydney, where its incidence (7.7 per 100 000 persons, 95% CI: 7.0-8.4) is significantly higher than the state average (5.2 per 100 000 persons, 95% CI: 5.0-5.5)[1].

Nearly 90% of hepatitis-B-related HCC in NSW occurs in people born overseas, with approximately 70% affecting Australians born in countries of high hepatitis B prevalence[2]. Migrants born in these countries are 6-12 times more likely to develop HCC than other Australians[3], explained by the strong association between hepatitis B infection acquired early in life and the subsequent development of hepatic cirrhosis and HCC[4]. In recent years, effective treatments for chronic hepatitis B (CHB) infection have achieved sustained suppression of viral replication and significant reductions in disease progression to cirrhosis, end-stage liver disease and HCC[5-7]. This opens unique opportunities to reduce CHB-related morbidity and mortality among the 350 million chronically infected people worldwide[8], provided that treatment costs are affordable at a population level. This is particularly relevant in the developing world, where the great majority of people with CHB reside[4].

Our previous modeling work showed that in Asian populations with CHB residing in Australia (representing > 50% of people diagnosed with CHB in Australia[9]), a strategy of HCC prevention is more cost-effective than HCC screening[10]. For people with CHB, we defined HCC prevention as an intervention comprising regular (6-monthly) patient follow-up, the institution of antiviral therapy in those with active disease, and HCC surveillance. Following the confirmation of CHB diagnosis, general practitioners (GPs) order the relevant investigations and stratify participants into discrete risk categories, based upon hepatitis B virus (HBV) DNA and alanine aminotransferase (ALT) levels (Figure 1). Low-risk patients [those with low HBV DNA (defined as < 20 000 IU/mL for participants aged < 50 years and < 2000 IU/mL for those aged > 50 years) and normal ALT levels [defined as < 1.5 times upper limit of normal (ULN)] are offered routine CHB surveillance, consisting of 6-monthly GP follow-up visits and testing for hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), viral load and ALT levels. Patients with normal ALT levels, but elevated viral loads (as defined above) are followed up by their local medical practitioners under a program of enhanced CHB surveillance (which in addition to routine blood tests also includes 6-monthly HCC surveillance using α fetoprotein (AFP) measurements and liver ultrasound (US) examinations. Patients with elevated ALT and viral load levels are referred to tertiary care for assessment and consideration of antiviral therapy (Figure 1). The general assumptions of the Markov model are summarized in Table 1.

These treatment strategies are at slight variance with those described in hepatitis B practice management guidelines, because they factor in age as a consideration for treatment initiation (program participants are aged ⩾ 35 years and treatment criteria change at age 50 years) and ALT cutoff levels are ⩾ 1.5 × ULN. Additionally, HCC screening is being offered to groups deemed at higher risk, rather than to all Asian males over the age of 40 years and Asian women over the age of 50 years, as it is recommended by the American Association for the Study of Liver Disease (AASLD) guidelines[11].

The modeling work informed the development of a program of CHB management targeting the area of Sydney with the highest burden of hepatitis-B-related HCC in Australia, located in Southwest Sydney. As the Gastroenterology Society of Australia uses viral loads > 2000 IU/mL as a cut-off for treatment initiation irrespective of age[12], we subsequently modified the viral load cut-off for the B positive program, to avoid confusion among primary care providers participating in the program.

In order to inform hepatitis B management and provide data to policy makers, we estimated B positive program costs using the original B positive screening and treatment algorithm and compared them with those incurred using the modified B positive algorithm (viral load cutoff of 2000 IU/mL for treatment initiation, irrespective of patient age) and to costs incurred when applying guidelines published by the American, European and Asia-Pacific Associations for the Study of Liver Disease (AASLD, EASL and APASL)[6,13,14] as well as those developed by an independent panel of hepatologists from the United States (referred to here as the United States Expert Group)[15]. We also determined the relative proportion of program costs attributable to CHB screening, drug treatment, CHB surveillance and HCC screening and surveillance incurred by applying each of these algorithms. Costs were calculated in Australian dollars (AUD).

MATERIALS AND METHODS

The B positive project targets Asian migrant communities in Southwest Sydney, but is inclusive of all individuals who meet eligibility criteria. Eligibility criteria include: confirmed CHB, age ⩾ 35 years, and attending a general practice in the target local government areas. To estimate the size of the eligible population, we used data provided by the Australian Bureau of Statistics 2006 National Cen-

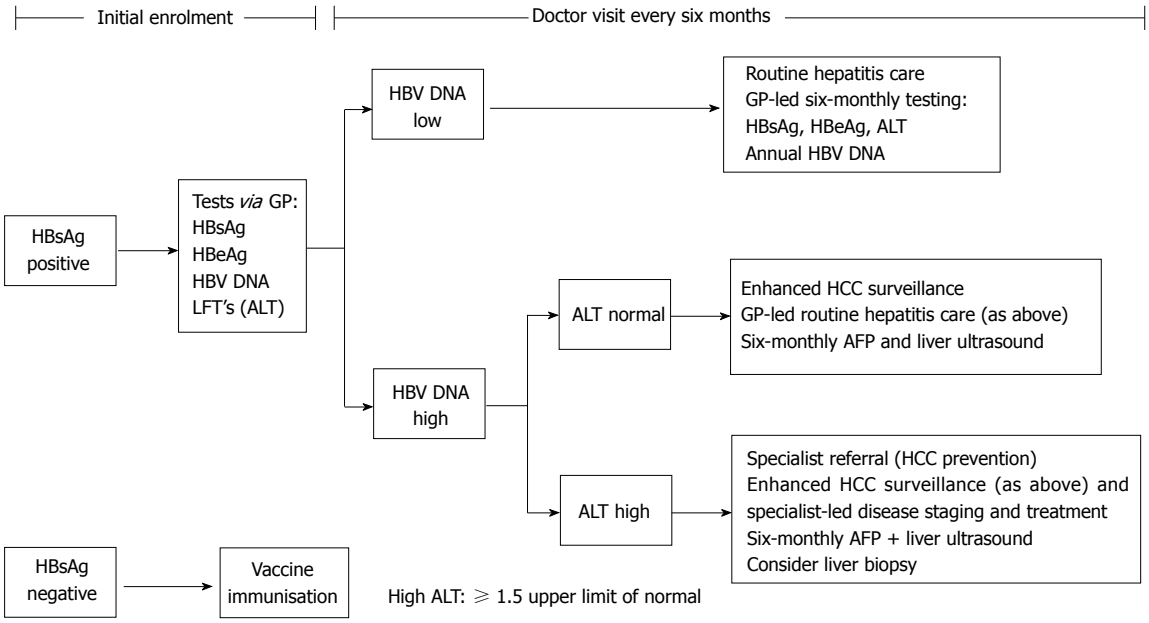

Figure 1 B positive program for chronic hepatitis B screening and treatment protocol. Depicts the algorithm used to stratify participants aged > 35 years into routine surveillance [for those with viral loads below 2000 IU/ mL and normal alanine aminotransferase (ALT)], enhanced hepatocellular cancer (HCC) surveillance (for elevated viral loads and normal ALT), and those at highest HCC risk (referred for specialist opinion and antiviral therapy). CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; GP: General practitioner; HBV: Hepatitis B virus; AFP: α fetoprotein; LFTs: Liver function tests.

sus on the number of local residents born in China, Hong Kong and Vietnam aged ⩾ 35 years. We applied HBV seroprevalence data on these numbers, based upon epidemiological estimates from the respective countries of birth[16].

We estimated the proportion of people in different CHB-related disease stages over the 50-year timeframe [CHB without cirrhosis; CHB with cirrhosis; CHB with liver failure; CHB and HCC; spontaneous HBsAg clearance and death due to CHB-related causes (liver failure or HCC), or death from other causes], using the assumptions of our previously published Markov model[10]. The model takes a health care funder perspective and discounts costs at 5% per annum. We estimated a program participation rate of 25%, informed by the experience of the New Zealand HBV screening program, which screened 27% of their eligible population[17]. Table 1 summarizes the key assumptions of our model. We used an ALT level of ⩾ 1.5 × ULN to define high-risk patients and estimated that about 50% of the target population have ALT levels < 1 × ULN, 12.5% have ALT levels of 1-1.5 × ULN, 12.5% between 1.5 and 2 × ULN and 25% levels > 2 × ULN, informed by a Hong Kong population-based study of CHB patients[18].

We estimated the proportion of the target population receiving antiviral treatment based upon the cutoffs for ALT and viral load recommended by the different guidelines for patients who are HBeAg negative (Tables 2 and 3). Briefly, in this population group the AASLD guidelines recommend treatment to be initiated when viral load exceeds 20 000 IU/mL and ALT levels are > 2 × ULN[6]. The APASL guidelines use the same ALT level cutoff, but a lower threshold (2000 IU/mL) for viral load[14], while the United States expert group and EASL use the 2000 IU/mL cutoff for viral load, but recommend treatment for patients with ALT levels exceeding the normal range[13,15].

The B positive program (unpublished) data suggested that 94% of those enrolled in the program were HBeAg negative at the time of enrolment (which corroborates the findings of Yuen *et al*[19], that in an Asian population, HBeAg seroconversion occurs around 35 years of age); consequently we compared estimated CHB program costs using treatment guidelines developed for HBeAg-negative populations.

We factored in the costs of CHB screening and follow-up provided by specialists and/or primary care providers, the costs of HCC screening, and CHB and HCC treatment, but not recruitment costs, or the costs of immunization for those susceptible.

RESULTS

The CHB population targeted by the B positive program in Southwest Sydney was estimated at 5800 patients. Assuming a 25% enrolment rate, about 1500 patients (1451 patients) with CHB in Southwest Sydney would be enrolled in the program. Nearly two-thirds (63%) of these patients would be followed up by their GPs under a program of routine surveillance (Figure 1). Half of the patient population would receive this type of management if using the other guidelines, except for the AASLD guidelines, where 70% of all patients would be receiving routine surveillance. The proportion of patients under enhanced surveillance (which is 23% for B positive and 31% for the modified B positive) would range from a low of 23% using the AASLD guidelines to a high of 37% using

Table 1 General assumptions of the Markov model of the B positive hepatocellular cancer prevention program

Assumption	How addressed and rationale
Participant recruitment	Target population age ⩾ 35 yr, HBsAg +ve for ⩾ 6 mo, born in China, Hong Kong, Vietnam
Contact testing and immunisation	Not factored into the model
Seroprevalence in target populations	10.7% for people born in China 10.5% for people born in Vietnam 7.7% for people born in Hong Kong (Nguyen *et al*[16])
Initial testing to confirm chronic hepatitis B	Not factored in the GP consultation calculations
Program participation rates	Base case assumption: 25% of eligible people are enrolled
HCC screening	All participants have AFP and liver US at enrolment Participants receiving enhanced surveillance have 6-monthly AFP and US Participants receiving treatment also have liver biopsy
Follow up requirements	Routine surveillance arm: 2 GP appointments/yr Enhanced HCC surveillance arm: 2 GP appointments/yr Interferon treatment: 6 specialist appointments/yr Entecavir treatment: 4 specialist appointments/yr Patients with HCC: assumed two monthly follow up
Viral load distribution	Based upon Risk Evaluation of Viral Load Elevation and Associated Liver Disease study data (Chen *et al*[20])
ALT level distribution	Based upon Hong Kong data (Yuen *et al*[18])
Progression rates through different disease stages	Constant
Treatment protocol	30% receive first line interferon (weekly for 12 mo); 30% seroconvert and receive no further treatment; 70% commence entecavir the following year 70% receive entecavir as first-line treatment; 20% seroconvert in first year and receive no further treatment; 80% continue lifelong entecavir
Patients with liver failure	Receive lifelong entecavir

Lists the general assumptions used in building the model, including treatment protocols and follow up and other relevant elements and data sources. HBsAg: Hepatitis B surface antigen; GP: General practitioner; AFP: α fetoprotein; ALT: Alanine aminotransferase; HCC: Hepatocellular cancer; US: Ultrasound.

Table 2 Participant distribution by disease stage at initial enrolment and management pathways, according to the B positive algorithm and hepatitis B treatment published guidelines *n* (%)

Treatment guideline	B positive	Modified B positive	EASL, United States experts	APASL	AASLD
HBV DNA level to treat	> 2000 if > 50 > 20 000 if < 50	> 2000	> 2000	> 2000	> 20 000
ALT (ULN)	> 1.5	> 1.5	> 1	> 2	> 2
Number receiving interferon	61 (4)	81 (6)	108 (8)	54 (4)	33 (2)
Number receiving entecavir	143 (10)	190 (13)	253 (17)	126 (9)	76 (5)
Total on treatment	204 (14)	271 (19)	361 (25)	181 (12)	109 (8)
Total under enhanced surveillance	340 (23)	452 (31)	361 (25)	542 (37)	326 (23)
Total under routine surveillance	907 (63)	728 (50)	728 (50)	728 (50)	1016 (70)
Total	1451 (100)	1451 (100)	1451 (100)	1451 (100)	1451 (100)

Tabulates the estimated number of participants undergoing different monitoring strategies and treatment options, according to recommendations put forward by Australian, Asia-Pacific, United States and European chronic hepatitis B treatment guidelines. ULN: Upper limit of normal; HBV: Hepatitis B virus; EASL: European Associations for the Study of Liver Disease; APASL: Asia-Pacific Associations for the Study of Liver Disease; AASLD: American Associations for the Study of Liver Disease.

the EASL and United States Expert Panel guidelines. The proportion of patients receiving antiviral treatment (14% for B positive) ranges from a low of 8% under AASLD to a high of 25% under the more stringent EASL and United States Expert Panel Group guidelines (Table 2).

Overall, the lowest program costs are associated with the application of AASLD guidelines, because the 20 000 IU/mL viral load cutoff for treatment initiation and ALT levels ⩾ 2 × ULN make fewer patients eligible for treatment. Treatment costs are highest when applying EASL and United States Expert Group guidelines, because they recommend treatment for all patients with viral loads > 2000 IU/ mL and ALT levels > 1 × ULN.

The total B positive program costs would amount to 13 979 224 AUD, or 9634 AUD per patient using the original B positive algorithm, ranging from a low of 6344 AUD for the AASLD guidelines to a high of 14 039 for the EASL and United States Expert Group recommendations (Table 3). The largest component of the cost structure relates to antiviral treatment, which represents over three-quarters (75.8%) of program costs if using EASL and United States Expert Group recommendations, approximately 70% (70.2%) for the modified B positive algorithm, 66.9% for the original B positive, 60% for the APASL guidelines, and just over 50% for the AASLD guidelines. Costs of CHB surveillance ranges from 17.5% for the modified B positive protocol to 30.1% of the program expenditure if using AASLD guidelines. The con-

Table 3 Calculated costs (in Australian dollars) of implementing a program of chronic hepatitis B management in hepatitis B e antigen-negative patients according to the B positive algorithms and published hepatitis B treatment guidelines, *n* (%)

Discounted costs of management strategies	B positive	Modified B positive	APASL	EASL, United States experts	AASLD
Cost/QALY (discounted)	13 465	15 770	11 746	19 622	8867
Total program cost (discounted)	13 979 224	16 372 320	12 194 905	20 371 117	9 205 680
Cost components					
Initial CHB screening cost	767 728 (5.5)	800 792 (4.9)	755 971 (6.2)	845 613 (4.2)	720 357 (7.8)
Drug treatment costs	9 347 662 (66.9)	11 493 535 (70.2)	7 360 940 (60.4)	15 447 510 (75.8)	4 951 419 (53.8)
CHB surveillance costs	2 827 093 (20.2)	2 866 053 (17.5)	2 866 053 (23.5)	2 866 053 (14.1)	2 767 073 (30.1)
HCC surveillance costs	917 783 (6.6)	1 092 983 (6.7)	1 092 983 (9.0)	1 092 983 (5.4)	647 874 (7.0)
Total cost per person in the program	9634	11 283	8404	14 039	6344
% change with equivalent unit costs/QALY	100	117	87	146	66

Tabulates the costs of chronic hepatitis B screening, surveillance and treatment as per Australian, Asia-Pacific, United States and European treatment guidelines scenarios. Costs are discounted by 5% and calculated in Australian dollars (AUD). EASL: European Associations for the Study of Liver Disease; APASL: Asia-Pacific Associations for the Study of Liver Disease; AASLD: American Associations for the Study of Liver Disease; HCC: Hepatocellular cancer; CHB: Chronic hepatitis B; QALY: Quality-adjusted life year.

tribution of the cost of HCC screening and surveillance remains relatively small in all scenarios, ranging from a low of 5.4% for the EASL and United States guidelines to 9% for the APASL guidelines.

Compared to the B positive algorithm, the lowest cost to achieve an equivalent unit cost/quality-adjusted life year is incurred by applying the AASLD guidelines (34% cost saving), followed by the APASL guidelines (13% cost saving); costs would be 17% higher with the modified B positive algorithm (because more patients aged 35-50 years would be in receipt of treatment) and 46% higher for the EASL and United States Expert Group recommendations.

DISCUSSION

This modeling exercise demonstrates that in a population-based CHB management program informed by current treatment guidelines, the majority of patients (ranging from 50 to 70%) have low viral loads (< 2000 IU/mL) and low ALT levels and may be effectively managed at the primary care level. Between a quarter and a third of patients have elevated viral loads and would benefit from more comprehensive follow-up, which we termed "enhanced surveillance", which could still effectively be delivered at the primary care level and free up specialist resources. These calculations suggest that the proportion of patients requiring tertiary-level assessment for consideration of antiviral therapy is variable, ranging from a low of 8% if the AASLD guidelines are followed to a high of 25% if the more stringent EASL and United States Expert Group guidelines are used. Program costs range from approximately 9 million AUD (if AASLD guidelines are used) to more than double that figure (20 371 117 AUD) if the EASL or United States Expert Group recommendations are applied. Correspondingly, this leads to variations in costs per patient enrolled, ranging from 6344 AUD (for AASLD guidelines) to 14 039 AUD (for EASL and United States Expert Group guidelines). The B positive algorithm steers a mid-course with regards to total (13 979 224-16 372 320 AUD) and per patient costs (9634-11 283 AUD, depending on whether the viral load cut-off is set at 2000 IU/mL for all patients, or only for those aged > 50 years.

The model assumes that all patients with elevated viral loads and ALT levels receive specialist assessment and liver biopsy to assess the degree of fibrosis prior to treatment initiation, although since November 2011, liver biopsy is no longer mandatory for treatment initiation.

In all modeled programs, the greatest contribution to cost is that of antiviral drug treatment (interferon or entecavir), accounting for 50%-75% of the program budget. By comparison, the costs of CHB surveillance are relatively low for a primary-care-based program (ranging from 14% to 30% of total costs); even lower are the costs associated with HCC screening and surveillance (ranging from 5% to 9%). The original B positive modeling did not include HCC surveillance in the subgroup presumed to have inactive disease; this is at variance with current published guidelines, which recommend HCC surveillance for all Asian men aged > 40 years or women aged > 50 years, irrespective of their disease stage or viral load[11]. Although the original intention was to balance cost containment with ensuring that HCC cases are not missed, it appears that the contribution of HCC screening to costs would remain modest even if the program embraces the recommended screening guidelines. Sherman in a recent review concedes that it may be possible that, as more information about HCC risk stratification becomes available, patients with long-term inactive disease may not require the same intensive HCC surveillance[11], something that is supported by the REVEAL data[20].

Our model has a number of limitations related to our assumptions and the lack of clear data in some areas. For example, we used a 1.5 × ULN cut-off for the ALT levels prompting treatment. The different ALT cut-off levels in various guidelines indicate that there is no agreement about what level of ALT should prompt treatment initiation, and information to clarify this is keenly awaited. Similarly, we assumed that all patients with elevated ALT levels would require antiviral therapy, although in reality, ALT elevation may not always relate to disease reactivation, but to other factors, such as high

body mass index, non-alcoholic fatty liver disease, chronic alcohol consumption or coexisting hepatitis C infection[21,22]. We also acknowledge that the rate of progression to HCC development is variable in different patient populations, being dependent on the degree of fibrosis, genotype, and other associated risk factors. Although the short-term goals of antiviral therapy have been achieved in recent clinical trials[22], more answers are needed as to the extent to which this affects liver cancer incidence and the number of cancer deaths. As this is also the major assumption that underlies our analysis, more data confirming the impact of treatment on HCC risk reduction would strengthen the economic model findings.

Our original model factored in the cost of liver biopsy for all patients being considered for antiviral therapy, reflecting the recommendations of guidelines referred to in the paper, suggesting that a liver biopsy is helpful for determining the degree of necroinflammation and fibrosis. However the relatively low cost of a liver biopsy and the relatively small numbers of patients in this subgroup (we estimated that only about 12.5% of patients have ALT levels in that range and even fewer also have low–medium viral loads) means that this procedure will have a minimal impact on overall program costs, although it may influence the number of patients willing to accept drug therapy.

The effectiveness and cost-effectiveness of different interventions need to be corroborated by other types of studies and real-life outcomes data utilized to validate the economic models. We are currently collecting program data for this purpose.

Ideally our findings would need to be compared to those of other studies using decision models and against real-life data from clinical studies, but available data are limited. A recent European review[23] identified only two studies addressing the cost-effectiveness of screening high-risk groups for CHB: both a United States[24] and a Dutch study[25] suggested that screening of migrant groups for HBV was both clinically effective and cost-effective findings that were corroborated by our Australian study[10].

A clinical study estimating the efficacy and cost of HCC screening in a clinic population in Australia[26] confirmed our model costs. We need to bear in mind however that direct comparisons between studies are of limited value, due to differences in study design, model assumptions, cut-offs used, and variable cost structures in different countries.

As the stated aim of this short paper was to examine the cost implications of utilizing different guidelines to treat patients with CHB, in order to assist funding decisions, we did not include a broader discussion of cost-effectiveness, effectiveness and efficacy of antiviral treatment, but agree that a more comprehensive review of effectiveness and cost-effectiveness may be warranted.

Although we incorporated a wide range of supporting evidence into our economic model, the relatively limited information available on the composition of cohorts of patients with CHB receiving treatment and the possible differences in treatment response in patients with HBeAg-negative disease (which has been less extensively studied) may limit the generalizability of our findings. In the absence of relevant data, we assumed that the effectiveness and durability of current interventions can be extrapolated over a lifetime horizon, but acknowledge that the lack of long-term evidence precludes confident estimates of treatment outcomes.

From a policy perspective, the high cost of antiviral therapy makes population-based CHB screening and treatment unaffordable for all but well-resourced countries. For example, Hutton *et al*[27] published their analysis of the cost-effectiveness of interventions aiming to combat hepatitis B in the United States and China. They found that in an American setting it is cost-effective to screen adult Asian and Pacific Islanders (APIs) for CHB, with a view to providing them with appropriate treatment, as well as to vaccinate close contacts. This work led to a change in United States public health policy on hepatitis B screening, with a recommendation that all adult APIs and adults born in areas of intermediate HBV prevalence be screened for CHB. The authors found that in a Chinese setting catch-up adolescent vaccination is cost-effective, but that drug treatment costs would need to be halved (to about 1000 United States dollars/year) before the benefits of vaccination would be surpassed by those of instituting treatment[27]. China's economic successes makes population level CHB screening and treatment a possibility in the future, but effective and inexpensive treatments are needed to reduce the burden of CHB in developing economies[4].

Our original economic model assumptions attempted to reflect the Australian practice prevalent in 2006 and 2007, when entecavir was becoming first-line treatment for CHB. At that time interferon was still in common use, therefore, we modeled 30% of the cohort as receiving first-line interferon.

However, the therapeutic landscape has changed in recent years, with interferon being used now in only about 10% of patients as first-line treatment for CHB. We therefore repeated our calculations estimating that 10% of patients receive first-line interferon and found the incremental total cost was only about 1.5% higher (data not shown), as a result of interplay between a more intensive and more costly specialist follow-up during interferon treatment, a small proportion of patients who clear the infection, and the overall small number of patients affected.

As both available resources and local clinical preferences guide drug treatment, generic lamivudine may be the only affordable antiviral for low-income countries in Asia and Africa, where CHB is most prevalent, because replacing it with a more potent antiviral agent is associated with a more than 10-fold increase in drug costs[28] .

Consequently, we repeated the analysis, substituting lamivudine for entecavir and assuming that the cost of lamivudine represented only a tenth of the cost of entecavir. This led to significant reductions in drug costs (ranging from 75.3% to 77.6%) and in overall program costs (ranging from 40.5% to 58.9% - data not shown), which further emphasizes the important role played by

drug costing in program feasibility.

Our modeling has provided estimates of the cost of CHB management programs that could be useful for policy makers and health care providers in different settings, to inform program development. It would appear that population-based CHB management programs targeting at-risk groups are affordable in high-resource settings, but remain unattainable for many of the world's population with CHB, living in low- or middle-resourced countries. The high cost of antiviral therapies represents the largest cost component of a CHB management program. It is hoped that reductions in the cost of antiviral drugs will lead to more equitable access to treatment and address the global burden of chronic hepatitis B.

COMMENTS

Background

Chronic hepatitis B (CHB) is a leading cause of cirrhosis and hepatocellular carcinoma (HCC), and in Australia, HCC incidence has been rising faster than that of any other cancer, mostly related to changing migration patterns over recent decades. A population-level disease control is predicated upon early disease detection, regular monitoring and timely institution of antiviral treatment for people with active disease, but antiviral treatment is unaffordable for the great majority of people with CHB who live in resource-limited countries. Therefore the cost and cost-effectiveness of CHB management programs is an important consideration for program funders and needs to be factored in by those tasked with guideline development. The authors previously carried out modeling work that showed that screening and treating migrants born in high prevalence countries is cost-effective, which corroborated the findings of research groups in the United States and the Netherlands. This paper examines the cost of treatment of hepatitis B applied to a hypothetical population of people with CHB diagnosis, treated according to CHB screening and treatment guidelines in common use in Asia (issued by the Asia-Pacific Society for the Study of Liver Disease), Australia (issued by the Gastroenterological Society of Australia), Europe (issued by the European Association for the Study of Liver Disease, EASL) and the United States (issued by the American Association for the Study of Liver Disease, AASLD, as well as by an expert United States advisory group).

Research frontiers

This work builds the body of evidence suggesting the cost-effectiveness of screening high-risk groups for hepatitis B, by examining the implications of implementing a public health program of screening and treatment using different treatment initiation parameters, as recommended by different guideline developers. The proportion of patients in this hypothetical cohort requiring antiviral therapy ranges from 8% (if the AASLD guidelines are used) to 25% (if EASL guidelines are applied). The most substantial component of program cost relates to antiviral treatment (representing up to 75% of program costs), while screening for CHB and cancer surveillance account for a small part of total costs. Treatment with generic lamivudine (instead of entecavir) leads to substantially lower total program costs, although this must be balanced against the greater suppression of viral replication and lack of drug resistance associated with entecavir treatment.

Innovations and breakthroughs

Here the authors propose and cost a scheme for the diagnosis and subsequent monitoring of patients from countries with high prevalence of hepatitis B, resident in Australia. In the B positive algorithm, antiviral treatment is offered for patients with alanine aminotransferase (ALT) elevation above 1.5 × normal, treading the "middle ground" between treating everyone with an elevated ALT level (which may have significant resource implications for countries with high disease prevalence, but limited resources) and treating only people with advanced disease (and running the risk of limiting the effectiveness of the program).

Applications

These findings are relevant for the design of interventions with the potential to make a significant impact on hepatitis B disease burden at a population level, in both well-resourced and low-resource settings. The authors hope that this type of work may be of interest to experts involved in CHB treatment guideline development, policy makers and clinicians working in areas with a large hepatitis B load.

Peer review

This is a study on the cost of treatment of hepatitis B, using Markov models. The authors propose a scheme for the diagnosis and subsequent monitoring of patients from countries with high prevalence of hepatitis B, resident in Australia. They also compared the relative cost with the proposed guidelines from other major hepatology associations. They found that the AASLD recommendations were more cost-effective. This type of study is important in view of the increasing cost of drug treatment of HBV infection but also of the increasing cost of diagnostic tests related to HCC surveillance.

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BRIEF ARTICLE

Anemia after gastrectomy for early gastric cancer: Long-term follow-up observational study

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Abstract

AIM: To identify the incidence and etiology of anemia after gastrectomy in patients with long-term follow-up after gastrectomy for early gastric cancer.

METHODS: The medical records of those patients with early gastric adenocarcinoma who underwent curative gastrectomy between January 2006 and October 2007 were reviewed. Patients with anemia in the preoperative workup, cancer recurrence, undergoing systemic chemotherapy, with other medical conditions that can cause anemia, or treated during follow up with red cell transfusions or supplements for anemia were excluded. Anemia was defined by World Health Organization criteria (Hb < 12 g/dL in women and < 13 g/dL in men). Iron deficiency was defined as serum ferritin < 20 μg/dL. Vitamin B_{12} deficiency was defined as serum vitamin B_{12} < 200 pg/mL. Iron deficiency anemia was defined as anemia with concomitant iron deficiency. Anemia from vitamin B_{12} deficiency was defined as megaloblastic anemia (mean cell volume > 100 fL) with vitamin B_{12} deficiency. The profile of anemia over 48 mo of follow-up was analyzed.

RESULTS: One hundred sixty-one patients with gastrectomy for early gastric cancer were analyzed. The incidence of anemia was 24.5% at 3 mo after surgery and increased up to 37.1% at 48 mo after surgery. The incidence of iron deficiency anemia increased during the follow up and became the major cause of anemia at 48 mo after surgery. Anemia of chronic disease and megaloblastic anemia were uncommon. The incidence of anemia in female patients was significantly higher than in male patients at 12 (40.0% *vs* 22.0%, P = 0.033), 24 (45.0% *vs* 25.0%, P = 0.023), 36 (55.0% *vs* 28.0%, P = 0.004), and 48 mo (52.0% *vs* 31.0%, P = 0.022) after surgery. Patients with total gastrectomy showed significantly higher incidence of anemia than patients with subtotal gastrectomy at 48 mo after surgery (60.7% *vs* 31.3%, P = 0.008). The incidence of iron deficiency was significantly higher in female patients than in male patients at 6 (35.4% *vs* 13.3%, P = 0.002), 12 (45.8% *vs* 16.8%, P < 0.001), 18 (52.1% *vs* 22.3%, P < 0.001), 24 (60.4% *vs* 20.9%, P < 0.001), 36 (62.5% *vs* 29.2%, P < 0.001), and 48 mo (66.7% *vs* 34.7%, P = 0.001) after surgery.

CONCLUSION: Anemia was frequent after gastrectomy for early gastric cancer, with iron deficiency being the major cause. Evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

Key words: Gastrectomy; Stomach neoplasms; Anemia; Iron deficiency; Vitamin B_{12} deficiency

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INTRODUCTION

Gastric cancer is the most common malignancy in Korea and the second most frequent cause of cancer-related death worldwide[1,2]. Curative resection has proven to be the only successful treatment modality for locally confined gastric cancer[3]. Anemia is a frequent complication after gastrectomy and deficiencies of iron, vitamin B_{12}, or folate, either alone or in combination, have been reported after gastric surgery[4-6]. However, these studies included relatively small numbers of patients, follow-up visits were not systematically scheduled, and the definition and biochemical markers of anemia were ambiguous or insufficient. There are limited reports concerning anemia in patients who undergo gastrectomy for early gastric cancer who have systematically scheduled serial follow-up visits, but do not receive supplements for anemia.

The incidence of gastric bypass surgery for morbid obesity is increasing, and anemia after bypass surgery has been reported[7-11]. Dietary life after gastric surgery in patients with gastric cancer may differ from that in patients with morbid obesity: in contrast to patients with obesity, patients having gastrectomy for gastric cancer do not restrict their dietary intake for weight reduction. The aim of this study was to identify the natural history of anemia after gastrectomy in a cohort of patients undergoing gastrectomy for early gastric cancer who were systematically followed up in the long term.

MATERIALS AND METHODS

Study population

This study was a retrospective chart review of the registry of all patients who had undergone gastrectomy for early gastric cancer at Seoul St Mary's Hospital, Seoul, Korea between January 2006 and October 2007. Patients with anemia in the preoperative workup, cancer recurrence, undergoing systemic chemotherapy, with other medical conditions that can cause anemia, or treated during follow up with red cell transfusions or supplements for anemia were excluded from the study.

Follow-up program

Patients with early gastric cancer were followed up at 3, 6, 9, 12, 18, 24, 48 and 60 mo after surgery. The follow-up program consisted of interim history taking, physical examination, imaging studies, endoscopic examination, hematology, and blood chemistry panels. Mean cell volume (MCV), serum iron, serum ferritin, total iron-binding capacity, serum vitamin B_{12}, and serum folate level were included in the follow-up program.

Definition of anemia and related conditions

Anemia was defined by World Health Organization criteria (Hb <12 g/dL in women and <13 g/dL in men)[9]. Iron deficiency was defined as serum ferritin < 20 μg/dL. Vitamin B_{12} deficiency was defined as serum vitamin B_{12} < 200 pg/mL. Iron deficiency anemia was defined as anemia with concomitant iron deficiency. Anemia of chronic illness was defined as anemia with serum ferritin > 20 μg/dL. Anemia from vitamin B_{12} deficiency was defined as megaloblastic anemia (MCV >100 fL) with vitamin B_{12} deficiency.

Statistical analysis

Continuous data are presented as the mean ± SD and categorical data are presented as proportions. The data were analyzed using the paired *t* test to assess the differences in continuous data during follow up and a χ^2 test or Fisher's exact test to assess the differences according to sex and the type of gastrectomy. Differences were considered significant if the *P* value was less than 0.05. All statistical analysis were performed using SAS software (SAS Institute, Cary, NC, United States).

RESULTS

Four hundred fifty-eight patients with early gastric adenocarcinoma underwent curative gastrectomy in our institution during the study period. Of these, 297 were excluded from the analysis (anemia in preoperative workup in 45 patients, systemic chemotherapy in 79, other medical conditions that can cause anemia in 13, follow-up loss in 86, insufficient biochemical profile for anemia in 73, red cell transfusion during the follow-up in one). Finally, 161 patients undergoing gastrectomy for early gastric cancer were analyzed. No patient in the study population received iron or vitamin B_{12} replacement therapy. Follow-up after surgery was possible at 3 mo in 159, 6 mo in 161, 12 mo in 161, 18 mo in 160, 24 mo in 158, 36 mo in 154, and 48 mo in 142.

The characteristics of the study population are shown in Table 1. The mean age was 56.2 ± 10.9 years (range, 23-78 years) and 113 patients were men (70.2%). Thirty-nine (24.2%) patients underwent Billroth I subtotal gastrectomy, 90 (55.9%) Billroth Ⅱ subtotal gastrectomy, and 32 (19.9%) total gastrectomy.

The incidence of anemia was 24.5% at 3 mo after surgery and increased to 37.1% at 48 mo after surgery. The incidence of iron deficiency anemia increased during the follow-up and became the major cause of anemia at 48 mo after surgery (Figure 1). Anemia of chronic disease and megaloblastic anemia were uncommon. Only one

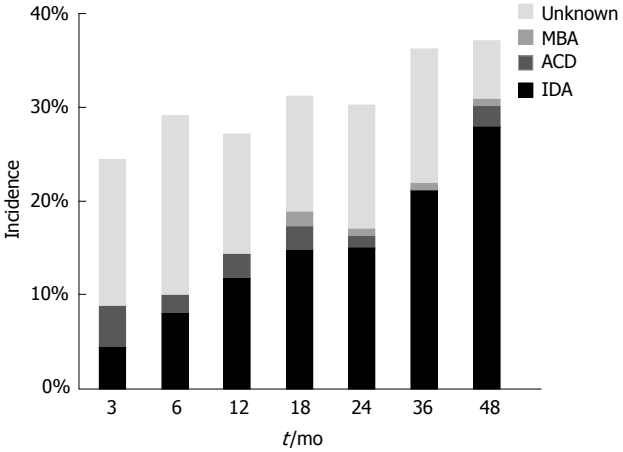

Figure 1 Incidence and distribution of anemia after gastrectomy. IDA: Iron deficiency anemia; ACD: Anemia of chronic disease; MBA: Megaloblastic anemia.

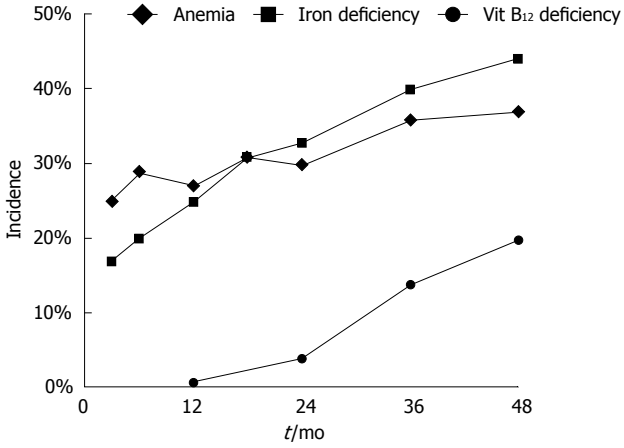

Figure 2 Incidence of anemia, iron deficiency state, and vitamin B12 deficiency state after gastrectomy.

Table 1 Patients characteristics (n = 161)

Variable	Value n (%)
Age (yr)[1]	56.2 ± 10.9
Gender	
Male	113 (70.2)
Female	48 (29.8)
Preoperative hemoglobin (g/dL)[1]	14.2 ± 1.1
Male	14.6 ± 0.9
Female	13.2 ± 0.8
Combined disease	
Hypertension	20 (12.4)
Diabetes mellitus	19 (11.8)
Pulmonary disease	2 (1.2)
Coronary artery disease	2 (1.2)
Chronic liver disease	2 (1.2)
Type of operation	
Billroth I subtotal gastrectomy	39 (24.2)
Billroth II subtotal gastrectomy	90 (55.9)
Total gastrectomy	32 (19.9)
Nodal Stage	
N0	143 (88.8)
N1	15 (9.4)
N2	2 (1.2)
N3	1 (0.6)

[1]Data are presented as mean ± SD.

patient had a picture consistent with megaloblastic anemia with vitamin B12 deficiency at 48 mo after surgery. The incidence of anemia, iron deficiency, and vitamin B12 deficiency increased during follow-up after surgery (Figure 2). The incidence during follow-up of iron deficiency without anemia was considerable. Most vitamin B12 deficiency occurred in patients who had total gastrectomy. At 48 mo after gastrectomy, the incidence of vitamin B12 deficiency was 3.2% in patients with Billroth I subtotal gastrectomy, 7.5% in Billroth II subtotal gastrectomy, and 76.9% in total gastrectomy. The incidence of vitamin B12 deficiency in patients with total gastrectomy was 0%, 16.1%, 50.0% and 76.9% at 12, 24, 36 and 48 mo after gastrectomy, respectively.

Serial follow-up hematology and blood chemistry data are shown in Table 2. Hemoglobin level, serum ferritin level, and vitamin B12 level decreased during the follow-up. The average serum albumin level was 3.3 ± 0.4 g/dL before surgery and recovered to 4.2 ± 0.3 g/dL at 3 mo after surgery.

The incidence of anemia in female patients was significantly higher than in male patients at 12 mo, 24 mo, 36 mo, and 48 mo after surgery (Figure 3A). Patients with total gastrectomy showed significantly higher incidence of anemia at 48 mo after surgery than patients with subtotal gastrectomy (60.7% *vs* 31.3%, P = 0.008) (Figure 3B). There was no significant difference in the incidence of anemia 48 mo after surgery between patients having Billroth I and Billroth II subtotal gastrectomy (18.8% *vs* 36.1%, P = 0.078). The incidence of iron deficiency was also significantly higher in female patients than in male patients at all times in the follow-up period except at 3 mo after surgery (Figure 3C). The incidence of iron deficiency was significantly higher in patients with total gastrectomy than those with in subtotal gastrectomy only at 36 mo after surgery (58.1% *vs* 35.0%, P = 0.024) (Figure 3D). There was no significant difference in the incidence of anemia and iron deficiency between patients with Billroth I and II subtotal gastrectomy at 48 mo after surgery (28.1% *vs* 45.8%, P = 0.095).

DISCUSSION

In this study, we identified the incidence and etiology of anemia after gastrectomy in a cohort of patients having gastrectomy for gastric cancer who were systematically followed up over the long term. We found that the incidence of anemia in the patients with gastrectomy increased during follow-up. Iron deficiency anemia became the major cause of anemia after gastrectomy, and megaloblastic anemia with vitamin B12 deficiency was rare.

In this study, anemia was relatively frequent after gastrectomy for early gastric cancer. Anemia was detected shortly after surgery and increased during the follow-up period. The incidence of anemia was 24.5% at 3 mo after surgery and increased to 37.1% at 48 mo after surgery. Anemia has been reported to occur in up to 60% of

Table 2 Biochemical marker after gastrectomy

	Before surgery	3 mo	6 mo	12 mo	18 mo	24 mo	36 mo	48 mo
Hemoglobin (g/dL)	14.2 ± 1.1	13.4 ± 1.2	13.3 ± 1.2[a]	13.4 ± 1.3	13.2 ± 1.3[a]	13.2 ± 1.4[a]	13.1 ± 1.5[a]	13.1 ± 1.6[a]
Serum ferritin (μg/L)		70.8 ± 59.1	63.9 ± 52.4[a]	55.2 ± 43.4[a]	49.3 ± 51.3[a]	43.3 ± 46.0[a]	34.7 ± 32.2[a]	33.7 ± 30.7[a]
Serum vitamin B_{12} (pg/mL)				1167.7 ± 597.6		1113.9 ± 629.4	773.7 ± 999.9[c]	560.3 ± 472.9[c]
Serum albumin (g/dL)	3.3 ± 0.4	4.2 ± 0.3	4.2 ± 0.4	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3

[a]$P < 0.05$ *vs* 3 mo; [c]$P < 0.05$ *vs* 12 mo.

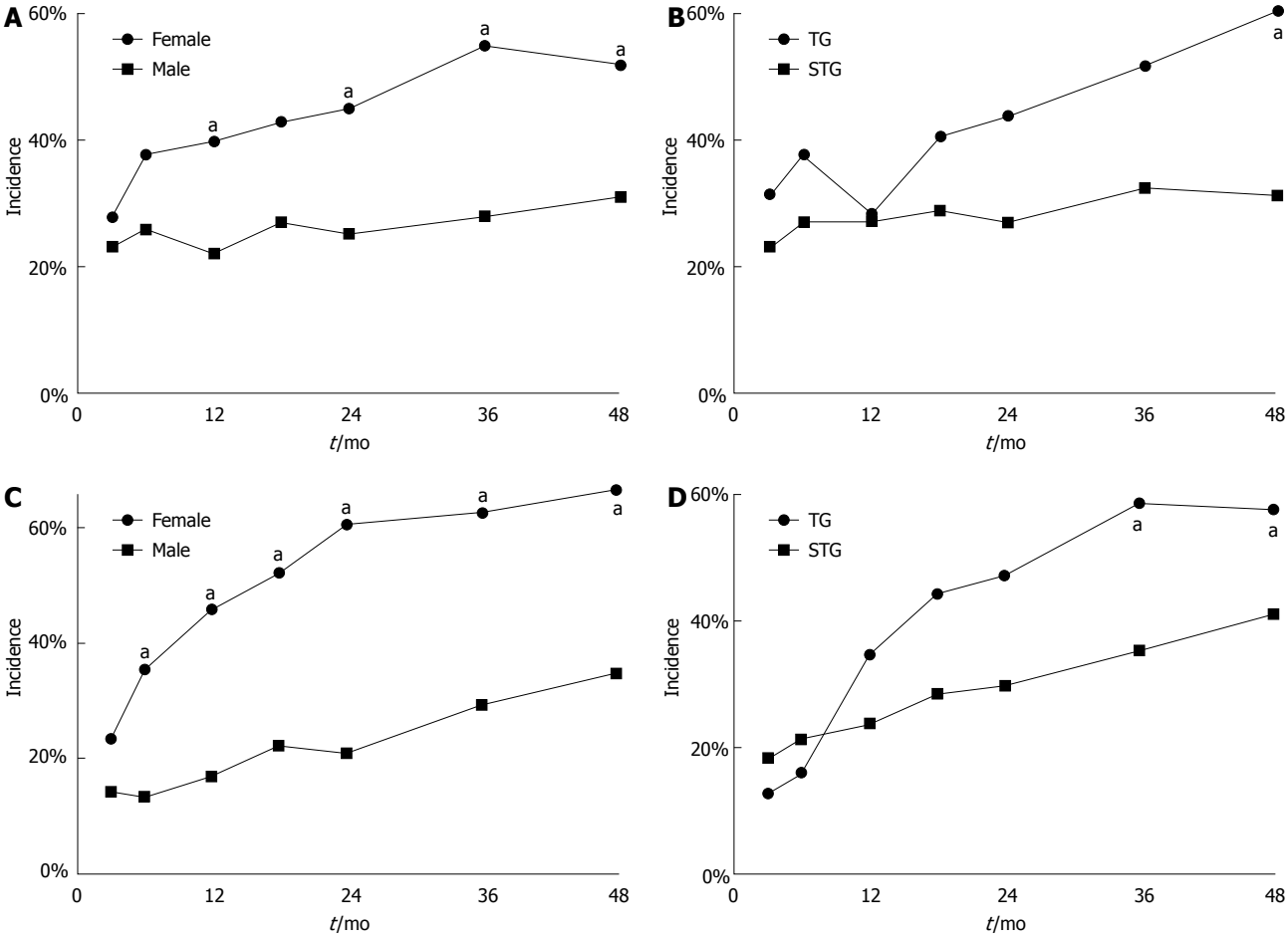

Figure 3 Incidence of anemia and deficiency state after gastrectomy. A: Significantly higher incidence of anemia in female patients than in male patients at 12, 24, 36 and 48 mo after surgery, [a]$P < 0.05$ *vs* female group; B: Significantly higher incidence of anemia in patients with total gastrectomy (TG) than in patients with subtotal gastrectomy (STG) at 48 mo after surgery, [a]$P < 0.05$ *vs* STG group; C: Significantly higher incidence of iron deficiency state in female patients than in male patients at all times in the follow-up period except at 3 mo after surgery, [a]$P < 0.05$ *vs* male group; D: Significantly higher incidence of iron deficiency in patients with TG than in patients with STG at 36 mo after surgery, [a]$P < 0.05$ *vs* STG group.

patients after partial gastrectomy[12,13]. The incidence of anemia after bypass surgery has been reported as from 5% to 64%[7-9,14-17]. A recent study of postsurgical anemia after gastrectomy reported that the incidence of anemia was 37.9%, 33.5% and 34.7% at 3, 6 and 12 mo after surgery, respectively[18].

Our study showed that iron deficiency anemia increased during follow-up and was the major cause of anemia at 48 mo after surgery. Serial follow-up showed that serum ferritin gradually decreased from 70.8 μg/L to 30.1 μg/L during follow up and the incidence of iron deficiency gradually increased to 44% at 48 mo after gastrectomy. Our results are similar to those of a previous study of patients after Roux-en-Y gastric bypass[19]. Iron deficiency anemia was reported to be very common in post-gastrectomy patients at twenty five to thirty years after surgery[20]. Iron deficiency after gastrectomy or bypass results from malabsorption of iron because of a surgically altered gastrointestinal tract. Inadequate oral intake or occult blood loss may also contribute to this condition. Iron is absorbed in the duodenum and proximal jejunum and its absorption is enhanced by gastric acid secretion. A lack of acidity results in impaired conversion of ingested ferric iron to absorbable ferrous iron[21]. Duodenal bypass also contributes to iron deficiency in patients with gastrectomy or bypass surgery. Atrophic gastritis

and *Helicobacter pylori* infection were reported to play an important role in iron deficiency after gastrectomy for cancer of the stomach[22]. In our study, the patients with Billroth I subtotal gastrectomy showed the lowest incidence of iron deficiency compared with all groups of patients with duodenal bypass (total gastrectomy and Billroth Ⅱ subtotal gastrectomy) at 48 mo after surgery (28.1% *vs* 48.6%, $P = 0.045$). Our study showed a higher incidence of anemia and iron deficiency in patients with total gastrectomy than in those with subtotal gastrectomy. This difference may originate from the more depleted nutritional status of patients with total gastrectomy[15,23,24]. In contrast to the subjects of other studies of severe obesity, our study population was patients with early gastric cancer and did not need weight reduction. They were recommended an adequate oral intake and serum albumin level was maintained at an adequate level. Therefore, our results show an etiology and natural course of postgastrectomy anemia that is close to reality.

The incidence of vitamin B_{12} deficiency gradually increased to 20% of patients in our study at 48 mo after gastrectomy. The majority of the patients with vitamin B_{12} deficiency at 48 mo were those with total gastrectomy. Vitamin B_{12} deficiency causes megaloblastic anemia and neurological symptoms, and is known to develop within 5 or 6 years after total gastrectomy, a delay that reflects the time needed to exhaust cobalamin stores after cobalamin absorption ceases[25]. Our study showed that 16.1%, 50.0% and 76.9% of the patients with total gastrectomy presented with vitamin B_{12} deficiency at 24, 36 and 48 mo after gastrectomy, respectively. Although the incidence of vitamin B_{12} deficiency at 48 mo after gastrectomy was high in our study, only one patient with vitamin B_{12} deficiency presented with megaloblastic anemia. Because 70% of patients with vitamin B_{12} deficiency at 48 mo after gastrectomy showed anemia and the majority of these were iron-deficient, the generation of macrocytosis by concomitant iron deficiency should be considered[26]. Vitamin B_{12} deficiency can develop within 2 years after total gastrectomy and vitamin B_{12} replacement may be considered for postgastrectomy patients with persistent anemia despite iron replacement or with neurological symptoms combined with vitamin B_{12} deficiency.

Our study has some limitations. This study was a single-center retrospective study. The symptoms of anemia and other nutritional parameters such as body weight or body mass were not investigated. However, a strength of our study is the tightly controlled long-term follow-up of the patient cohort after gastrectomy for early gastric cancer, with systematically and prospectively scheduled serial follow-up visits and without any supplement for anemia. Our results enabled us to understand the development of post-gastrectomy anemia.

In conclusion, anemia was frequent after gastrectomy for early gastric cancer and iron deficiency was the major cause. Evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

COMMENTS

Background

Curative resection has proven to be the only successful treatment modality for locally confined gastric cancer. Anemia is a frequent complication after gastrectomy. The present study identified the natural history of anemia after gastrectomy in a cohort of patients undergoing gastrectomy for early gastric cancer who were systematically followed up in the long term.

Research frontiers

Anemia was frequent after gastrectomy for early gastric cancer, with iron deficiency being the major cause.

Innovations and breakthroughs

Anemia is a common complication of gastrectomy, particularly in female patients and it worsens as follow-up lengthens in a cohort with long-term follow-up for 48 mo. The incidence of iron deficiency gradually increased during follow up and became the major cause of anemia. Megaloblastic anemia is uncommon although the incidence of vitamin B_{12} deficiency gradually increased after gastrectomy, particularly particularly after total gastrectomy.

Applications

The results of this study suggest that evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

Peer review

This study examined anemia in a large cohort of patients undergoing gastrectomy for early gastric cancer. The results of this study can serve as a benchmark in the follow up of patients undergoing partial or total gastrectomy for gastric carcinoma.

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BRIEF ARTICLE

Association of chronic viral hepatitis B with insulin resistance

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Abstract

AIM: To investigate the relationship between chronic viral hepatitis B (CVHB) and insulin resistance (IR) in Korean adults.

METHODS: A total of 7880 adults (3851 men, 4029 women) who underwent a comprehensive medical examination were enrolled in this study. Subjects diagnosed with either diabetes mellitus, or any other disorder that could influence their insulin sensitivity, were rejected. Anthropometry, metabolic risk factors, hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B core antibody, fasting plasma glucose and insulin were measured for all subjects. Homeostasis model assessment (HOMA), quantitative insulin check index (QUICKI), and Mf_{fm} index were used for determining insulin sensitivity. Each participant was categorized into a negative, recovery, or CVHB group. To compare variables between groups, a t-test and/or one-way analysis of variance were used. Partial correlation coefficients were computed to present the association between insulin resistance and other variables. Multiple logistic regression analysis was used to assess the independent association between CVHB and IR.

RESULTS: The mean age of men and women were 48.9 and 48.6 years, respectively. Subjects in the CVHB group had significantly higher waist circumference [(86.0 ± 7.7 cm *vs* 87.3 ± 7.8 cm, $P = 0.004$ in men), (78.3 ± 8.6 cm *vs* 80.5 ± 8.5 cm, $P < 0.001$ in women)], cystatin C [(0.96 ± 0.15 mg/dL *vs* 1.02 ± 0.22 mg/dL, $P < 0.001$ in men), (0.84 ± 0.15 mg/dL *vs* 0.90 ± 0.16 mg/dL, $P < 0.001$ in women)], fasting insulin [(5.47 ± 3.38 μU/mL *vs* 6.12 ± 4.62 μU/mL, P < 0.001 in men), (4.57 ± 2.82 μU/mL *vs* 5.06 ± 3.10 μU/mL, $P < 0.001$ in women)] and HOMA index [(1.24 ± 0.86 *vs* 1.43 ± 1.24, $P < 0.001$ in men), (1.02 ± 0.76 *vs* 1.13 ± 0.87, $P = 0.033$ in women)] compared to control group. The HOMA index revealed a positive correlation with body mass index (BMI) ($r = 0.378$, $P < 0.001$), waist circumference ($r = 0.356$, $P < 0.001$), percent body fat ($r = 0.296$, $P < 0.001$), systolic blood pressure ($r = 0.202$, $P < 0.001$), total cholesterol ($r = 0.134$, $P < 0.001$), triglycerides ($r = 0.292$, $P < 0.001$),

cystatin C (r = 0.069, P < 0.001) and uric acid (r = 0.142, P < 0.001). The QUICKI index revealed a negative correlation with BMI (r = -0.254, P < 0.001), waist circumference (r = 0-0.243, P < 0.001), percent body fat (r = -0.217, P < 0.001), systolic blood pressure (r = -0.132, P < 0.001), total cholesterol (r = -0.106, P < 0.001), triglycerides (r = -0.205, P < 0.001), cystatin C (r = -0.044, P < 0.001) and uric acid (r = -0.096, P < 0.001). For subjects identified with IR, the odds ratio of an accompanying diagnosis of chronic hepatitis B was 1.534 (95% CI: 1.158-2.031, HOMA index criteria) or 1.566 (95% CI: 1.124-2.182, QUICKI criteria) after adjustment for age, gender, BMI, and amount of alcohol consumption.

CONCLUSION: Our study demonstrates that CVHB is associated with IR. CVHB may need to be monitored for occurrence of IR and diabetes mellitus.

Key words: Hepatitis B; Insulin resistance; Diabetes mellitus, type 2; Metabolic syndrome

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INTRODUCTION

Insulin resistance (IR) is the principal indication for development of metabolic syndrome and type 2 diabetes[1,2]. IR appears as a consequence of the inability of insulin to induce the appropriate effect on glucose metabolism. Inordinately large amounts of insulin are required to achieve a normal response in a state of IR. A hyperinsulinemic state causes several clinical abnormalities to appear in the blood vessels, kidneys, and liver, and these represent the major features of metabolic syndrome[3].

Metabolic syndrome generally refers to a combination of metabolic diseases such as abdominal obesity, high blood pressure, dyslipidemia and elevated blood glucose, that appear together in an individual patient[1]. Because metabolic syndrome is recognized as a serious risk factor for cardiovascular disease, prevention and comprehensive management are important in treating this condition[4].

Hepatitis B is one of the most common health problems and it is estimated that, of the world's total population, one third (over 2 billion people) have been infected with hepatitis B virus (HBV)[5]. Approximately two thirds of chronic viral hepatitis B (CVHB) patients live in Asia and the Pacific Islands. HBV infection may cause acute and/or chronic hepatitis and premature death from liver cirrhosis, liver failure or hepatocellular carcinoma[6]. Moreover, CVHB infection is related to other diseases such as polyarteritis nodosa (PAN)[7], glomerulonephritis (GN)[8], serum sickness-like syndrome (prodrome)[9], arthritis[10], and acrodermatitis[11].

Recently, an experimental study suggested that hepatitis B X protein (HBx) impairs the hepatic insulin signaling pathway, and that HBV infection is associated with IR[12]. A previous clinical studies also suggest that hyperinsulinemia occurs in CVHB and hepatitis C[13], and this association has been elucidated in hepatitis C virus (HCV) infection[14,15]. HCV may disturb the insulin signaling pathway by activation of the tumor necrosis factor (TNF) system[16]. IR has been proposed as an important risk factor in patients with chronic hepatitis C, mainly due to its relationship to steatosis development[17] and fibrosis progression[18], and non-response to peginterferon plus ribavirin[19]. However, the effect of HBV infection on human insulin sensitivity remains unclear. In this study, we tested the hypothesis that HBV infection may associate with IR and metabolic syndrome, by comparing incidence of IR and prevalence of metabolic syndrome between HBV-infected study participants and a healthy control group.

MATERIALS AND METHODS

Study subjects

This consecutive study conducted at the Center for Health Promotion, Pusan National University Hospital in Busan, South Korea. Data for this study were obtained from 7880 Koreans (3851 men, 4029 women) who underwent a comprehensive medical examination between January 2007 and September 2008. The study participants were eligible if they met all of the following criteria: age ⩾ 18 years, no history of diabetes and hypertension requiring medication, negative for anti-hepatitis C antibody, serum aspartate aminotransferase or alanine aminotransferase (ALT) < 80 IU/L, serum gamma-glutamyl transferase (GGT) < 80 mg/dL, serum creatinine < 1.5 mg/dL, prostate specific antigen < 5.0 ng/mL, α-fetoprotein < 10.0 IU/mL, carcinoembryonic antigen < 5.0 ng/mL (for smokers) or < 2.5 ng/mL (for non-smokers), WBC count < 10 000/μL, and high sensitivity C-reactive protein (hs-CRP) < 1 mg/dL.

Measurements

The health checkup which provided our study data included a physical examination, anthropometric measurements and blood tests. Height and weight were measured to the nearest 0.1 cm and 0.1 kg using standard protocol with subjects wearing a light gown and without shoes. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured at the narrowest point between the lower border of the rib cage and the iliac crest, at the end of a normal expiration of breath and to the nearest 0.1 cm.

Percentage of body fat and total fat mass were measured by bioelectric impedance analysis (Inbody 3.0, Biospace Co, Ltd, Korea). Blood pressure was measured using the right arm of subjects assuming a sitting position, and after they had rested for at least 10 min. By use of an automated blood pressure measurement device (BP-203RV Ⅱ, Colin Corp, Aichi, Japan). Medication history, alcohol intake and smoking habits were obtained by patient interview. The questions relating to alcohol intake included descriptions of the type of alcohol beverage consumed, the weekly frequency of alcohol consumption, and the amount consumed daily. Smoking status was classified as either non-smoker or smoker (former or current). Blood samples were obtained from an antecubital vein after 12 h fasting, typically between 8 and 9 AM. The blood samples were subsequently analyzed at a certified laboratory at Pusan National University Hospital. Lipid profiles, uric acid, and GGT concentrations were measured using an autoanalyzer with the enzymatic colorimetric method (Hitachi7600, Hitachi Ltd, Japan). Cystatin C was measured by turbidimetric immunoassay (HBI Co, Ltd, Korea) using the Modular Analytics E170 (Roche Diagnostics, Switzerland). Hs-CRP was measured using a Behring Nephelometer (DadeBehring, Germany). Fasting plasma glucose was measured by the glucose oxidase method using a Synchron LX 20 (Beckman Coulter, Fullerton, CA, United States). Fasting insulin was measured using a radioimmunoassay (Diagnostic Product Corporation, Los Angeles, CA, United States) with antibody-coated tubes. The mean intra- and interassay coefficient of variation (CV) values were 4.2% and 6.3%, respectively.

Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody, and hepatitis B core antibody were measured by enzyme-linked immunosorbent assay (Bio Focus Co, Ltd, Korea). Hepatitis B viral status was classified into three groups (negative/recovery/CVHB), according to serologic patterns. Insulin sensitivity was estimated using homeostasis model assessment (HOMA)-IR [fasting insulin (μIU/mL) × fasting glucose (mg/dL)/405][20], quantitative insulin check index (QUICKI) {1/[log glucose (mg/dL) + log insulin (μU/mL)]}[21,22] and Mf_{fm} index ($\exp^{2.63 - [0.28 \times \ln(\text{insulin})] - [0.31 \times \ln(\text{triglycerides})]}$)[23].

Definition of metabolic syndrome

The prevalence of metabolic syndrome reported in this study was estimated using definitions proposed in 2005 by the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI)[24] and the International Diabetes Federation criteria[25] for the diagnosis of metabolic syndrome. We defined central obesity as a waist circumference ⩾ 90 cm in males and ⩾ 85 cm in females, according to geography-specific cut points for waist circumference[26].

Ethical approval

All participants gave informed consent, and this study was approved by the Institutional Review Board at Pusan National University Hospital and is in accordance with the Declaration of Helsinki (E2010-055).

Table 1 Baseline characteristics of study subjects

Variables	Men (n = 3851)	Women (n = 4029)
Age (yr)	48.9 ± 10.7	48.6 ± 10.4
BMI (kg/m^2)	24.5 ± 2.8	23.5 ± 2.9
Abdominal circumference (cm)	86.5 ± 7.4	79.2 ± 8.4
Percentage body fat (%)	22.7 ± 5.1	30.1 ± 5.7
Systolic BP (mmHg)	126.3 ± 15.3	122.3 ± 15.4
AST (IU/L)	23.8 ± 8.6	20.9 ± 10.5
ALT (IU/L)	26.8 ± 16.6	19.0 ± 11.5
GGT (IU/L)	38.0 ± 20.1	20.0 ± 12.6
Fasting plasma glucose (mg/dL)	91.6 ± 14.7	88.3 ± 14.1
Fasting insulin (μU/mL)	5.48 ± 3.49	4.64 ± 2.78
Total cholesterol (mg/dL)	196.3 ± 33.3	195.8 ± 35.2
Triglycerides (mg/dL)	138.0 ± 80.9	103.8 ± 63.6
HDL-cholesterol (mg/dL)	50.8 ± 12.5	59.8 ± 14.1
LDL-cholesterol (mg/dL)	124.8 ± 29.5	121.0 ± 31.8
high-sensitivity CRP (mg/dL)	0.15 ± 0.47	0.11 ± 0.37
TSH (μU/mL)	1.79 ± 1.79	2.29 ± 2.15
Uric acid (mg/dL)	5.88 ± 1.25	4.20 ± 0.91

Differences between men and women were statistically significant except for age and total cholesterol (P < 0.05 by t test). BMI: Body mass index; BP: Blood pressure; AST: Aspartate transferase; ALT: Alanine transferase; GGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; TSH: Thyroid stimulating hormone.

Statistical analysis

To compare variables between groups, a t test and/or one-way analysis of variance followed by a Scheffé post hoc test or Kruskal-Wallis test were used as appropriate. Pearson partial correlation coefficients were computed to present the association between fasting plasma glucose concentration and other variables after adjustments for age, gender and alcohol consumption. Using multiple logistic regression analysis and adjusting for age, gender and alcohol intake, we estimated the existence of any independent association between IR and HBV status. Statistical analysis was performed using SPSS 12.0 for Windows. A P value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

RESULTS

Baseline characteristics of study subjects

The subjects were classified as men (n = 3851) and women (n = 4029), and their baseline clinical characteristics were compared (Table 1). The mean age of men and women were 48.9 years and 48.6 years, respectively. Age and total cholesterol level were not statistically different between men and women (P > 0.05). Men had significantly higher results for BMI, abdominal circumference, systolic blood pressure, aspartate aminotransferase, ALT, GGT, fasting plasma glucose, insulin, triglycerides, low-density lipoprotein-cholesterol, hs-CRP, and uric acid (P < 0.001).

Metabolic characteristics according to hepatitis groups

The metabolic data of study participants are shown in Table 2. In both men and women, subjects in the CVHB group were significantly older with larger waist circum-

Table 2 Means and frequencies of metabolic risk factors associated with hepatitis B virus status in men and women (mean ± SD)

Variables	Negative (n = 1292)	Recovery from hepatitis B (n = 1956)	Chronic hepatitis B (n = 603)	P value[1]
Hepatitis B virus status in men				
Age (yr)	44.4 ± 11.7	51.4 ± 9.4	50.8 ± 10.7	0.000
Body mass index (kg/m^2)	24.5 ± 2.9	24.5 ± 2.7	24.7 ± 2.9	0.493
Waist circumference (cm)	86.0 ± 7.7	86.5 ± 7.0	87.3 ± 7.8	0.004
Percentage body fat (%)	22.5 ± 5.7	22.8 ± 4.6	23.0 ± 5.0	0.094
Systolic blood pressure (mmHg)	126.4 ± 15.5	126.3 ± 15.0	126.1 ± 15.6	0.922
Total cholesterol (mg/dL)	196.0 ± 33.2	196.8 ± 33.1	195.5 ± 34.5	0.680
Triglyceride (mg/dL)	142.8 ± 84.5	135.6 ± 79.2	135.2 ± 78.2	0.029
HDL-cholesterol (mg/dL)	51.0 ± 12.7	50.9 ± 12.8	50.4 ± 11.4	0.602
Cystatin C (mg/L)	0.96 ± 0.15	0.98 ± 0.16	1.02 ± 0.22	0.000
Fasting glucose (mg/dL)	90.7 ± 15.3	92.1 ± 14.4	91.9 ± 14.4	0.039
Fasting insulin (μU/mL)	5.47 ± 3.38	5.29 ± 3.10	6.12 ± 4.62	0.000
HOMA index	1.24 ± 0.86	1.22 ± 0.80	1.43 ± 1.24	0.000
QUICKI index	0.386 ± 0.065	0.386 ± 0.044	0.385 ± 0.134	0.922
Mf$_{fm}$ index	8.41 ± 2.43	8.58 ± 2.31	8.39 ± 2.63	0.072
Hepatitis B virus status in women				
Age (yr)	45.9 ± 10.7	50.9 ± 9.3	51.6 ± 10.3	0.000
Body mass index (kg/m^2)	23.2 ± 3.0	23.7 ± 2.7	24.0 ± 2.9	0.000
Waist circumference (cm)	78.3 ± 8.6	79.8 ± 8.0	80.5 ± 8.5	0.000
Percentage body fat (%)	29.6 ± 6.8	30.4 ± 4.6	31.0 ± 4.9	0.000
Systolic blood pressure (mmHg)	121.3 ± 15.4	123.0 ± 15.5	124.5 ± 14.3	0.000
Total cholesterol (mg/dL)	193.3 ± 35.4	197.9 ± 35.0	198.1 ± 34.3	0.000
Triglyceride (mg/dL)	102.0 ± 63.5	106.4 ± 66.2	100.3 ± 47.5	0.061
LDL-cholesterol (mg/dL)	118.3 ± 31.6	123.2 ± 31.8	123.7 ± 31.7	0.000
Cystatin C (mg/L)	0.84 ± 0.15	0.87 ± 0.15	0.90 ± 0.16	0.000
Fasting glucose (mg/dL)	88.0 ± 14.6	88.7 ± 13.7	88.3 ± 12.9	0.357
Fasting insulin (μU/mL)	4.57 ± 2.82	4.62 ± 2.67	5.06 ± 3.10	0.010
HOMA index	1.02 ± 0.76	1.03 ± 0.67	1.13 ± 0.87	0.033
QUICKI index	0.401 ± 0.056	0.398 ± 0.051	0.392 ± 0.045	0.013
Mffm index	9.77 ± 2.62	9.58 ± 2.53	9.36 ± 2.25	0.006

[1]Analysis of variance except triglyceride (Kruskal-Wallis test). HDL: High-density lipoprotein; LDL: Low-density lipoprotein; HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index.

Table 3 Partial correlation coefficients of insulin sensitivity index to metabolic parameters after adjusting for age, gender, and alcohol consumption

	BMI	WC	BFP	SBP	TC	TG	HDL-C	Cys-C	Uric acid
FPG	0.150	0.151	0.114	0.138	0.087	0.141	-0.102	-0.108	-0.010
Insulin	0.395	0.368	0.313	0.196	0.132	0.293	-0.206	0.105	0.168
HOMA index	0.378	0.356	0.296	0.202	0.134	0.292	-0.202	0.069	0.142
QUICKI index	-0.254	-0.243	-0.217	-0.132	-0.106	-0.205	0.164	-0.044	-0.096
Mf$_{fm}$ index	-0.374	-0.366	-0.318	-0.199	-0.258	-0.628	0.386	-0.116	-0.203

BMI: Body mass index; WC: Waist circumference; BFP: Percent of body fat; SBP: Systolic blood pressure; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; Cys-C: Cystatin-C; FPG: Fasting plasma glucose; HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index. All correlation coefficients are statistically significant (P < 0.001).

ferences, and had higher percentages of body fat, cystatin C, fasting insulin, and HOMA index compared to other groups. There were significant differences between men in the CVHB group and negative group in terms of fasting plasma glucose, but no significant differences were observed for women. QUICKI (P < 0.05) and Mf$_{fm}$ index (P < 0.01) results were significantly lower for women in the CVHB group.

Correlation of insulin sensitivity index with metabolic factors

The HOMA index revealed a positive correlation with BMI (r = 0.378, P < 0.001), waist circumference (r = 0.356, P < 0.001), percent body fat (r = 0.296, P < 0.001), systolic blood pressure (r = 0.202, P < 0.001), total cholesterol (r = 0.134, P < 0.001), triglycerides (r = 0.292, P < 0.001), cystatin C (r = 0.069, P < 0.001) and uric acid (r = 0.142, P < 0.001) (Table 3). QUICKI and Mf$_{fm}$ index produced a negative correlation with BMI, waist circumference, percent body fat, systolic blood pressure, total cholesterol, triglycerides, cystatin C, and uric acid (P < 0.001).

Association of insulin resistance and chronic hepatitis B

For the presence of IR, adjusted odds ratios for the CVHB group was 1.534 (95%CI: 1.158-2.031, HOMA index criteria) or 1.566 (95% CI: 1.124-2.182 in QUICKI

Table 4 Logistic regression analysis with insulin resistance as a dependent variable

Variables	Insulin resistance					
	HOMA index criteria			QUICKI criteria		
	β	SE	Odds ratio (95% CI)	β	SE	Odds ratio (95% CI)
Age (yr)	0.009	0.005	1.009 (0.999-1.019)	0.016	0.006	1.016 (1.005-1.028)
Gender						
Men	0.487	0.109	1.627 (1.314-2.015)	0.452	0.131	1.571 (1.215-2.031)
Women			1.000			1.000
Body mass index (kg/m^2)	0.316	0.017	1.372 (1.328-1.417)	0.347	0.020	1.746 (1.663-1.833)
Alcohol consumption (kcal)	0.000	0.000	1.000 (0.999-1.000)	0.000	0.000	1.000 (0.999-1.000)
Hepatitis B virus status						
Negative			1.000			1.000
Recovery from hepatitis B	-0.027	0.112	0.974 (0.782-1.212)	-0.075	0.135	0.928 (0.712-1.210)
Chronic hepatitis B	0.428	0.143	1.534 (1.158-2.031)	0.449	0.169	1.566 (1.124-2.182)

HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index.

criteria) (Table 4). Adjusting factors included age, gender, BMI and amount of alcoholic consumption.

DISCUSSION

In this study, CVHB was observed to be associated with IR in subjects free of prior diabetes mellitus. CVHB independently predicted a clinically significant increase in the odds ratio for the development of IR. These results indicate that patients with CVHB may need to be carefully monitored for occurrence of IR and diabetes mellitus. As the present study reports basic data on the association between CVHB and IR in a large community population, these findings support previous proposals that CVHB infection is related to IR.

HBV is the prototype member of a steadily growing family of viruses called hepadnaviruses[27]. It is a partially double stranded virus that uses reverse transcriptase in its replication cycle. CVHB infection is a common health issue in Asia and the Pacific Islands. In Korea, approximately 3.7% of total population are affected in chronic hepatitis B[28]. Most were infected directly from their mother during birth or through contact between children. CVHB infection may increase the occurrence of hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma[29]. In addition, CVHB infection is related to diseases such as PAN, GN, and arthritis[7-9]. Moreover, there is experimental evidence that CVHB infection increases the appearance of both IR and associated diabetes mellitus. A recent animal study suggested that HBx impairs the insulin signaling pathway[12]. These findings provide the basis of a hypothesis for mechanism and are consistent with our study results.

IR is assumed to be caused by an inadequate glucose metabolism capacity which leads to more insulin to be secreted to achieve the same biologic response[30]. Hyperinsulinemia may induce a large variety of abnormalities in blood vessels, kidneys, and muscles, and is the major pathogenesis associated with metabolic syndrome. Diabetes mellitus and metabolic syndrome are also independent risk factors for atherosclerotic disease[31]. Thus, early screening of high risk groups is very important to successful health promotion. The gold standard parameter for determining insulin sensitivity is the hyperinsulinemic euglycemic clamp technique. The HOMA model[20], QUICKI[21], and Mf_{fm} index[23] used in this study show good correlation with the clamp technique and are easily utilized in primary practice.

Previous studies have proposed association of HBV infection and IR. One previous, retrospective study proposed that maternal HBsAg carrier status was a risk factor for development of gestational diabetes[32]. Sangiorgio *et al*[33] also reported increased frequency of HCV and HBV infection in type 2 diabetic patients. One study reported concordant results using the HOMA model that concluded that hyperinsulinemia occurs in chronic viral hepatitis B and hepatitis C[13]. However, another previous study reported that HBV carriers were not associated with IR[34]. But that study had limitations due to small number of study subjects and high prevalence of fatty liver disease in the subjects.

The mechanism IR plays in CVHB infection remains unknown. There are four proteins that originate from the HBV genome including polymerase, a surface protein, a core protein, and the HBx protein. Among these proteins, HBx may be most closely associated with hepatic steatosis, inflammation, and HBV-related disease[34]. Previous reports proposed that hepatic steatosis and systemic inflammation are associated with IR[35]. HBx protein can induce hepatic steatosis and inflammation, thus CVHB infection is possibly associated with an impaired insulin signaling pathway[36]. A recent report concluded that chronic inflammation had effect on IR[37]. HBV may induce activation of proinflammatory cytokines TNF-α, interleukin (IL)-6, and IL-1β associated with fat accumulation[29]. Hepatic steatosis has already been demonstrated to be related to IR, and this association has been clearly identified in HCV infection. HCV proteins present due to infection may also disturb the insulin signaling pathway. IR with chronic hepatitis C has also been related to steatosis development and fibrosis progression[13-15].

The strength of this study was inclusion of healthy volunteers, which provided greater validity compared to hospital-based or institutional based populations. Other strengths included the large sample size, characterization of multiple confounders that influence IR, and the availability of 3 IR index or insulin sensitivity markers which are validated and widely used, and were also used for determining the degree of IR in previous studies.

This study had several limitations. Because of the cross-sectional nature of this study, it was difficult to prove a causal relationship between HBV infection and IR. Also, our results may not be able to be generalized to other ethnic groups because the present study was conducted exclusively using ethnic Koreans. A weakness in terms of clinical data is that daily variability of insulin within individuals is high, and a single, daily sample may not accurately characterize the actual level. Moreover, there are numerous other factors that influence IR such as condition of skeletal muscles, engagement in physical activity, and severity of liver injuries. Future studies that overcome these limitations are needed to confirm our findings.

In conclusion, CVHB may be associated with IR as identified by HOMA-index, QUICKI, and Mf_{fm} index results. These findings should be explored further.

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COMMENTS

Background

Insulin resistance (IR) is the principal indication for development of metabolic syndrome and type 2 diabetes. Chronic viral hepatitis B (CVHB) is one of the most common health problems and previous clinical studies also suggest that hyperinsulinemia occurs in CVHB. However, the effect of hepatitis B virus (HBV) infection on human insulin sensitivity remains controversial. The authors therefore investigated the hypothesis that HBV infection may associate with IR and metabolic syndrome, by comparing incidence of IR between HBV-infected subjects and healthy group.

Research frontiers

There are four proteins that originate from the HBV genome including polymerase, a surface protein, a core protein, and the hepatitis B X protein (HBx) protein. Among these proteins, HBx may be most closely associated with hepatic steatosis, inflammation, and HBV related disease. Moreover, recent experimental study suggested that HBx impairs the hepatic insulin signaling pathway.

Innovations and breakthroughs

CVHB was observed to be associated with IR in subjects free of prior diabetes mellitus. CVHB independently predicted a clinically significant increase in the odds ratio for the development of IR. These results indicate that patients with CVHB may need to be carefully monitored for occurrence of IR and diabetes mellitus.

Applications

CVHB infection is a common health issue in Asia and the Pacific Islands. CVHB may need to be monitored for occurrence of insulin resistance and diabetes mellitus.

Terminology

Homeostasis model assessment index is calculated by equation of [fasting insulin (μIU/mL) × fasting glucose (mg/dL)/405], quantitative insulin check index is calculated by equation of {1/[log glucose (mg/dL) + log insulin (μU/mL)]}, Mf_{fm} index is obtained by equation of $(\exp^{2.63 - [0.28 \times \ln(\text{insulin})] - [0.31 \times \ln(\text{triglycerides})]})$.

Peer review

This study investigated the association of insulin resistance and chronic viral hepatitis B. The authors evaluated a numerous population of HBV infected patients and compared their metabolic features with control group. This manuscript reinforce further evaluations on a real clinical impact of this association.

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BRIEF ARTICLE

Treatment of functional dyspepsia with sertraline: A double-blind randomized placebo-controlled pilot study

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Abstract

AIM: To evaluate sertraline, a selective serotonin reuptake inhibitor in the treatment of patients with functional dyspepsia.

METHODS: Consecutive tertiary hospital patients with a clinical diagnosis of functional dyspepsia (FD) according to the Rome Ⅱ criteria with a Hong Kong dyspepsia index (HKDI) of greater than 16 were recruited. Patients commenced enrolment prior to the inception of the Rome Ⅲ criteria for functional dyspepsia. All patients were ethnic Chinese, had a normal upper endoscopy and were *Helicobacter pylori* negative prior to enrolment. Study patients were randomized to receive sertraline 50 mg or placebo daily for 8 wk. HKDI symptom scores, quality of life, hospital anxiety and depression (HAD) scale and global symptom relief were evaluated before, during and after treatment. Adverse effects were monitored during and after treatment.

RESULTS: A total of 193 patients were randomized in the intention to treat (ITT), and 150 patients were included in the per protocol (PP) analysis. In both the ITT and PP, there was no difference in the primary outcome of global dyspepsia symptoms between the sertraline and placebo groups at week 8. In the ITT analysis, 98 and 95 patients were randomized to the sertraline and placebo groups respectively. A total of 43 patients withdrew from the study (22.3%) by week 8, with 23 of the 24 drop-outs in the sertraline group occurring prior to week 4 (95.8%). In contrast, in the placebo arm, 11 of 19 patients dropped out by week 4 (57.9%). Utilizing the last response carried forward to account for the drop-outs, there were no differences between the sertraline and placebo groups at baseline in terms of the HKDI, HKDI 26.08 ± 6.19 *vs* 26.70 ± 5.89, $P = 0.433$; and at week 8, HKDI 22.41 ± 6.36 *vs* 23.25 ± 7.30, $P = 0.352$ respectively. In the PP analysis, 74 and 76 patients were randomized to the sertraline and placebo groups respectively. At baseline, there were no statistically significant differences between the sertraline and placebo groups, HKDI 25.83 ± 6.313 *vs* 27.19 ± 5.929 respectively, $P = 0.233$; however by week 8, patients in the sertraline group demonstrated a statistically significant difference in their Hong Kong Dyspepsia Index compared to placebo, HKDI 20.53 ± 6.917 *vs* 23.34 ± 7.199, $P = 0.02$, respectively). There was also no statistically significant difference in overall quality of life measures or the HAD scale related to treatment in either the ITT or PP analysis at week 8.

CONCLUSION: This pilot study, the first to examine sertraline, a selective serotonin reuptake inhibitor, for the management of FD, did not find that it was superior to placebo.

Key words: Dyspepsia; Chinese; Gastrointestinal diseases; Drug therapy; Sertraline; Selective serotonin reuptake inhibitors

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INTRODUCTION

Functional dyspepsia (FD) is defined as persistent or recurrent pain and/or discomfort centered in the upper abdomen for at least 12 wk in the preceding 12 mo according to the Rome Ⅱ criteria in the absence if structural disease[1]. The Rome Ⅲ criteria, published in 2006, further refines FD into epigastric pain syndrome and/or postprandial distress syndrome with the criteria fulfilled in the last 3 mo with symptoms onset at least 6 mo prior to diagnosis, again in the absence of structural disease[2,3]. The prevalence of dyspepsia in the Asia Pacific region varies from 10% to 20%, with a FD prevalence of 7.9%-12% which is lower than that seen in the west[4-6]. FD or non-ulcer dyspepsia is a significant cause of morbidity and work-related productivity lost[7]. The pathogenesis of FD is not known. A number of studies have shown an important role of psychological factors in the pathogenesis of this condition[8-10]. We have demonstrated previously that anxiety and depression are important co-factors in its pathogenesis[11]. There is no definitive treatment for this condition. Acid suppression therapy has been shown to be ineffective for the treatment of this condition in Chinese patients[12] despite benefit of proton pump inhibitor therapy in patients with ulcer-like and reflux like dyspepsia[13]. *Helicobacter pylori* (*H. pylori*) eradication confers only small benefit relative to placebo[14] and studies of itopride, a dopamine D2 antagonist with acetyl cholinesterase effects although initially promising, conferred no benefit in a subsequent and larger study[15,16]. Visceral hypersensitivity appears to be important in the pathogenesis of FD, as evidenced by a small study utilizing capsaicin to generate a desensitization of gastric nociceptive C fibers[17]. Similarly, antidepressants were investigated in FD for their ability to modulate visceral hypersensitivity[18]. Earlier antidepressant therapy studies demonstrated some effectiveness in the treatment of functional gastrointestinal symptoms, however a recent student utilizing venlafaxine (a selective serotonin and noradrenaline reuptake inhibitor) did not show any benefit[19-23]. In terms of antidepressants studied in FD, tricyclic compounds are the class of antidepressants best studied for this application, however selective serotonin reuptake inhibitors (SSRIs) are more commonly used in clinical practice because of their safer side-effect profile. The precise mechanism of action of SSRIs in the treatment of depression is not fully understood. However, long-term treatment with SSRIs has been reported to down regulate the serotonin transmitter responsible for serotonin reuptake as well as serotonergic receptors[24], which may down regulate visceral hypersensitivity.

An open label study found that the SSRI, fluoxetine, was superior to no treatment in depressed patients with FD, however had methodological flaws including the open label nature of the study[25]. To date, there are no published randomized controlled studies on the effect of sertraline for the treatment of FD. We performed a double-blind, randomized, placebo-controlled trial consisting of 8 wk of therapy in Chinese patients with FD. We aimed to assess the efficacy of SSRI in the treatment of FD and to identify potential responders to SSRI therapy in subgroups of patients with dyspepsia as their chief symptom.

MATERIALS AND METHODS

Patient enrollment

Consecutive patients referred to the Department of Medicine, Queen Mary Hospital, Hong Kong between June 2002 and June 2008 were screened for enrolment. FD was defined as persistent or recurrent dyspepsia (pain or discomfort centered in the upper abdomen) with no evidence of organic disease, chronic severe constipation, or irritable bowel syndrome to explain the symptoms, for at least 12 wk, which need not be consecutive, within the preceding 12 mo, in accordance with the Rome Ⅱ criteria[26]. The Rome Ⅲ criteria for FD had not yet been conceived when the study was commenced[3]. Patients aged 18-80 years with symptoms of dyspepsia within two weeks prior to the endoscopy visit were eligible for the study. Informed written consent was obtained from all patients. Patients were also required to have a dyspepsia score of greater than 16 by our validated questionnaire[27] and have had no prior investigations performed for this episode of dyspepsia within the 6 mo prior to the study. All enrolled patients were ethnic Chinese. Exclusion criteria included patients who were pregnant or breast feeding, had a history of alcohol or drug abuse; recent malignancy or significant medical illnesses or concurrent medication, which may interact with or contra-indicate the use of sertraline. Patients with a history of or current anti-depressant use were excluded. Patients with classical heartburn or acid regurgitation as their only symptom without epigastric discomfort or pain were also excluded to avoid recruitment of patients with non-erosive gastro-oesophageal reflux disease. All patients had normal upper endoscopy. The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (EC 1774-02).

Study protocol

Patients were randomized to receive either sertraline 50

mg or placebo once daily for eight weeks. Randomization was performed by drawing a sealed envelope that contained a pre-assigned randomized treatment generated by computer on entry to the study. Both the investigators and patients were blinded to the assigned treatment throughout the study. The sertraline and placebo capsules were identical in appearance. Patients were given a diary in which they recorded side effects and symptoms during therapy. After enrolment by gastroenterologists, patients returned for follow up at four and eight weeks where one of two gastroenterologists assessed their symptoms and quality of life.

Dyspepsia symptoms were assessed by a locally validated dyspepsia questionnaire, the Hong Kong dyspepsia index (HKDI) which consisted of 12 questions (epigastric pain, upper abdominal bloating, upper abdominal dull ache, epigastric pain before meals, epigastric pain when anxious, vomiting, nausea, belching, acid regurgitation, heartburn, feeling of acidity in the stomach, loss of appetite) graded on a five point Likert scale as follows: 1 (none), no symptoms; 2 (mild), symptoms can be easily ignored; 3 (moderate), awareness of symptoms but easily tolerated; 4 (severe), symptoms sufficient to cause interference with normal activities; and 5 (incapacitating), incapacitating symptoms with an inability to perform daily activities and/or require days off work. Test-retest reproducibility and internal consistency were good, with an intra-class correlation coefficient of 0.89 and Cronbach's alpha coefficient of 0.90. A cut off score of $\geqslant$ 16 was discriminative between controls and dyspeptic patients. Moreover, the HKDI score was significantly correlated to patients who reported a subjective improvement in symptoms and those who reported no change or worsening after therapeutic intervention (Kendall's *tau* = 0.21, P = 0.02)[27]. Patients were then sub-classified into four dyspepsia subgroups according to their predominant symptoms: (1) ulcer-like dyspepsia-predominant epigastric pain; (2) dysmotility-like dyspepsia-predominant discomfort that may be characterised by upper abdominal fullness, early satiety, bloating, or nausea; (3) reflux-like dyspepsia-predominant reflux symptoms (heartburn or acid regurgitation); and (4) unspecified-symptoms do not fulfill the criteria for ulcer-like, dysmotility-like, or reflux-like dyspepsia. Although reflux-like dyspepsia was discarded in the Rome II criteria, we felt that a certain proportion of patients with FD still belong to that particular subgroup and there is considerable overlap between FD and non-erosive or negative endoscopy reflux disease[28,29]. Furthermore, inclusion of reflux-like dyspepsia allows comparison with previous randomized controlled trials[30].

Quality of life was assessed by a locally validated questionnaire [Chinese translated form of 36-item short-form (SF-36)][31]. The SF-36 consisted of 36 items to measure eight aspects of psychological general well being (physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional, and mental health). A generic quality of life instrument was utilized to assess general well being as at the commencement of this study, there were no dyspepsia specific quality of life questionnaires validated in the Chinese language. The symptoms pertaining to anxiety and depression were assessed by the hospital anxiety and depression (HAD) scale questionnaire which consists of 14 questions. Finally, subjective global symptom relief was graded by patients, from a scale of 1 to 5, representing the spectrum from complete resolution of symptoms to worsening of symptoms, respectively.

Study intervention: Sertraline

The SSRI utilized in this study was sertraline (Zoloft, Pfizer Corporation) at a dose of 50 mg orally daily. Study participants were provided with pre-sealed boxes containing either sertraline or placebo and were asked to take a capsule per day for 8 wk in total. Patients were provided with a 4 wk supply of capsules and were contacted weekly to ensure compliance with treatment. Patient compliance was checked by counting returned study medications. Subjects who took less than 75% of the study medication were excluded from the final per protocol (PP) analysis.

Statistical analysis

Mean dyspepsia score, the eight aspects of the SF-36 scores and the two HAD scores before and after treatment were collected on Excel (Microsoft) databases in the two treatment groups. The change in mean HKDI, SF-36 and HAD scores from baseline, at the four and eight week visit were calculated and compared between the sertraline and the placebo groups. Patient diaries, detailing the presence and severity of symptoms, were also compared between groups at weeks 4 and 8. The primary end point was defined as an improvement in clinical symptoms at week 8. Secondary endpoints included an improvement or resolution of the clinical symptoms, or an improvement in any of the scales including HKDI, SF-36 or HAD at week 8. Continuous variables were expressed as mean ± SD, and categorical data expressed as percentages. Continuous variables were compared using Student's t tests. Categorical variables were compared using Fisher exact or χ^2 tests as appropriate. The Mann-Whitney test was used for data with a skewed distribution. The intention to treat (ITT) analysis included all patients who had taken at least one tablet. In the PP analysis, patients with poor drug compliance (< 75% intake of any study drugs) and drop outs (due to adverse effects) were excluded. Multiple logistic regression analysis was performed to determine independent factors (age, sex, *H. pylori* status, smoking, alcohol intake, dyspepsia duration, predominant symptoms, and type of treatment given) associated with treatment response.

All calculated P values were two-sided and P values < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS Ver. 16.0 for Windows (SPSS Inc., Chicago, IL, United States).

Power of the study

This was a pilot study, so assuming a placebo response

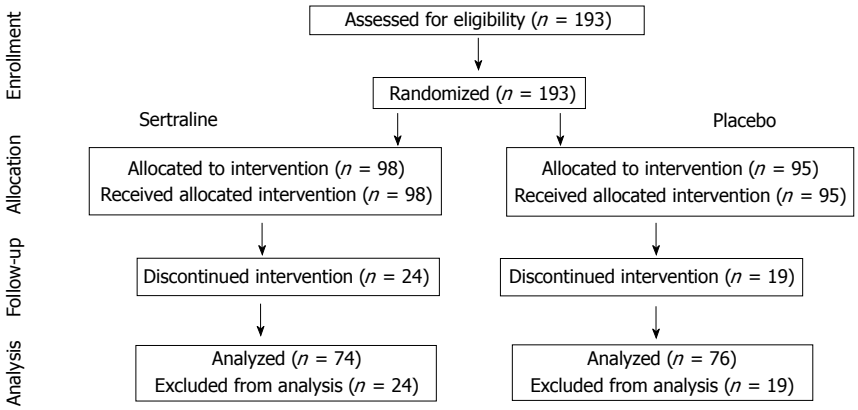

Figure 1 Study patient flow chart.

Table 1 Demographics of study patients

	Sertraline	Placebo	*P* value
Number of patients	98	95	
Age (yr)	43.0	41.6	0.515
Sex (male)	27	27	1.000
Current smokers (%)	3.2	7.3	0.122
Alcohol (%)	6.2	8.3	0.295
H. pylori positive (%)	8.4	7.3	0.843
NSAID use (%)	3.1	2.6	1.000
Predominant symptom *n* (%)			
Ulcer like	17 (44.7)	21 (55.3)	
Dysmotility like	60 (49.2)	62 (51.8)	
Reflux like	8 (57.1)	6 (42.9)	
Non-specific	13 (68.4)	6 (31.6)	

H. pylori: *Helicobacter pylori*; NSAIDs: Non-steroidal anti-inflammatory drugs.

rate of 25%-30%, a drug response rate of 30 % above placebo and a drop out rate of 20%[11], 166 patients will be needed to demonstrate 95% confidence interval with power of 0.8 and alpha of 0.05 (i.e., 166 patients with dyspepsia with 83 patients in each arm). It was aimed to recruit 190 patients.

RESULTS

Baseline demographics

We recruited 193 eligible patients (patients for the ITT analysis). A total of 98 patients were randomized to receive sertraline 50 mg and 95 patients were randomized to receive placebo (Figure 1).

All recruited patients were ethnic Chinese. Baseline characteristics of the patients in the two treatment groups are given in Table 1. 75.5% and 80% of patients took more than 75% of the medications in the sertraline and placebo groups, respectively. Poor compliance patients, those who refused follow up, and those who discontinued treatment because of adverse events were excluded from the PP analysis ($n = 150$).

Baseline characteristics of the patients and their dyspepsia subtypes are listed in Table 1. Mean age of these patients was 42.4 years (range: 18-71 years) with a median dyspepsia score of 26.5 (range: 17-46). There were 54 males (mean age 45.2 years) and 139 females (mean age 41.4 years). Mean age, sex distribution, smoking history, alcohol consumption and H pylori positivity at baseline were similar between the treatment groups (Table 1). Baseline mean HKDI score, SF-36 and HAD scales in the PP analysis (Table 2) assessments were similar between the treatment groups.

Dyspepsia scores

In the PP analysis at week 8, 28.4% *vs* 27.6%, of patients experienced complete resolution of their dyspepsia symptoms, whilst 64.9% *vs* 59.2% of patients experienced no difference in their dyspepsia symptoms in the sertraline and placebo groups ($P = 0.511$ for difference between the two cohorts) respectively. Sub group analysis for complete response at weeks 4 and 8 was also unrevealing. Complete response was similar between the treatment groups at weeks 4 and 8.

In the PP analysis the baseline mean HKDI score was 25.83 (SD = 6.313) and 27.19 (SD = 5.929) for sertraline and placebo arms respectively ($P = 0.233$). Mean HKDI score improved in all groups at week 4 compared to baseline. HKDI score in the sertraline group improved the most but was not statistically significant. By week 8, the sertraline group had a mean HKDI score of 20.53 (SD = 6.917), whilst the placebo group's mean dyspepsia score was 23.34 (SD = 7.199), ($P = 0.02$). The change in HKDI between week 0 to 8 was 5.3 and 3.85 in the treatment and placebo groups respectively ($P < 0.001$ for both sertraline and placebo groups). There were no consistent significant differences in the parameters of the quality of life assessment and the HAD scale at week 8 (Table 2).

For the ITT analysis, where the method utilized was the last response carried forward, the mean HKDI at week 0 was 26.08 and 26.70 (SD = 6.19 and 5.89, $P = 0.433$) for sertraline and placebo cohorts respectively. Although improvement of the HKDI was seen at weeks 4 and 8, the results were not statistically significant at (HKDI week 8 = 22.41 and 23.25, SD = 6.36 and 7.30 for sertraline and placebo respectively, $P = 0.352$). Again, there was no consistent significant differences in the parameters of quality of life assessment, HAD scale or complete responses at week (data not shown).

Table 2 Dyspepsia index, 36-item short-form score and hospital anxiety scale results

	Week 0	*P* value	Week 4	*P* value	Week 8	*P* value
Mean dyspepsia score						
Sertraline	25.83	0.124	22.59	0.740	20.53	0.02
Placebo	27.19		22.94		23.34	
SF36-physical functioning						
Sertraline	81.79	0.585	79.30	0.39	75.61	0.15
Placebo	83.11		81.63		80.39	
SF36-role physical						
Sertraline	57.91	0.30	52.62	0.40	52.70	0.22
Placebo	52.11		57.87		62.17	
SF36-bodily pain						
Sertraline	49.10	0.002	54.22	0.89	53.97	0.50
Placebo	41.06		53.78		51.63	
SF36-general health						
Sertraline	33.76		39.06		35.39	
Placebo	32.54	0.65	41.85	0.34	34.72	0.84
SF36-vitality						
Sertraline	47.69	0.57	47.91	0.73	49.05	0.68
Placebo	46.76		48.88		50.33	
SF36-social function						
Sertraline	67.75		72.38		68.41	
Placebo	67.68	0.98	72.19	0.96	69.74	0.73
SF36-role emotional						
Sertraline	51.82		53.49		52.70	
Placebo	50.75	0.85	54.19	0.90	51.32	0.85
SF36-mental health						
Sertraline	58.12		54.89		65.24	
Placebo	58.59	0.83	61.57	0.03	64.37	0.69
HAD scale						
Anxiety score						
Sertraline	14.27	0.52	13.58		14.29	
Placebo	13.88		13.66	0.90	13.68	0.41
HAD scale						
Depression score						
Sertraline	15.51	0.14	15.50	0.88	14.27	0.45
Placebo	14.84		15.56		13.70	

HAD: Hospital Anxiety and Depression Scale; SF36: 36-item short-form.

Adverse events

At week 8, a total of 43 patients (24 on sertraline and 19 on placebo) discontinued treatment. The main reasons for discontinuation of the study medication were drug side effect (41.2%), no reason given (41.9%) or other reason which included the development of conditions for which sertraline could interfere with prescribed treatment (7%) (Table 3). Of particular interest is the pattern of withdrawal from the study, 23 of 24 patients withdrawing from the sertraline group did so before week 4, representing 95.8% of all drop-outs from the sertraline group. By contrast, in the placebo group, approximately half of the patients withdrew prior to week 4 (57.9%), whilst the other half withdrew prior to week 8. Patients experiencing drug adverse effect were noted to have multiple symptoms including insomnia, constipation and agitation, however there was no significant difference in the rate of adverse effects experienced by the two cohorts. Nine percent of all study patients withdrew from the study without explanation (were lost to follow up).

Table 3 Default patient profile *n* (%)

	Default week 4	Default week 8	Reason for default week 8		
			No reason given[1]	Adverse effect of drug[1]	Other[1]
Sertraline	23	24	7 (16.3)	14 (32.6)	3 (7)
Placebo	11	19	11 (25.6)	8 (18.6)	0 (0)

[1]Represents percentage of all default patients; $P = 0.259$ for drug adverse effect between sertraline and placebo groups.

Factors associated with response

Age, sex, *H. pylori* status, smoking, alcohol consumption, and dyspepsia duration were not associated with response to sertraline. Multiple logistic regression analysis did not identify any independent predictors of favorable outcome.

A *post hoc* analysis comparing reflux like dyspepsia *vs* all other types of dyspepsia showed similar results to the PP analysis, in the non reflux like group the HKDI was 25.70 and 27.00 at week 0, whilst at week 8, HKDI was 20.85 and 23.42 respectively ($P = 0.004$). Similarly, the SF 36, HAD scales and complete response rates did not show any statistically significant differences (data not shown). In the reflux like group where $n = 14$, HKDI was 28.88 and 28.00 at week 0, and at week 8, HKDI was 21.00 and 23.80 ($P = 0.426$).

DISCUSSION

We have reported a double-blind, randomized, placebo-controlled pilot study of sertraline 50 mg *vs* placebo for the treatment of FD. We found that there was a statistically significant improvement in the mean HKID score at week 8 in the sertraline group compared to the placebo group in the PP but not the ITT analysis. There were also no differences in measures of quality of life, depression and anxiety and subjective global symptom resolution.

This study is a pilot study examining the effect on sertraline in patients with FD. The sertraline dose that was administered is a clinically relevant dose (the initial treatment dose for depression and obsessive-compulsive disorder and in some studies, depression)[32]. The trial duration of 8 wk seems adequate given that a steady-state plasma sertraline level is expected after 1 wk with once daily dosing and the beneficial effects of antidepressants in functional gastrointestinal disorders are often observed after shorter treatment duration than in depression[33]. However, longer term follow-up may yield more significant results given that individual responses to sertraline can vary and up to 12 wk may be required to see the full onset of action. Furthermore, although the dosage of sertraline utilized was appropriate for the reasons cited above, several studies have indicated that due to ethnic differences, Chinese patients may tolerate lower doses better[33,34].

One of the limitations of the study is the drop out rate, 17.6% at week 4 and 22.3% at week 8. Our drop

out rates are similar to those observed when antidepressants are utilized in functional gastrointestinal disorders[35]. There are many factors which could account for the drop out rate in our study. Sertraline's known side effects include sleep disturbance, headache, tremors, agitation and gastrointestinal upset[24]. In our study, adverse drug effect was the cause of study withdrawal in 41.2% of cases. Interestingly, of the patients who dropped out, 41.9% withdrew from the study without giving a reason. Multiple studies have indicated a cultural bias in the Chinese population against a diagnosis of psychiatric or functional disorders[36-38] and the authors hypothesize that the cultural stigma attached to treatment for a functional disorder with a known anti-depressant would contribute significantly to the drop out rate. Furthermore, the majority of the drop outs in the sertraline group occurred at week four, which could possibly be explained by the short term side effects of sertraline usually seen during the run in period of SSRIs[39] which could be mitigated by more intensive support and education during the first few weeks of treatment.

Another limitation of the study is the failure to discern a difference in the generic quality of life measures utilized between the sertraline and placebo cohorts. This may ostensibly be a reflection of the fact that generic quality of life instruments are not sensitive enough to detect changes in overall well being in patients with FD particularly with treatment[40]. This has been seen with other gastrointestinal disorders and has resulted in the development of disease specific quality of life instruments[41,42].

Finally, the most important limitation of the study is the failure to find a difference in global symptom scores in the ITT analysis, and only a small difference in the HKDI, but not global symptoms score in PP analysis. The authors believe this small finding suggests a possible benefit of sertraline in patients with FD, but perhaps this study was under-powered to detect this difference due to the unexpectedly high dropout rate, particularly in the first 4 wk when SSRI adverse effects are at their maximal, and for this reason warrants further larger studies utilizing sertraline to clarify the issue. We assert that our findings are important given that clinicians not uncommonly are utilizing SSRIs to treat FD despite the fact that to date, our study included, there is no strong justification for its use[43].

In conclusion, our data suggest that an SSRI, sertraline was not superior to placebo for the management of FD in Chinese patients. Further studies are warranted to confirm these results as this study was likely under powered to determine an effect in the context of a higher than expected drop out rate and there is a suggestion that with more support, a longer follow up period, and perhaps a reduction in the dose of sertraline in Chinese patients an effect may be seen.

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COMMENTS

Background

Early studies with tricyclic antidepressants demonstrated efficacy in the treatment of functional dyspepsia yet there have been no double-blind, randomized, placebo-controlled trials examining the role of selective serotonin reuptake inhibitors (SSRIs) in this condition.

Research frontiers

SSRIs, the most commonly utilized antidepressant in clinical practice, may improve the symptoms of functional dyspepsia through modulation of visceral hypersensitivity. In this study, the authors examine the effects of sertraline, a SSRI, on global symptoms, a locally validated dyspepsia index, the 36-item short-form (SF-36) and the hospital anxiety and depression scale.

Innovations and breakthroughs

Tricyclic antidepressant medication has been shown to be efficacious in functional dyspepsia, however tricyclic antidepressant medications have significant side effects, prompting the study of the utility of newer antidepressants in this condition. Venlafaxine, a selective serotonin and noradrenaline reuptake inhibitor did not show any benefit in functional dyspepsia however an open label study of fluoxetine, a SSRI, found benefit. This is the first double-blind, randomized, placebo-controlled study examining an SSRI in the treatment of functional dyspepsia.

Applications

This study found that treatment with the SSRI, sertraline, improved the Hong Kong dyspepsia index score at week 8 compared to baseline but did not find overall improvement in global dyspepsia symptoms, SF-36 or the hospital anxiety and depression scale, possibly due to the higher than expected drop out rate in the sertraline group by week 4. Larger studies are warranted to further examine the effects of sertraline in functional dyspepsia.

Peer review

This is a nicely designed study showing that SSRI sertraline is of no benefit in functional dyspepsia. The authors acknowledge the main limitation of a negative study represented by the scarce numerosity and high drop out rate, based on the optimistic calculations adopted to evaluate sample size. A more realistic hypothesis will substantially raise the number of patients needed to be included.

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BRIEF ARTICLE

Transcatheter arterial chemoembolization for gastrointestinal stromal tumors with liver metastases

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Abstract

AIM: To evaluate the efficacy and safety of transcatheter arterial chemoembolization (TACE) for gastrointestinal stromal tumor (GIST) with liver metastases after the failure of tyrosine kinase inhibitors (TKIs).

METHODS: Patients with histologically confirmed CD117-positive GIST with liver metastases who were resistant and/or intolerant to prior imatinib and/or sunitinib and who received TACE for at least one treatment cycle or only best supportive care and TKI reintroduction were eligible for the study. The patients were divided into two groups: those in TACE group received TACE treatment containing 5-20 mL iodized oil and 40-80 mg doxorubicin hydrochloride and TKI reintroduction or best supportive care, those in control group only received TKI reintroduction or best supportive care. The primary end-point was overall survival and the secondary end-points were, progression-free survival (PFS), response rates, and safety.

RESULTS: Sixty patients admitted between June 2008 and October 2011 were eligible for this study, including 22 in TACE group and 38 in control group. In the TACE group, 12 (54.5%) achieved liver partial response, 5 (22.7%) had stable disease, and 5 (22.7%) had liver progressive disease. Disease control rate of liver metastases was 77.3% in the TACE group and 39.5% in the control group. The median liver PFS in TACE group was 47.1 wk (95% CI: 23.9-70.3). The median PFS in TACE group was longer than in control group (30.0 wk, 95% CI: 20.1-39.9 *vs* 12.9 wk, 95% CI: 11.9-13.9) (P = 0.0001). The median overall survival in TACE group was also longer than in control group (68.5 wk, 95% CI: 57.4-79.6 *vs* 25.7 wk, 95% CI: 23.2-28.2) (P = 0.0001). TACE treatment significantly reduced the risk of death (hazard ratio: 0.109). Patients without extrahepatic metastases treated with TACE had significantly better prognosis. Most of the adverse events were of grade 1 or 2 and tolerable.

CONCLUSION: TACE is effective and well tolerated in GIST patients with liver metastases after TKI failure, and it may be an optional treatment for this disease.

Key words: Gastrointestinal stromal tumor; Liver metastases; Transcatheter arterial chemoembolization; Tyrosine kinase inhibitor failure; Overall survival

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and account for about 2% of gastrointestinal tract tumors[1,2]. Tyrosine kinase inhibitors (TKIs) imatinib and sunitinib have demonstrated efficacy against GISTs, and are referred to as the first- and second-line therapeutic drugs[3-5]. However, resistance to such kind of TKIs remains a substantial problem. Around 4%-5% patients showed evidence of primary resistance and nearly half of the patients will experience secondary resistance within two years[4,6,7]. At present, there is still no standard treatment for metastatic GIST after imatinib and sunitinib failure. The National Comprehensive Cancer Network (NCCN) guideline (2010)[8] recommended considering reintroduction of a TKI for palliation of symptoms in patients with GIST progression despite prior imatinib and sunitinib.

Liver is the most common site of metastasis from GISTs, with a reported incidence of 55%-72% in patients with recurrence, and metastatic liver disease is a major determinant of patient survival[9,10]. Some studies[11-14] have shown favorable results of transcatheter arterial chemoembolization (TACE) for GIST with liver metastases. However, there are few studies about the role of TACE in the treatment of GIST patients after TKIs failure, moreover, there is no control study comparing TACE with best supportive care (BSC) and/or TKI reintroduction. Herein we retrospectively analyzed the survival benefit of TACE, BSC and/or TKI reintroduction in the patients with liver metastatic GISTs treated in the Peking University Cancer Hospital when resistance and/or intolerance occurred to imatinib and/or sunitinib.

MATERIALS AND METHODS

Study design

It is an open, retrospective, controlled study to evaluate the efficacy and safety of TACE in Chinese GIST patients with liver metastases after TKI treatment failure. Patients with histologically confirmed CD117-positive GIST with liver metastases who were resistant and/or intolerant to prior imatinib and/or sunitinib and who received TACE for at least one treatment cycle or only BSC and TKI reintroduction were eligible for the study. Following a restrospective review of the medical records of the patients seen at our hospital between June 2008 and October 2011, a total of 60 patients were found to meet the study criteria. There were 22 in TACE treatment group and 38 in BSC/TKI reintroduction group, which served as control group.

Patient characteristics: The following demographic and clinicopathological information was retrospectively obtained from the patient records: gender, age, extent of liver disease, and extrahepatic metastases.

Treatment: Data of TKI reintroduction and TACE treatment, including dose of TKI, interval between TKI and TACE, TACE procedure, and cycles of TACE, were collected.

Follow-up: Overall survival (OS) and progression-free survival (PFS) were acquired.

Study end-points

The primary end-point was OS and the secondary end-points were PFS, disease control rate (DCR) of liver metastases defined as a combination of complete response + partial response (PR) + stable disease (SD), and safety. Response rate was evaluated every 6 wk. OS was defined as the time from the first TACE or BSC/TKI reintroduction to the occurrence of death from any cause. The PFS was defined as the time from the first time of TACE or BSC/TKI reintroduction to the occurrence of disease progression or death from any cause. Disease control rate was assessed by Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria version 3.0.

Statistical analysis

All the statistical analyses were performed using the SPSS 15.0 (SPSS Inc., Chicago, IL, United States). PFS and OS curves were constructed by the Kaplan-Meier method and compared with log-rank test. In order to adjust for confounding variables, we used Cox proportional hazards models to estimate the simultaneous effects of prognostic factors on survival. Frequency and percentage descriptions were used for categorical variables and the χ^2 test was used to compare the incidence of different events. If the theoretical frequency was lower than 1, F test was conducted. Continuous variables were expressed as mean ± SD and mean differences between two groups were compared using Student's t test.

RESULTS

Patient characteristics

There were 45 males and 15 females with a median age of 55.0 years (95% CI: 51.8-58.2). All the patients at registration were assessed to have the Eastern Cooperative Oncology Group (ECOG) performance status grade 0-2 and had received imatinib treatment. Among them, 35 took sunitinib after imatinib failure prior to TACE or BSC/TKI introduction treatment. Thirty-four (56.7%) had liver metastases and the others had extrahepatic metastases. Clinical features of the patients in the two groups are shown in Table 1.

In TACE group, 15 (68.2%) had an extent of liver involvement within 50%, 6 (27.3%) within 50%-70%,

Table 1 Clinicopathologic features of the patients

Clinicopathologic features	TACE *n* = 22 (%)	Control *n* = 38 (%)	Statistical test	*P* value
Sex			$\chi^2 = 0.310$	0.578
Male	16 (72.7)	29 (76.3)		
Female	6 (27.3)	9 (23.7)		
Age (yr)	53.0 (49.3-59.6)	55.0 (48.0-62.0)	$U = 5.000$	0.279
ECOG PS			$\chi^2 = 2.344$	0.126
0-1	16 (72.7)	20 (52.6)		
2	6 (27.3)	18 (47.4)		
Primary location			$\chi^2 = 0.012$	0.994
Stomach	9 (40.9)	15 (39.5)		
Small intestinal	9 (40.9)	16 (42.1)		
Other	4 (18.2)	7 (18.4)		
Number of liver lesions			$\chi^2 = 1.805$	0.406
1	8 (36.4)	8 (21.1)		
2-5	9 (40.9)	20 (52.6)		
> 5	5 (22.7)	10 (26.3)		
Extrahepatic metastases			$\chi^2 = 0.083$	0.773
Yes	9 (40.9)	17 (44.7)		
No	13 (59.1)	21 (55.3)		
Sunitinib second-line therapy before TACE			$\chi^2 = 0.992$	0.319
Yes	11 (50.0)	24 (63.2)		
No	11 (50.0)	14 (36.8)		
TKI reintroduction			$\chi^2 = 1.778$	0.182
Yes	10 (45.5)	24 (63.2)		
No	12 (54.5)	14 (36.8)		

TACE: Transcatheter arterial chemoembolization; ECOG PS: The Eastern Cooperative Oncology Group performance status; TKI: Tyrosine kinase inhibitors.

and 1 more than 70%. Eight patients (36.4%) had only 1 liver metastasis, 9 (40.9%) had 2-5 liver metastases, and the others had more than 5. The mean TACE treatment cycles received by all the patients in TACE group was 2.64, with 6 (27.3%) receiving only one TACE, and 16 (72.7%) received more than one TACE. Fifteen patients (68.2%) showed a good blood supply of liver metastases.

Treatment in TACE group

TACE protocol: Eligibility criteria for TACE included well-preserved hepatic and renal function, the Child-Pugh classification within A and B, adequate hematologic function, and ECOG performance status of 0-2. Patients with high-risk factors, such as portal vein occlusion, no hepatopetal flow, massive ascites, encephalopathy, or active cardiac failure, were excluded.

Local anesthesia was obtained with 1% lidocaine. After the introduction of a selective catheter through the femoral artery using the Seldinger technique, the localization of the hepatic arteries was checked with celiac and mesenteric arteriography. This was performed to define vascular anatomy. Next, indirect portography was performed to outline the portal circulation in the venous phase. A 5 French catheter was placed in the celiac trunk to identify the hepatic artery. Depending on the size, location, and arterial supply to the tumor, a micro-catheter was advanced further into the segmental feeding arteries to perform embolization. An emulsion containing 5-20 mL iodized oil and 40-80 mg doxorubicin hydrochloride was used according to the tumor size. Additional embolization was performed using 1-2 mm diameter gelated sponge particles according to the status of blood supply. The ideal embolization end-point is the stasis of flow in tumor-feeding branches. Follow-up abdominal imaging (computed tomography) was generally performed two months after the first embolization. The follow-up images were assessed by two radiologists (Cao K and Cui Y) and compared with the baseline images to assess response.

TKI reintroduction: Ten patients received TKI reintroduction during the intermittent period of TACE. Among them, 6 patients took imatinib 400 mg/d and 4 patients took sunitinib 37.5 mg/d. The interval between TKI therapy and TACE was 2 wk.

Treatment in control group

All the patients in control group had GIST resistant to imatinib and 35 had tumor resistant to sunitinib. Among them, 9 patients received imatinib 400 mg/d and 15 received sunitinib 37.5 mg/d reintroduction, and the others only received best supportive care. Efficacy was evaluated every 6-8 wk according to the RECIST criteria.

Response rate: All the patients had measurable metastatic disease according to the RECIST criteria and tumor assessment was performed at least once. In TACE group, 12 (54.5%) achieved liver PR, 5 (22.7%) had SD, and 5 (22.7%) showed liver disease progression (PD) after TACE treatment. The DCR of liver metastases was 77.3%. In addition, 8 patients had PD when all the lesions were evaluated, and the DCR of all the lesions was 63.6%. In the control group, 12 patients receiving TKI reintroduction and 3 patients receiving BSC had SD, and the others had PD. The DCR in the control group was 39.5%.

PFS: As of May 2012, 19 (86.0%) patients in TACE group had liver metastasis progression. The median liver PFS of all 22 patients was 47.1 wk (95% CI: 23.9-70.3). In control group, all the patients had tumor progression. The median PFS in TACE group was longer than in control group (30.0 wk, 95% CI: 20.1-39.9 *vs* 12.9 wk, 95% CI: 11.9-13.9) ($P = 0.0001$, Figure 1A).

OS: As of May 2012, 4 patients in TACE group and 2 patients in control group were alive, and deaths occurred because of tumor progression. The median OS in TACE group was longer than in control group (68.5 wk 95% CI: 57.4-79.6 *vs* 25.7 wk 95% CI: 23.2-28.2) ($P = 0.0001$, Figure 1B). TACE significantly reduced the risk of death in GIST patients with liver metastases according to the Cox proportional hazards regression model [hazard ratio (HR): 0.109; 95% CI: 0.044-0.271].

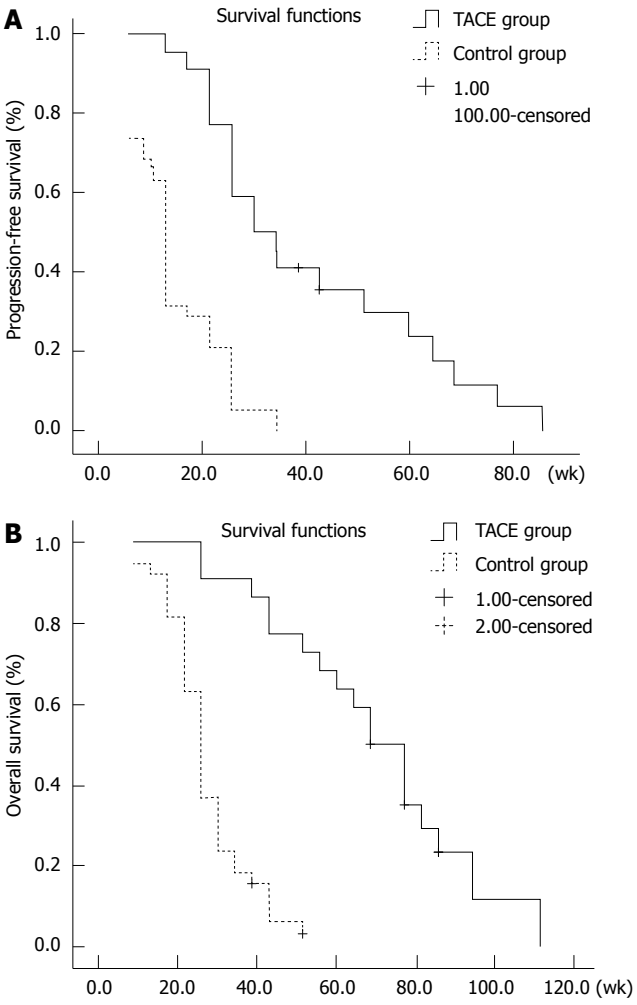

Figure 1 The median progression-free survival (A) and overall survival (B) were longer in transcatheter arterial chemoembolization group than in control group (*P* = 0.0001). TACE: Transcatheter arterial chemoembolization.

Univariate and multivariate analysis

Results of the univariate and multivariate analysis are summarized in Table 2. The results showed the *P* value of number of liver metastases is 0.086. The patients without extrahepatic metastases and the patients treated with TACE were the two factors significantly associated with good survival. The two factors led to a reduction of death risk by 53.7% (HR: 0.463, $P = 0.007$) and 58.5% (HR: 0.415, $P = 0.005$), respectively.

In TACE group, univariate and multivariate analysis showed that absence of extrahepatic metastases, more than one session of TACE, and DCR of more than 3 mo after TACE were significantly associated with a good survival ($P = 0.006$, $P = 0.02$, $P = 0.012$).

Adverse events

Most patients in TACE group developed post-embolization complications, which included abnormal liver function, abdominal pain, fever and nausea. The incidence of fever, alanine aminotransferase increase and nausea in TACE group was higher than in control group ($P < 0.05$). However, the majority of adverse events were of grade 1-2, and in most cases, these symptoms were effectively resolved with supportive measures. No patient died within 1 mo after TACE. Other adverse events included anemia, neutropenia, thrombocytopenia, ascites, pleural effusion and hemorrhage (Table 3). No one discontinued treatment because of adverse events.

Table 2 Univariate analysis by each variable

Variable	*n*	OS (wk)	*P* value
Gender			0.133
Male	45	34.3	
Female	15	25.7	
Primary tumor location			0.825
Stomach	24	25.7	
Intestine	25	34.3	
Others	11	30.0	
ECOG PS			0.102
0-1	36	35.8	
2	24	24.5	
Number of liver metastases			0.079
1	16	42.9	
2-5	29	25.7	
> 5	15	38.6	
Extrahepatic metastases			0.005
Yes	26	25.7	
No	34	42.9	
TKI reintroduction			0.657
Yes	34	30.0	
No	26	30.0	
TACE treatment			0.0001
Yes	22	68.5	
No	38	25.7	

OS: Overall survival; ECOG PS: The Eastern Cooperative Oncology Group performance status; TKI: Tyrosine kinase inhibitors; TACE: Transcatheter arterial chemoembolization.

DISCUSSION

There is still no standard treatment for the GIST patients after imatinib and sunitinib failure. TKI reintroduction, BSC or drugs in clinical trial are recommended for these patients. Some studies[15-19] reported that the novel TKIs had potential activity against metastatic GIST, but the efficacy remains to be validated in prospective randomized controlled trials. Liver is the most common metastatic site of GIST and some patients even have only liver metastases other than other diseases till death. Resection of liver metastases has improved the overall survival[20-22], again the efficacy of resection should be further confirmed by prospective clinical trials. Some retrospective studies[11-14] showed that TACE may be potentially effective for GIST resistant to TKI. In this study, the patients with liver metastatic GIST receiving TACE after imatinib and/or sunitinib failure gained better PFS and OS than the patients receiving TKI reintroduction or BSC. In the sunitinib phase Ⅲ trial[23], the median time to progression of the patients receiving placebo was only 6.4 wk. The results demonstrated that TACE may benefit the patients with liver metastases.

In TACE group, 68.2% patients had good blood

Table 3 Adverse events in the two groups

Adverse events	All grades (%)			Grade 3-4 (%)		
	TACE group (n = 22)	Control group (n = 38)	P value	TACE group (n = 22)	Control group (n = 38)	P value
Fever	20 (90.9)	5 (13.2)	0.0001	2 (9.1)	0 (0)	0.061
Fatigure	16 (72.7)	28 (73.7)	0.936	5 (22.7)	8 (21.1)	NA
Abnormal ALT	16 (72.7)	6 (15.8)	0.0001	5 (22.7)	0 (0)	0.005
Nausea	14 (63.6)	14 (36.8)	0.045	1 (4.5)	0 (0)	0.367
Ascites	5 (22.7)	10 (26.3)	0.757	0 (0)	0 (0)	NA
Diarhoea	4 (18.2)	5 (13.2)	0.712	0 (0)	0 (0)	NA
Hemorrhage	3 (8.3)	4 (10.5)	0.700	1 (4.5)	2 (5.3)	1.000
Neutropenia	12 (54.5)	16 (42.1)	0.352	3 (12.6)	3 (7.9)	0.659
Anemia	7 (31.8)	24 (63.2)	0.019	3 (12.6)	6 (15.8)	1.000
Thrombocytopenia	7 (31.8)	10 (26.3)	0.649	2 (10.5)	1 (2.6)	0.548

TACE: Transcatheter arterial chemoembolization; ALT: Alanine aminotransferase; NA: Not applicable.

supply of liver metastases. Some suspensions such as iodizedoil (lipiodol) can occlude small tumor vessels and cause obstruction in the vascular bed of liver metastases. Unresectable or metastatic GIST resists the conventional cyto-toxic chemotherapy[9,24,25], so cyto-toxic drugs are not recommended in TACE. Further to a earlier report[24] which showed that doxorubicin had slight efficacy in metastatic GIST, it has been reported recently that the chemo-embolization with doxorubicin elusion with the iodized oil demonstrated a potential efficacy[11-14]. Lipiodol and microspheres concentrate and prolong the retention of the chemotherapeutic agent (doxorubicin) in the tumor[26].

The results of this study showed that TACE significantly reduced death risk by 89.1%. In the subgroup analysis, DCR of more than 3 mo after TACE was correlated to good survival, indicating the benefit of TACE with regard to the overall survival. In the univariate and multivariate analysis, absence of extrahepatic metastases and TACE treatment were the independent prognostic factors. The similar results were seen in subgroup analysis in TACE group. These results showed that the patients without extrahepatic metastases can enjoy a longer survival after TACE. At the same time, a single session of TACE may not be adequate enough to control liver metastases. The results were consistent to the earlier report[11]. However, bias of the patient selection may exist in this retrospective study. More prospective trials are expected to confirm the efficacy of TACE in this group of patients. In this study, all cases enrolled had advanced GIST with relatively larger liver lesions after the TKI failure. TACE still yielded a good control rate in this group of patients. Whether TACE procedure should be recommended earlier even before TKI failure warrants future studies.

NCCN guideline recommended considering reintroduction of a TKI for palliation of symptoms in patients with GIST progression despite prior imatinib and sunitinib. Does TKI reintroduction combining with TACE improve PFS and OS of GIST with liver metastases, especially for the patients with extrahepatic metastases? In this study it seemed that the patients receiving combined TACE and TKI reintroduction had longer overall survival than those receiving TACE alone, but there was no statistical significance (P = 0.638). This may be attributed to the small case number in this study. However, TKI reintroduction did not increase the incidence of complication during TACE treatment. The interval time of 2 wk between TKI and TACE is appropriate. For the patients without extrahepatic metastases, the combined TACE and TKI reintroduction may be an optional method of treatment.

Many patients in TACE group suffered from post-embolization complications, such as abdominal pain, fever and nausea. Most of them were of grade 1 or 2, and 22.7% patients were of grade 3. But all the adverse events were ameliorated within 1-2 wk with supportive measures. No adverse events out of expectation happened and no one discontinued treatment because of severe adverse events. The combined TACE and TKI reintroduction was well tolerated in the majority of the patients.

In summary, TACE may be an optional treatment for GIST with liver metastases after TKI failure. TACE can better benefit the patients without extrahepatic metastases.

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COMMENTS

Background

There is still no standard treatment for metastatic gastrointestinal stromal tumor (GIST) after imatinib and sunitinib failure. Liver is the most common site of metastasis from GISTs and liver metastasis is one of the major causes of death in these patients. The authors evaluated the efficacy and safety of transcatheter arterial chemoembolization (TACE) in GIST with liver metastases after the failure of tyrosine kinase inhibitors.

Research frontiers

There are few studies about the role of TACE in the treatment of GIST patients after tyrosine kinase inhibitors (TKIs) failure, moreover, there is no control study comparing TACE with best supportive care (BSC) and/or TKI reintroduction.

Innovations and breakthroughs

This study is the first controlled report to evaluate the therapeutic effect of TACE combining with BSC and/or TKI reintroduction in GISTs with liver metastases after TKI failure. The results of the paper showed that TACE improved progression-free survival and overall survival of GIST patients with liver metastases after TKI failure as compared with those receiving only BSC and or TKI reintroduction.

Applications

This study provided some evidences that TACE may be an optional treatment for GIST with liver metastases after TKI failure. TACE can better benefit the patients without extrahepatic metastases.

Terminology

GIST is the most common mesenchymal tumor of the gastrointestinal tract and TKI is the standard treatment for metastases GIST. TACE is the abbreviation of transcatheter arterial chemoembolization. TACE is the use of vascular embolizing material combined with cytotoxic drugs to induce tumor ischemic necrosis and prolonged drug transit time, and often used in treatment of hepatocellular carcinoma.

Peer review

This is a nice review of a unique series of patients with metastatic GIST. The authors should specify what the chemotherapy in the TACE procedure actually is. It appears only to be lipid.

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BRIEF ARTICLE

Liver-protecting effects of omega-3 fish oil lipid emulsion in liver transplantation

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Abstract

AIM: To investigate the liver-protecting effect of parenteral nutrition (PN) support with omega-3 fatty acids in a randomized controlled clinical trial.

METHODS: Sixty-six patients with the diagnosis of end-stage liver disease or hepatic cellular carcinoma were admitted to the Affiliated Drum Tower Hospital, Nanjing University, China for orthotopic liver transplantation. The patients were randomly divided into two groups: PN group (n = 33) and polyunsaturated fatty acid (PUFA) group (n = 33). All patients received isocaloric and isonitrogenous PN for seven days after surgery, and in PUFA group omega-3 fish oil lipid emulsion replaced part of the standard lipid emulsion. Liver function was tested on days 2 and 9 after surgery. Pathological examination was performed after reperfusion of the donor liver and on day 9. Clinical outcome was assessed based on the post-transplant investigations, including: (1) post-transplant mechanical ventilation; (2) total hospital stay; (3) infectious morbidities; (4) acute and chronic rejection; and (5) mortality (intensive care unit mortality, hospital mortality, 28-d mortality, and survival at a one-year post-transplant surveillance period).

RESULTS: On days 2 and 9 after operation, a significant decrease of alanine aminotransferase (299.16 U/L ± 189.17 U/L *vs* 246.16 U/L ± 175.21 U/L, P = 0.024) and prothrombin time (5.64 s ± 2.06 s *vs* 2.54 s ± 1.15 s, P = 0.035) was seen in PUFA group compared with PN group. The pathological results showed that omega-3 fatty acid supplement improved the injury of hepatic cells. Compared with PN group, there was a significant decrease of post-transplant hospital stay in PUFA group (18.7 d ± 4.0 d *vs* 20.6 d ± 4.6 d, P = 0.041). Complications of infection occurred in 6 cases of PN group (2 cases of pneumonia, 3 cases of intra-abdominal abscess and 1 case of urinary tract infection), and in 3 cases of PUFA group (2 cases of pneumonia and 1 case of intra-abdominal abscess). No acute or chronic rejection and hospital mortality were found in both groups. The one-year mortality in PN group was 9.1% (3/33), one died of pulmonary infection, one died of severe intra-hepatic cholangitis and hepatic dysfunction and the other died of hepatic cell carcinoma recurrence. Only one patient in PUFA group (1/33, 3.1%) died of biliary complication and hepatic dysfunction during follow-up.

CONCLUSION: Post-transplant parenteral nutritional support combined with omega-3 fatty acids can significantly improve the liver injury, reduce the infectious morbidities, and shorten the post-transplant hospital stay.

Key words: Fish oil lipid; Liver; Transplantation; Paren-

teral nutrition; Metabolism

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INTRODUCTION

Liver transplantation has dramatically improved the prognosis of end-stage liver disease. The progress made in the immunosuppressive regimens and surgical techniques has yielded a better outcome of the patients, and the 5-year survival after liver transplantation is 70%-80%[1]. The liver recipients with liver insufficiency are in fact known to experience a higher incidence of severe protein/calorie malnutrition, which is associated with a greater risk of postoperative complications and mortality in patients undergoing liver transplantation[2,3]. And ischemia/reperfusion (I/R) injury associated with liver transplantation often leads to hepatic dysfunction despite the improvement in surgical techniques and perioperative medication. I/R injury of the liver is an event involving multiple factors, such as hypoxia during inflow occlusion of the liver and inflammatory reactions after reperfusion[4,5], and the mechanisms of the reperfusion injury, including the release of inflammatory cytokines, the generation of oxygen free radicals, Kupffer cell activation and leukocyte-endothelial cell interaction[6,7]. Based on the pathophysiology of hepatic I/R injury, the current study particularly focused on various perioperative approaches to protect the liver from these inflammatory reactions and microcirculatory disturbances.

Omega-3 (N-3) fatty acids, which are derived from fish oil, are essential polyunsaturated fatty acids (PUFAs) for humans. Omega-3 fatty acids exert anti-inflammatory and immunomodulatory properties through their ability to modulate the synthesis of different eicosanoids[8,9]. Perioperative administration of omega-3 fatty acids reduces plasma and tissue levels of the eicosanoids, specific leukotrienes, thromboxanes, and prostaglandins, all of which have pro-inflammatory effects[10,11]. Recent studies described that supplementation with omega-3 fatty acids decreases the rate of inflammatory complications, the length of hospital stay, and the mortality after major abdominal surgeries[12-14]. Their protective effects on hepatic I/R injury and inflammatory responses have been increasingly investigated.

In this study, we investigated the liver-protecting effects of parenteral nutrition (PN) supplemented with omega-3 fish oil lipid emulsion in patients undergoing liver transplantation.

MATERIALS AND METHODS

Ethics

This study was carried out in the Department of Hepatobiliary Surgery of the Affiliated Drum Tower Hospital, Medical School of Nanjing University, China according to the principles and guidelines of the Helsinki Declaration of 1975 revised in 2000. The protocol was approved by the Ethics Committee of the Affiliated Drum Tower Hospital. All patients fully understood the objective and adverse reactions of the study, and signed the written informed consent voluntarily prior to study enrollment.

Patients and randomization

From January 2006 to July 2010, we prospectively investigated 66 patients (45 men and 21 women; mean age, 51.6 years; range, 34-64 years) who underwent orthotopic liver transplantation in the Department of Hepatobiliary Surgery at the Affiliated Drum Tower Hospital of Medical School of Nanjing University. Recipients with manifest metabolic diseases (e.g., diabetes mellitus and hyperthyroidism) or severe renal abnormality were excluded. No acute rejection, primary transplanted liver dysfunction or second operation, which may affect the evaluation of liver function, were seen during the first 9 d after transplantation. The selection criteria for donors in this study included: (1) age < 50 years, matched ABO blood group and no history of chronic liver disease; (2) no evidence of malignant tumor, viral hepatitis or other viral infections; (3) no cirrhosis, mass or severe fatty degeneration of the donor liver seen during organ harvesting; and (4) liver biopsies of each donor liver taken before transplantation were reviewed by two pathologists. Donor livers with normal pathology or mild fatty change (10%-30%) were included in this study (Figure 1).

After transplantation, these patients were randomized into two groups based on the randomization chart generated by the Statistical Analysis System (SAS): (1) PN group (33 patients), without supplementation of omega-3 fatty acids in addition to routine treatment; and (2) PUFA group (33 patients), with PN supplemented with omega-3 fatty acids in addition to routine treatment.

This is a randomized controlled clinical study carried out in the Department of Hepatobiliary Surgery of our hospital. Nutrition Risk Screening 2002 (NRS 2002) scoring system was used, and the post-operative NRS 2002 score was ≥ 3 in all the patients, which meant that all the patients need nutritional support.

Treatment

The PN was given around the clock for seven days from the second day after operation. The two nutritional support groups were isonitrogenous and isocaloric. Nitrogen intake was 0.16 g/kg body weight per day, caloric intake was 104.5 kJ/kg per day, and lipid intake was 1.0 g/kg per day. The nonprotein calories were provided with dextrose (4.0 g/kg per day) and fat emulsion in a ratio of 2:1. The only source of lipids in PN group was

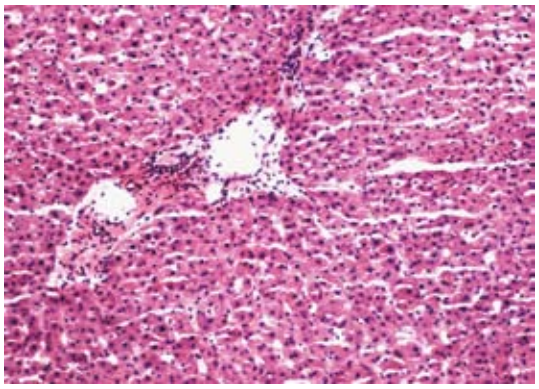

Figure 1 Pathology of liver biopsy of donor liver taken before transplantation. Mild steatosis was observed in donor liver. (Hematoxylin and eosin staining; paraffin-embedded 5 μm thick sections; ×200).

the standard lipid emulsion (20% emulsion, with a ratio of long-chain triglycerides to medium-chain triglycerides of 1:1, Huarui Pharmaceuticals, Jiangsu, China), and in PUFA group omega-3 fish oil lipid emulsion (Omegaven, 10%, 2 mL/kg per day, Fresenius Kabi Co., Austria) replaced part of the standard lipid emulsion. Both groups received 1.0 g amino acid/kg per day, and they were administered a commercially available branched-chain amino acid solution (Branched-chain amino acid solution 20%, Huarui Pharmaceuticals, Jiangsu, China). The ratio of nonprotein calories to nitrogen in both nutritional support groups was 653 kJ:1 g. The omega-3 fish oil lipid emulsion-containing solutions were prepared by the clinical pharmacist under aseptic condition and adjusted according to the weight of each individual patient. The amino acids, fat emulsion and dextrose mixture with electrolytes, vitamins, and trace elements were administered through a central venous catheter. As soon as the bowel function returned on days 3 or 4 after transplantation, all patients in the two groups were given liquid carbohydrate and cow's milk protein.

The surgical treatment was standardized, and modified piggyback orthotopic liver transplantation was performed by three groups of surgeons using the same approach. After operation, all the patients in the two groups were treated with the same antibiotics and antivirutics, and 20 g of albumin was administered intravenously daily for five days to prevent any complications caused by hypoalbuminemia.

Assessment

Venous heparin blood samples were obtained on days 1 (the day before transplantation), 2 and 9 after surgery and liver function assessment was made. Serum total bilirubin (TB), direct bilirubin (DB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and prothrombin time (PT) were measured by an automatic biochemical analyzer (HITACHI 7600, Japan).

Liver biopsy with fine needle was conducted after reperfusion of donor liver and on day 9 after surgery, respectively. Hepatic specimens for light microscopy were fixed with formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination. Portal inflammation in the liver biopsy specimens was semiquantified by calculating inflammatory cells in portal tracts based on the Knodell histology activity index (HAI)[15]. Portal inflammation was scored as 0, no portal inflammation; 1, mild (sprinkling of inflammatory cells in < 1/3 of portal tracts); 3, moderate (increased inflammatory cells in 1/3-2/3 of portal tracts); and 4, marked (dense packing of inflammatory cells in > 2/3 of portal tracts).

The assessment of clinical outcome was based on post-transplant investigations as shown by: (1) post-transplant mechanical ventilation; (2) total hospital stay; (3) infectious morbidities (pneumonia, intra-abdominal abscess, central line sepsis, wound infection, and urinary tract infection); (4) acute and chronic rejection; (5) mortality (intensive care unit mortality, hospital mortality, 28-d mortality, and survival at one year post-transplant surveillance period).

These post-transplant parameters were investigated and documented daily during the patients' post-transplant hospital stay and the period of one-year postoperative follow-up.

Statistical analysis

The results were expressed as mean ± SD. Data were analyzed using the SAS. Differences between means were evaluated using Student *t* test when normal distribution was confirmed by Shapiro-Wilks test. When the hypothesis of normal distribution was rejected, differences between groups were tested by nonparametric statistics using Mann-Whitney test for unpaired samples and Wilcoxon criteria for paired samples. Fisher's exact test was used for analysis of categorical values when appropriate. A P value of < 0.05 was considered significant.

RESULTS

A total of 66 patients were enrolled in this study, including 33 patients in PN group and 33 patients in PUFA group. The mean age of the subjects was 51.6 years (range, 34-64 years). The clinical diagnosis of these patients included: hepatic cell carcinoma (27 cases), post-hepatitis B liver cirrhosis (35 cases), alcoholic liver cirrhosis (1 case), primary biliary liver cirrhosis (2 cases) and congenital polycystic liver (1 case). Demographic and clinical data (including age, sex, clinical diagnosis, Child-Pugh classification of hepatic function, warm ischemic time, cold ischemic time, operative time, anhepatic phase and post-operative immunosuppression) are summarized in Table 1. With respect to warm ischemic time, cold ischemic time, operative time, anhepatic phase, ratio of Child-Pugh classification, immunosuppression and clinical diagnosis, there were no significant differences between the two groups in any of these above param-

Table 1 Clinical data of the enrolled patients

	PN group	PUFA group
Sex (M/F)	23/10	22/11
Age, yr	48.62 ± 14.61	51.52 ± 12.41
Clinical diagnosis		
Hepatic cell carcinoma	13	14
Post- hepatitis B liver cirrhosis	17	18
Alcoholic liver cirrhosis	1	0
Primary biliary liver cirrhosis	1	1
Congenital polycystic liver	1	0
Child-Pugh classification (A/B/C)	14/10/9	13/10/10
Warm ischemic time (min)	3.91 ± 1.16	4.15 ± 1.32
Cold ischemic time (min)	524.28 ± 132.83	506.56 ± 151.26
Operation period (min)	651.27 ± 181.42	626.39 ± 192.86
Anhepatic phase (min)	119.81 ± 82.35	142.15 ± 58.75
Immunosuppressive therapy (FK506 + P/CSA + P/CSA + P + MMF)	22/11/0	21/11/1

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; P: Prednisone; CSA: Ciclosporin A; MMF: Mycophenolate mofetil; FK506: Tacrolimus.

eters ($P > 0.05$).

Liver function assessment

No significant difference of pre-operative liver function was observed between the two groups. On days 2 and 9 after operation, a significant decrease of ALT (299.16 U/L ± 189.17 U/L *vs* 246.16 U/L ± 175.21 U/L, $P = 0.024$) and PT (5.64 s ± 2.06 s *vs* 2.54 s ± 1.15 s, $P = 0.035$) was seen in PUFA group compared with PN group. And there was no significant decrease of the following parameters tested on days 2 and 9: AST (116.31 U/L ± 42.19 U/L *vs* 121.09 U/L ± 53.14 U/L, P=0.682), TB (93.93 μmol/L ± 45.49 μmol/L *vs* 87.20 μmol/L ± 61.12 μmol/L, $P = 1.439$), DB (42.74 μmol/L ± 17.36 μmol/L *vs* 36.22 μmol/L ± 21.63 μmol/L, $P = 0.815$) and LDH (156.12 U/L ± 89.20 U/L *vs* 119.10 U/L ± 69.72 U/L, $P = 1.112$) in PUFA group compared with PN group (Table 2).

Light microscopy

The histological examination after reperfusion revealed some swelling hepatocytes and inflammatory cell infiltration in the portal areas, and no significant difference of numerical score of portal inflammation was observed between the two groups.

Histological examination on day 9 in PN group revealed more inflammatory cells aggregating in hepatic sinusoid lumen, extensive swelling and some balloon-like degeneration of hepatocytes, extensive congestion, and bilirubin deposit in the hepatic plasma (Figure 2A). These were ameliorated markedly by parenteral nutritional support with omega-3 fatty acids (Figure 2B), and the numerical score of portal inflammation was significantly lowered in PUFA group (Table 3). There was no sign of acute rejection in both groups.

Clinical outcome

There was no significant difference of post-transplant mechanical ventilation between the two groups ($P > 0.05$). Compared with PN group, the post-transplant hospital stay was significantly shortened in PUFA group ($P < 0.05$). Infectious complications occurred in 6 cases of PN group (2 cases of pneumonia, 3 cases of intra-abdominal abscess and 1 case of urinary tract infection), and in 3 cases of PUFA group (2 cases of pneumonia, 1 case of intra-abdominal abscess). No acute or chronic rejection and hospital mortality were found in the two groups. All patients were followed up, and the one-year mortality in PN group was 9.1% (3/33), one died of pulmonary infection, one died of severe intra-hepatic cholangitis and hepatic dysfunction and the other of hepatic cell carcinoma recurrence. Only one patient in PUFA group (1/33, 3.1%) died of biliary complication and hepatic dysfunction during follow-up (Table 4).

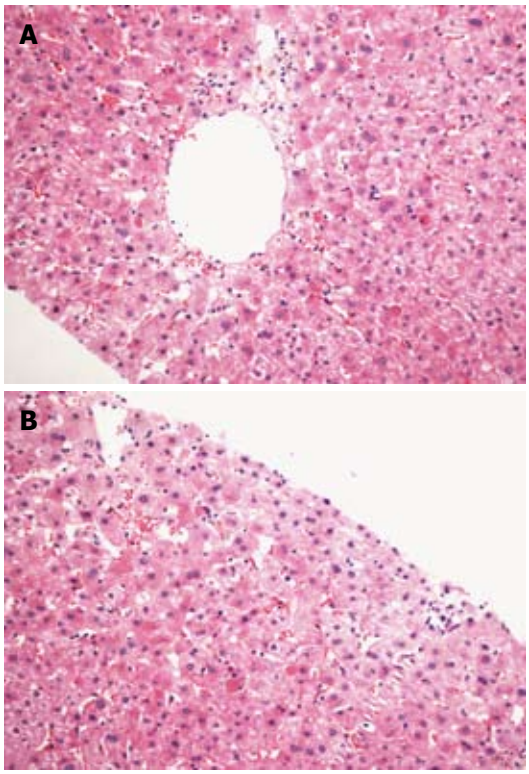

Figure 2 Histological appearance of the transplanted liver on day 9 after surgery. A: Extensive swelling, balloon-like degeneration and necrosis of hepatocytes, extensive congestion and bilirubin deposit were observed in the hepatic plasma in parenteral nutrition group; B: The results of histological examination were ameliorated markedly in the polyunsaturated fatty acid group. (Hematoxylin and eosin staining; paraffin-embedded 5 μm thick sections; ×200).

DISCUSSION

An impairment of nutritional status is a frequent finding in patients with end-stage liver disease. Malnutrition adversely affects the prognosis of these patients and is associated with the morbidity and mortality after liver transplantation[16]. Malnutrition has been shown to be the only independent risk factor for the length of stay in the intensive care and the total number of days spent in the hospital, and the liver recipient's nutritional status

Table 2 Effect of parenteral nutritional support with Omega-3 fatty acids on liver function

	Normal value	Group	Day 1	Day 2	Day 9	Decrease (Day 2-Day 9)
ALT (U/L)	5-40	PN group	198.16 ± 117.13	401.32 ± 215.35	155.16 ± 108.41[b]	246.16 ± 175.21
		PUFA group	227.16 ± 121.17	410.98 ± 201.64	101.82 ± 71.24[b]	299.16 ± 189.17[c]
AST (U/L)	8-40	PN group	95.12 ± 61.79	203.25 ± 73.49	82.16 ± 46.16[b]	121.09 ± 53.14
		PUFA group	115.62 ± 81.27	185.12 ± 42.16	68.81 ± 24.32[b]	116.31 ± 42.19
TB (μmol/L)	5-20.5	PN group	92.16 ± 42.15	158.32 ± 65.41	71.12 ± 55.12[a]	87.20 ± 61.12
		PUFA group	116.82 ± 61.65	160.34 ± 68.24	66.41 ± 61.52[b]	93.93 ± 45.49
DB (μmol/L)	1.7-6.8	PN group	52.15 ± 32.95	76.46 ± 31.28	40.24 ± 26.69[b]	36.22 ± 21.63
		PUFA group	47.39 ± 27.19	81.25 ± 26.32	38.51 ± 19.87[b]	42.74 ± 17.36
LDH (U/L)	109-245	PN group	266.25 ± 132.42	476.25 ± 98.15	357.15 ± 192.52[a]	119.10 ± 69.72
		PUFA group	226.45 ± 172.24	416.38 ± 151.14	260.26 ± 111.32[b]	156.12 ± 89.20
PT (s)	10-15	PN group	18.76 ± 3.21	17.16 ± 4.05	14.62 ± 3.87[a]	2.54 ± 1.15
		PUFA group	19.12 ± 4.16	17.81 ± 3.82	12.17 ± 3.69[b]	5.64 ± 2.06[c]

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DB: Direct bilirubin; LDH: Lactate dehydrogenase; PT: Prothrombin time. [a]$P < 0.05$, [b]$P < 0.01$ *vs* day 2; [c]$P < 0.05$ *vs* PN group.

Table 3 Effect of parenteral nutritional support with Omega-3 fatty acids on numerical score of portal inflammation

Group	Day 0	Day 9
PN group	3.3 ± 0.5	2.7 ± 0.9
PUFA group	3.6 ± 0.4	1.8 ± 0.6[a]

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid. [a]$P < 0.05$ *vs* PN group.

Table 4 Effect of parenteral nutritional support with Omega-3 fatty acids on clinical outcome

	PN group	PUFA group
Post-transplant mechanical ventilation (h)	12.1 ± 5.1	10.8 ± 5.4
Post-transplant hospital stay (d)	20.6 ± 4.6	18.7 ± 4.0[a]
Infectious morbidities	6/33	3/33[a]
Acute/chronic rejection	0/33	0/33
ICU mortality	0/33	0/33
Hospital mortality	0/33	0/33
28-d mortality	0/33	0/33
One-year mortality	3/33	1/33

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; ICU: Intensive care unit. [a]$P < 0.05$ *vs* PN group.

also influences the incidence of post-transplant complications and may therefore increase the costs of liver transplant[17,18]. After liver transplantation, surgical stress, postoperative fasting, and the possible occurrence of complications suggest the need for nutritional support. The primary goal of the nutrition support in the immediate post-transplant period is to provide adequate nutrition to promote recovery and replenishment of the depleted nutrient stores. Although most transplant centers use the similar post-transplant nutritional support as for other major abdominal operations, few studies have elucidated the role of postoperative nutritional support in the liver recipients.

Enteral nutrition is safer and less expensive than PN, and enteral nutrition has the potential advantage of maintaining intestinal trophism more effectively[19]. This effect may help prevent bacterial translocation and enteric-origin infections in patients treated with transplantation[20,21]. All patients in this study resumed their daily oral diet postoperatively as soon as bowel function returned to maintain intestinal trophism, but the recipients could not endure a large amount of liquid diet even with nasoenteric tube at the early phase after transplantation because of obvious abdominal pain, distention or diarrhea in our previous experience. The bowel function in all the patients in this study returned on day 3 or 4 after transplantation, and PN support discontinued on day 8 after surgery when the patients were able to maintain an adequate oral intake.

Omega-3 fatty acids mainly act as eicosapentaenoic acids (EPA) and docosahexaenoic acids (DHA), both had anti-inflammatory effects. EPA and DHA reduce the release of arachidonic acid-derived pro-inflammatory eicosanoids, and generate a group of lipid mediators called resolvins (E- and D-series) and protectins with potent anti-inflammatory and inflammation resolution properties[22,23]. Studies with experimental models of liver reperfusion injury have reported the beneficial actions of n-3 PUFA-derived resolvins and protectins in preventing liver DNA damage and oxidative stress, thus significantly ameliorating the necroinflammatory liver injury and hepatic steatosis[24-26]. The liver-protecting effects of postoperative PN support supplemented with omega-3 fatty acids were evaluated in this study. Liver enzyme of ALT released after I/R was significantly suppressed by the supplements of omega-3 fatty acids. PT, as an important parameter in evaluating the synthesis function of liver, was significantly decreased in PUFA group. And the results of histological examination on day 9 revealed that the hepatocyte injury and inflammatory cell aggregation were ameliorated markedly in PUFA group. PUFA therapy could also decrease the infectious morbidities, and shorten the post-transplant hospital stay significantly. The possible mechanisms of omega-3 fatty acids include down-regulation of the inflammatory responses to surgery and immune modulation rather than a sole nutritional effect.

Some of the patients exhibited an obvious nitrogen accumulation disorder reflected by either encephalopathy or an excessive rise in blood urea nitrogen in the immediate postoperative period. Branched-chain amino acids were chosen for this study because it can promote protein synthesis in patients with chronic liver diseases and avoid the additional metabolic load of transplanted liver[27]. Medium-chain triglycerides were included in the regimen to avert glucose intolerance and deposits in the transplanted liver. Based on the results of this study, we think that post-transplant nutritional support in the form of a solution enriched with branched-chain amino acids, dextrose, medium-chain triglycerides and omega-3 fatty acids might offer a benefit in terms of preserved liver function and better clinical outcome, including the decreased infectious morbidities and post-transplant hospital stay.

In conclusion, we have shown that omega-3 fatty acids-supplemented PN significantly improves the injury of transplanted liver, decreases the infectious morbidities, and shortens the post-transplant hospital stay.

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COMMENTS

Background

The liver recipients with liver insufficiency are known to experience a higher incidence of severe protein/calorie malnutrition, and malnutrition is associated with a greater risk of postoperative complications and mortality in patients undergoing liver transplantation. And ischemia/reperfusion injury associated with liver transplantation often leads to hepatic dysfunction despite the improvements in surgical techniques and perioperative medication.

Research frontiers

Omega-3 fatty acids exert anti-inflammatory and immunomodulatory properties through their ability to modulate the synthesis of different eicosanoids. Recent studies have described that supplementation with omega-3 fatty acids decreases the rate of inflammatory complication, the length of hospital stay, and the mortality after major abdominal surgeries.

Innovations and breakthroughs

Although most transplant centers use the similar post-transplant nutritional support as for other major abdominal operations, few studies have elucidated the role of postoperative nutritional support in the organ recipient. Based on the results of this study, the authors have shown that post-transplant nutritional support in the form of a solution enriched with branched-chain amino acids, dextrose, medium-chain triglycerides and omega-3 fatty acids might offer a benefit in terms of preserved liver function and better clinical outcome, including the decreased infectious morbidities and post-transplant hospital stay.

Applications

This study has shown that omega-3 fatty acids-supplemented parenteral nutrition (PN) significantly improves the injury of transplanted liver, decreases the infectious morbidities, and shortens the post-transplant hospital stay. The nutritional support strategy is recommended in patients undergoing liver transplantation.

Peer review

The manuscript evaluates the potential for supplementation with polyunsaturated fatty acid to ameliorate hepatic injury associated with reperfusion and PN. It provides evidences about an efficient nutritional support strategy for liver transplanted patients.

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BRIEF ARTICLE

Colorectal cancer lymph node staining by activated carbon nanoparticles suspension *in vivo* or methylene blue *in vitro*

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Abstract

AIM: To investigate whether activated carbon nanoparticles suspension (ACNS) or methylene blue (MB) can increase the detected number of lymph nodes in colorectal cancer.

METHODS: Sixty-seven of 72 colorectal cancer patients treated at our hospital fulfilled the inclusion criteria of the study which was conducted from December 2010 to February 2012. Seven patients refused to participate. Eventually, 60 patients were included, and randomly assigned to three groups (20 in each group): ACNS group (group A), MB group (group B) and non-stained conventional surgical group (group C). In group A, patients received subserosal injection of 1 mL ACNS in a 4-quadrant region around the mass. In group B, the main artery of specimen was identified and isolated after the specimen was removed, and 2 mL MB was slowly injected into the isolated, stretched and fixed vessel. In group C, no ACNS and MB were injected. All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and palpating the adipose tissue.

RESULTS: No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage, degree of differentiation, postoperative complications and peritoneal drainage retention time. The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient was significantly higher in group A than in group C (26.8 ± 8.4 *vs* 12.2 ± 3.2, $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 *vs* 12.2 ± 3.2, $P < 0.001$). However, there was no significant difference between group A and group B. There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B and 11 patients of group C, without significant differences among the three groups. Eleven of the 60 patients had insufficient number of detected lymph nodes (< 12). Only one patient with T_{4a} rectal cancer had 10 lymph nodes detected in group B, the other 10 patients were all from group C. Based on the different diameter categories, the number of detected lymph nodes in groups A and B was significantly higher than in group C. However, there was no statistically significant difference between group A and group B. The metastatic lymph nodes were not significant different among the three groups. Similarly, tumor location, T stage and tumor differentiation did not affect the staining results. Body mass index was a minor influencing factor in the two different staining methods. The stained lymph nodes can easily be identified from the mesenteric adipose tissues, and the staining time for lymph nodes was not significantly different compared with unstained group. None of the patients in groups A and B had drug-related complications.

CONCLUSION: Both activated carbon nanoparticles suspension *in vivo* and methylene blue *in vitro* can be used as tracers to increase the detected number of lymph nodes in colorectal cancer.

Key words: Nanotechnology; Activated carbon nanoparticles suspension; Methylene blue; Lymph nodes; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death among men and women in the United States with an estimated 143 460 new cases and 51 690 deaths in 2012 according to the statistics of American Cancer Society[1]. Accurate lymph node metastasis staging is of prognostic and therapeutic importance in patients with CRC. Previous researches have found that the number of lymph nodes evaluated after surgical resection was positively associated with the survival of CRC patients[2,3]. However, population-based data suggest that lymph node evaluation is not adequate in the majority of patients with CRC[4,5]. In addition, computed tomography (CT)[6], even positron emission tomography[7], are not efficacious enough in identifying nodal status. Therefore, a variety of techniques, including lymph node staining or radionuclide scan[8], have been applied to make lymph nodes retrieval more efficient.

The biological application of nanoparticles is a rapidly developing area of nanotechnology[9,10]. Particles have been observed passing through the lymphatic vessels but not the blood capillaries mainly due to the difference in permeability. Activated carbon nanoparticles suspension (ACNS), using smooth carbon particles at a diameter of 21 nm added with suspending agents, is a stable suspension of carbon pellets of 150 nm in diameter. ACNS is obviously inclined to the lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black. This unique selective biodistribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers and thermotherapy[11-13].

In our previous study[14], 62 patients with CRC were divided into two groups. The experimental group, using a simple lymphatic staining method, was injected with methylene blue (MB) into the regional main blood vessels immediately after specimens were resected *in vitro*. More and smaller lymph nodes could be detected, which significantly improved the lymph node harvest of resected colorectal specimens. However, the detection sensitivity for lymph node metastasis was low and the staining could not be done *in vivo* before the destruction of the lymphoid structures. Therefore, we conducted a randomized controlled trial to test whether MB and ACNS as tracers can increase the detected number of lymph nodes in the systematic nodal dissected tissues from CRC resection and compare the staining effect of the two methods in order to choose the best one for further clinical application.

MATERIALS AND METHODS

Patient selection

This trial was performed in the Department of Surgical Oncology, Second Affiliated Hospital Zhejiang University College of Medicine, China from December 2010 to February 2012. The study was approved by the Ethics Committee of Zhejiang University. Informed consent was obtained from all the patients. Inclusion criteria were as follows: 18-80 years of age; endoscopic biopsy confirmed; performance status of 0-1 on the Eastern Cooperative Oncology Group scale; good compliance; able to tolerate radical resection; adequate hematologic function [white blood cell (WBC) count > 4000/mL, absolute neutrophil count > 1500/mL, platelet count > 100 000/mL, and hemoglobin > 10 g/dL]; normal hepatic function [bilirubin < 1.5 the upper-normal limits (UNL) and alanine aminotransferase or aspartate aminotransferase < 2.5 UNL]; and normal renal function (creatinine < 1.5 mg/dL). Exclusion criteria included: clinical stage Ⅳ CRC according to the American Joint Committee on Cancer (AJCC); patients received chemotherapy, radiotherapy or biological therapy prior to surgery; previous abdominal surgery; significant neurological or mental disorder. Of the 72 CRC patients, 67 fulfilled the inclusion criteria. Seven patients refused to participate. The enrollment was completed when 60 patients were included. The patients were randomly allocated to three groups (20 patients in each group): ACNS group (group A), MB group (group B) and non-staining conventional surgical group (group C).

Surgical technique

All surgical procedures were completed by the same team of surgeons. Each patient was administrated with 2 g cephalosporin for antibiotic prophylaxis within 30 min before surgery, and the same dose was repeated if the operation lasted more than 2 h.

In group A, patients received subserosal injection of 1 mL ACNS (Chongqing LUMMY Pharmaceutical Co., Chongqing, China) in a 4-quadrant region around the mass (Figure 1A). To avoid surgical destruction of the lymphatic system along the bronchi and vessels, we waited for 10 min after injection. In group B, MB was injected by the same methods (Jiangsu Jumpcan Medicines Group, Taixing, Jiangsu Province, China), however, staining effect was very poor partly because MB was quickly absorbed *in vivo* and then excreted with urine.

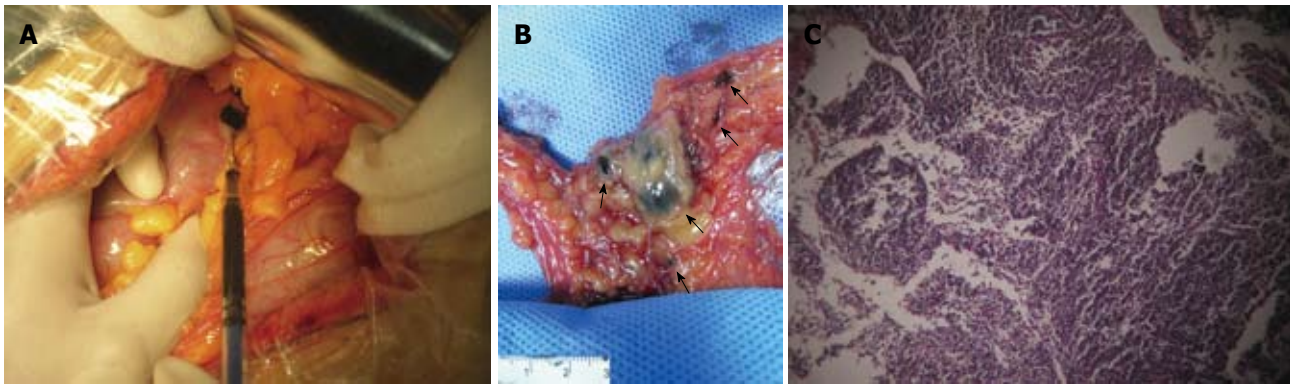

Figure 1 Activated carbon nanoparticles suspension *in vivo* is effective as a tracer in colorectal cancer lymph node detection. A: Subserosal injection of activated carbon nanoparticles suspension (ACNS) into a 4-quadrant region around the mass; B: Lymph nodes can easily be identified from the mesenteric adipose tissues, arrow points to the black dyed lymph nodes; C: ACNS migrating to the lymph node (hematoxylin and eosin, ×100).

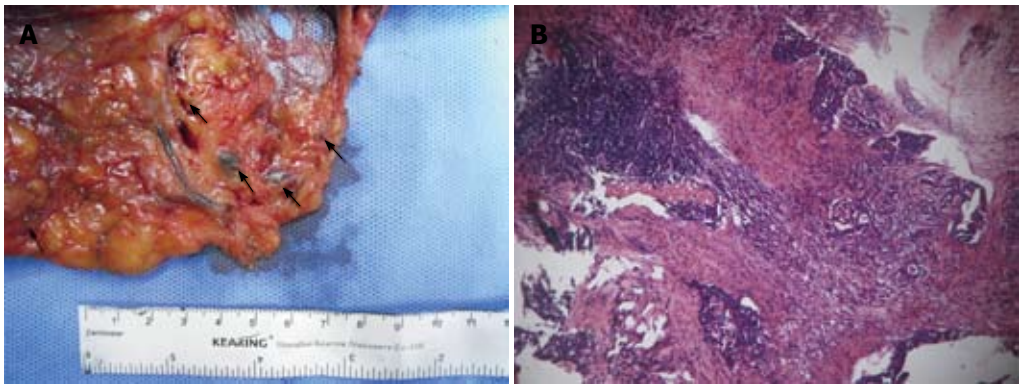

Figure 2 Methylene blue *in vitro* used as a tracer in colorectal cancer lymph node detection. A: The main artery of the specimen was isolated and methylene blue (MB) was injected into the vessel. Lymph nodes can easily be identified, arrow points to the blue dyed lymph nodes; B: MB migrating to the lymph node (hematoxylin and eosin, ×100).

Therefore, we established a method for lymph nodes staining *in vitro*. After the specimen was dissected, we immediately identified the main artery of the specimen and isolated it at the root for 1 cm, and injected 2 mL MB slowly into the isolated, stretched and fixed vessel (Figure 2A). We also waited for 10 min after injection. In group C, neither ACNS nor MB was used for the staining.

All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and palpating the adipose tissues. After identification and excision, all the black or blue nodes were collected for subsequent pathological examinations. Postoperative pain was relieved by intravenous opioid administration.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 for Windows (SPSS, Inc, Chicago, IL, United States). Factors considered to be possible determinants of the number of lymph nodes examined were first checked with the analysis of variance analysis and the influence of possible determinants was then tested in regression analysis. Kruskal-Wallis test was used when there was heterogeneity of variance. *P* value (two-tailed) of less than 0.05 was considered statistically significant.

RESULTS

Patient population

The 60 patients were randomly assigned to three groups. No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage and degree of differentiation. There was also no statistically significant difference in postoperative complications and peritoneal drainage retention time (Table 1).

Lymph nodes detected

The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient in group A was significantly higher than in group C (26.8 ± 8.4 *vs* 12.2 ± 3.2, $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 *vs* 12.2 ± 3.2, $P < 0.001$). However, there was no significant difference between group A and group B (26.8 ± 8.4 *vs* 23.8 ± 6.9, $P > 0.1$) (Figure 3A). There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B, and 11 patients of group C, without significant difference among the three groups ($P > 0.1$). According to the

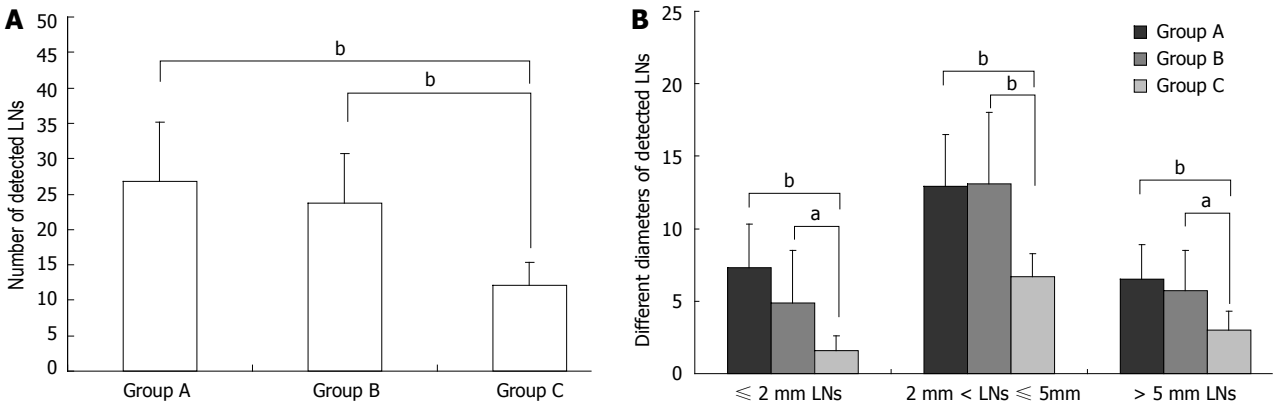

Figure 3 Lymph nodes detected in the three groups. A: The mean number of lymph nodes detected; B: Distribution of lymph node diameters. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* group A. LN: Lymph node.

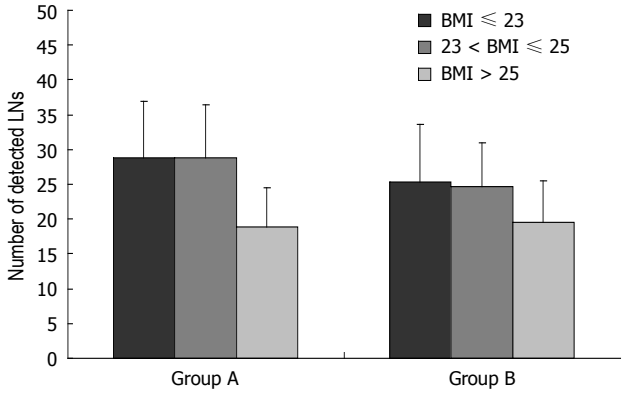

Figure 4 Body mass index as an influencing factor in the two different staining methods. LN: Lymph node; BMI: Body mass index.

AJCC guideline, more than 12 detected lymph nodes are required for the accurate clinical staging. In this study, 11 of the 60 patients had insufficient number of detected lymph nodes. Among them, only one patient in group B with T_{4a} rectal cancer had 10 lymph nodes detected, the other 10 patients were all from group C ($P < 0.001$). According to the different diameter range, lymph nodes (LNs) detected were divided into three categories: LNs ≤ 2 mm, 2 mm < LNs ≤ 5 mm, and LNs > 5 mm. We analyzed the different diameter categories of detected lymph nodes and metastatic lymph nodes in each group, and found that the number of detected lymph nodes was significantly higher in groups A and B than in group C. However, there was no statistically significant difference between group A and group B. Data is shown in Figure 3B. There was no significant difference in metastatic lymph nodes between each group ($P > 0.05$). Similarly, tumor location, T stage and tumor differentiation exerted no influence on the staining results ($P > 0.05$). Mesenteric hypertrophy may affect the lymph node detection by different staining methods[15]. To explore whether the mesenteric hypertrophy affects the staining results in our trial, patients in the two staining groups were re-classified according to the Asian body mass index (BMI) criteria as BMI ≤ 23 kg/m^2, 23 kg/m^2 < BMI ≤ 25 kg/m^{2}and BMI > 25 kg/m^2. Statistical analysis showed that BMI was a minor influencing factor in the two different staining methods while mesenteric hypertrophy did not influence the staining results (Figure 4). Using either ACNS (Figure 1B) or MB (Figure 2A), stained lymph nodes can easily be visualized from the mesenteric adipose tissues, and the staining time for lymph nodes was not significant different compared with unstained group ($P > 0.05$). Hematoxylin and eosin stained micrograph confirmed that ACNS migrates to the lymph nodes (Figure 1C), so does the MB (Figure 2B). None of the patients injected with either ACNS or MB had drug-related complications.

Table 1 Clinicopathological details of the 60 patients in the three groups

	Group A	Group B	Group C
No. of patients	20	20	20
Age (yr)	57.5 ± 11.5	58.9 ± 17.8	64.9 ± 7.4
Gender			
Male	14	14	13
Female	6	6	7
Tumor location			
Right colon	2	2	4
Transverse colon	1	1	1
Left colon	5	1	1
Sigmoid	5	4	4
Rectum	7	12	11
Tumor diameter (mm)	5.0 ± 1.7	4.7 ± 2.3	3.7 ± 1.1
T-stage			
T1	0	1	0
T2	2	4	4
T3	6	7	7
T4	12	8	9
Degree of differentiation			
Well	7	7	8
Moderate	10	11	11
Poor	3	2	1
Postoperative complications			
Bleeding	1	0	0
Infection	0	1	0
Fistula	1	1	2
Abdominal tube drainage (d)	6.7 ± 2.8	6.1 ± 3.3	7.8 ± 3.6

DISCUSSION

Lymph nodes status of colorectal cancer played a vital role in tumor staging, classification, postoperative sequential treatment and prognosis. The number of detected lymph nodes is a significant prognostic factor in colon cancer patients[16]. AJCC and College of American Pathologists recommend at least 12 lymph nodes detected for more accurate diagnosis of stage Ⅱ CRC[17]. However, recent studies indicated that lymph node detection rate was still low in CRC[4], which can not accurately reflect the patient's disease status. There are many factors affecting the lymph node detection, including patient's age, gender, tumor grade, extent of surgical resection and the pathologist's expertise[18]. Palpation is still the most important method for lymph node detection[19]. Numerous studies have been conducted to improve the methods for lymph node detection. Cawthorn *et al*[20] used xylene alcohol clearance technique to facilitate the identification of lymph nodes (23.1 ± 1.18 *vs* 10.5 ± 0.6). Quadros *et al*[21] performed lymphoscintigraphy using technetium-99 m-phytate and patent blue to detect lymph nodes of rectal adenocarcinoma patients, which significantly increase the lymph nodes detection rate, particularly lateral pelvic lymph node metastasis. However, these techniques were not widely used in clinical practice because they are time-consuming, labor-intensive and toxic to doctors. This clinical trial used the novel nanomaterials ACNS and MB *in vivo* or *in vitro*. The results suggested that both staining techniques can significantly improve the lymph node detection compared with the conventional palpation method (26.8 ± 8.4 *vs* 23.8 ± 6.9 *vs* 12.2 ± 3.2). In our research, none of the patients had insufficient number of detected lymph nodes in ACNS stained group, only one patient in MB stained group, but 10 patients did so from unstained group C. Statistical results showed that in different diameter categories, the number of the detected lymph nodes was significantly higher in both the two stained groups than in the unstained group (Figure 4), especially in the ACNS group. This demonstrated the obvious advantages of the two staining methods in detecting the smaller diameter lymph nodes. Micrometastasis is defined as cohesive deposits of tumor cells of 2 mm or less, but larger than 0.2 mm. This definition has been extended in the AJCC 7th edition to include non-cohesive infiltrate of > 200 cells as micrometastasis[22]. No definitive conclusions have been drawn about the role of sentinel lymph node biopsy and micrometastasis on the prognosis of CRC[23,24]. Taking into account the advantage of the detection for lymph node micrometastasis in this study, our team will further study the effects of sentinel lymph node biopsy and micrometastasis in the prognosis of CRC patients. In this trial, we failed to find significant differences between the two staining methods with regard to the total number of detected lymph nodes, lymph node diameters or lymph node metastasis, which may be attributed to the limitations of sample size.

The biological application of nanoparticles is a rapidly developing area of nanotechnology that raises new possibilities in the diagnosis[25] and treatment of human cancers[26,27]. Nanoparticles are being developed for contrast at T1-weighted magnetic resonance imaging[28], radionuclides for single photon emission computed tomography and positron emission tomograhpy[29], iodine for CT[30] and gas-containing bubbles for ultrasonography[31]. Several nanotechnologies have been used to improve the delivery of chemotherapeutic agents to cancer cells[32,33], which promoted the microdosing clinical studies[34]. A review by Schroeder *et al*[35] showed that nanoparticle therapies will improve the outcome for patients with metastatic cancer. Using ACNS combined with preoperative lymphoscintigraphy and intraoperative gamma probe detection, the detecting sensitivity was 100% for internal mammary sentinel node biopsy of breast cancer patients[36]. A clinical trial from him[37] showed that ACNS and MB both were effective as tracers in lymph nodes detection for non-small cell lung cancer. However, ACNS staining in colorectal cancer lymph nodes detection remains largely unexplored. MB is being widely used in clinical practice, however, when it was used in lymph nodes staining in CRC *in vivo*, it played a minor role in lymph nodes detection because it was absorbed and excreted too quickly. We thus used a modified method as described in the Surgical Technique, injected MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved much better staining results in lymph node detection. An ideal tracer should possess the following properties: not influenced by external conditions, easy to perform, effective and free from side-effects. We found that T stage, degree of tumor differentiation, tumor location, and BMI had minor influence on the two staining methods. In this study, black-stained or blue-stained lymph nodes can be easily identified from the mesenteric adipose tissues, and no side-effect was found among patients, surgeons and pathologists. However, the results obtained in this study may be limited by the sample size. Further studies with a larger sample will be conducted.

In conclusion, both ACNS *in vivo* and MB *in vitro* are effective as a tracer in increasing the detected number of lymph nodes in CRC. They may play a key role in the studies of sentinel lymph nodes biopsy and micrometastasis in CRCs.

COMMENTS

Background

Accurate lymph node metastasis staging has important prognostic and therapeutic implications in patients with colorectal cancer (CRC). Activated carbon nanoparticles suspension (ACNS) is obviously inclined to lymphatic system. The unique selective biodistribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers, and thermotherapy. Additionally, methylene blue (MB), as an efficacious and cost-effective tracer, was widely used in clinical practice.

Research frontiers

Numerous studies have been conducted to improve the methods of lymph node detection. The biological application of nanoparticles is a rapidly developing area of nanotechnology. However, ACNS as a tracer for lymph nodes detection

in CRC patients has not been reported. This study found that ACNS *in vivo* is effective in increasing the detected number of lymph nodes in CRC. Similarly, the authors used a modified method, injecting MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved analogous clinical effects. Moreover, the two staining methods were mildly influenced by T stage, degree of tumor differentiation, tumor location, and body mass index.

Applications

This is the first research that compares the staining effects of ACNS and MB in colorectal cancer lymph nodes detection. Both ACNS *in vivo* and MB *in vitro* are effective as tracers in increasing the detected number of lymph nodes in CRC.

Terminology

ACNS is a stable suspension of carbon pellets with a diameter of 150 nm, which is obviously inclined to lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black.

Peer review

The topic is very interesting and is of great clinical importance as the number of removed and histologically analyzed lymph nodes does determine the prognosis of CRC patients. An easy, time- and cost-effective method to help the surgeons' work is needed.

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CASE REPORT

Inverted Meckel's diverticulum as a cause of occult lower gastrointestinal hemorrhage

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Abstract

Meckel's diverticulum is a common asymptomatic congenital gastrointestinal anomaly, but rarely it can present with hemorrhage. Over the last few years inverted Meckel's diverticulum has been reported in the literature with increasing frequency as an occult source of lower gastrointestinal hemorrhage. Here, we report a case of a 54-year-old male, who was referred for surgical evaluation with persistent anemia and occult blood per rectum after a work up which failed to localize the source over 12 mo, including upper and capsule endoscopy, colonoscopy, enteroclysis, Meckel scan, and tagged nuclear red blood cell scan. An abdominal computed tomography scan showed a possible mid-ileal intussusception and intraluminal mass. During the abdominal exploration, inverted Meckel's diverticulum was diagnosed and resected. We review the literature, discuss the forms in which the disease presents, the diagnostic modalities utilized, pathological findings, and treatment. Although less than 40 cases have been reported in the English literature from 1978 to 2005, 19 cases have been reported in the last 6 years alone (2006-2012) due to improved diagnostic modalities. Successful diagnosis and treatment of this disease requires a high index of clinical suspicion, which is becoming increasingly relevant to general gastroenterologists.

Key words: Inverted Meckel's diverticulum; Gastrointestinal hemorrhage; Lower gastrointestinal bleeding; Intussusceptions

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INTRODUCTION

Meckel's diverticulum is the most common congenital abnormality in the gastrointestinal tract, but is usually asymptomatic[1]. When symptomatic it may present with hemorrhage in association with ectopic gastric and/or pancreatic mucosa, intestinal obstruction, intussusceptions, or inflammation. This abnormality can also present in the setting of an inverted diverticulum causing a lower gastrointestinal bleed. There is no role for non-operative

management in inverted Meckel's diverticulum, which mandates early surgical removal. Here, we present a case which first underwent an extensive diagnostic evaluation of persistent gastrointestinal hemorrhage over a 12-mo period before this entity was diagnosed and then appropriately treated. The aim of this report is to alert surgeons and gastroenterologists of this important source of persistent gastrointestinal hemorrhage which requires a high clinical suspicion to diagnose because of how difficult it is to detect. We conducted an extensive review of the previously reported cases in the literature, and discuss the presentations of this readily curable disease, the utility of various diagnostic modalities, pathological findings, and the appropriate management.

CASE REPORT

A 54-year-old male with no significant past medical history was seen by a gastroenterologist for anemia and stools positive for occult blood. An extensive diagnostic evaluation was undertaken over a 12-mo period before the patient was referred for surgical evaluation. He had a negative upper gastrointestinal and capsule endoscopy, and a negative colonoscopy, despite adequate bowel preparation. Enteroclysis revealed a polypoid lesion in the mid jejunum. The differential diagnosis at that point included tumor, lipoma, carcinoid, or sarcoma. He underwent a Meckel scan, which was negative. Given the continued anemia and occasional bright red blood per rectum, he underwent a tagged nuclear red blood cell scan, which failed to demonstrate an acute hemorrhagic source. An abdominal computed tomography (CT) scan was obtained, which demonstrated a possible mid-ileal intussusception and an intraluminal mass. The patient was referred for a surgical evaluation.

The patient underwent an exploratory laparoscopy which demonstrated a hard intraluminal mass in the mid-ileum. The remainder of the small bowel was normal to the level of the ligament of Treitz. A 5 cm mini midline-laparotomy was performed overlying the small bowel lesion. The lesion containing portion of the small bowel was delivered out of the abdomen and resected. Small bowel continuity was established *via* a stapled side-to-side anastomosis.

On gross examination, the specimen consisted of a segmental resection of the small bowel with a small dimple shaped defect penetrating through the bowel wall. This defect was proven to be patent by a probe. Opening of the specimen revealed a 5.0 cm × 1.8 cm × 1.5 cm polypoid lesion that terminated in a club-shaped head (Figure 1). The small bowel mucosa was contiguous with that of the stalk and extended to within 1.5 cm of the tip of the lesion. The dumbbell shaped tip was covered by attenuated mucosa that was granular in appearance. A small area of erythema was seen at the tip and appeared grossly consistent with an area of hemorrhage.

Microscopic sections revealed well-defined intestinal mucosa that surrounded the core of the lesion (Figure 2). The most internal component of the core consisted of the serosa and the muscle of the bowel wall. These layers were circumscribed by the intestinal submucosa and mucosa that became attenuated toward the tip of the specimen. The mucosa seen at the tip was that of intestinal-type with scattered paneth cells (exocrine serous cells) and goblet cells. No heterotopic gastric or pancreatic tissue was identified. The patient had an uneventful post-operative recovery. He was discharged on post-operative day three, and never had further episode of lower gastrointestinal hemorrhage nor any complications or sequelae during the subsequent two years of follow up.

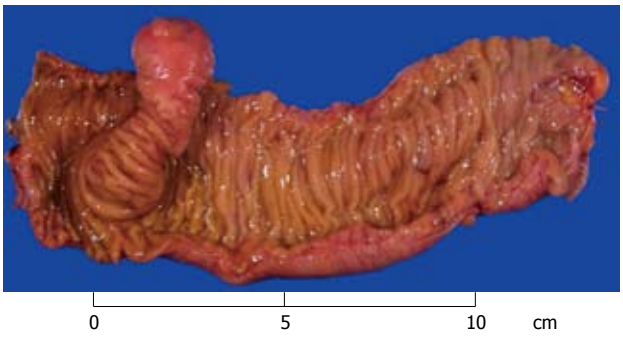

Figure 1 Gross specimen of the inverted Meckel's diverticulum arising in the segmental resection of the small bowel.

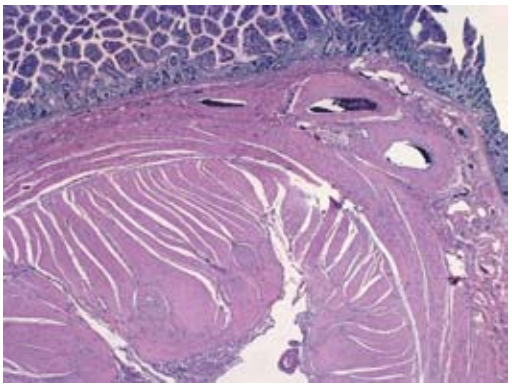

Figure 2 Histopathology reveals a central core consisting of serosa and muscle. The cross section reveals small intestinal mucosa lining the fragment.

DISCUSSION

Meckel's diverticulum is the most common congenital anomaly in the gastrointestinal tract[1]. In an autopsy series, the incidence was reported as 1%-3%[2]. Johann F Meckel, a famous anatomist, was the first to describe this entity. In 1808, he stated that the diverticulum comprised a remnant of the vitelline duct, the duct between the intestinal tract and the yolk sac[3]. In normal human embryology, the vitelline duct closes by the 10th week of gestation. If it persists, presentations include incidental meckel's diverticulum, fibrous cord connecting the bowel to the anterior abdominal wall, persistent omphalenteric fistula, enterocystoma, torsion, and intussusception[4]. Although the location of the diverticulum varies, they have been classically described on the antimesenteric surface of

Table 1 Clinical characteristics, diagnostic modalities, and pathological finding of patients with inverted Meckel's diverticulum

Clinical characteristics	*n* (%)
Gender[4-7,9-12,14-43]	
Male	41/59 (69)
Female	18/59 (30)
Signs and symptoms[4-7,9-12,14-34,36-43]	
Bleeding	48/59 (81)
Abdominal pain	41/59 (69)
Intussusception	23/59 (39)
Anemia	47/59 (80)
Diagnostic modalities[4-7,9-12,14-34,36-43]	
Positive upper endoscopy	2/58 (3)
Positive lower endoscopy	4/59 (7)
Positive abdominal ultrasonography	12/13 (92)
Positive tagged RBC scan	0/3 (0)
Positive Meckel's scan	0/3 (0)
Positive barium enema	0/0
Positive enteroclysis	7/7 (100)
Positive upper GI series with small bowel follow through	18/21 (86)
Positive abdominal CT scan	24/24 (100)
Pathologic findings[4-7,9-12,14-34,36-43]	
Ulceration	40/59 (68)
Ectopic gastric tissue only	18/59 (31)
Ectopic pancreatic tissue only	13/59 (22)
Ectopic gastric and pancreatic tissue	4/59 (7)
No ectopic tissue	24/59 (41)

GI: Gastrointestinal; CT: Computed tomography; RBC: Red blood cells.

the ileum within 100 cm of the ileocecal valve[5]. Possible complications include hemorrhage, obstruction, diverticulitis, hernia, tumor, and inflammation, one of which an estimated 2% of those with Meckel's diverticulum will develop[1,2]. The presentation of these complications often produces a complex constellation of recurrent symptoms consistent with obstruction, chronic abdominal pain and lower gastrointestinal hemorrhage which commonly delays diagnosis and definitive surgical treatment[5].

An inverted Meckel's diverticulum is a condition where the Meckel's diverticulum literally inverts on itself; however, the pathophysiology underlying this rare phenomenon is not clearly understood. One theory is that there is abnormal peristalsis of the bowel segment in the proximity of the Meckel's diverticulum, possibly due to the tissue present at the base of the diverticulum itself, which causes the diverticulum to invert. Because of the inversion of the Meckel's divertiuculum it may be difficult for diagnostic studies which rely on access to the lumen of diverticula, such as capsule endoscopy and colonoscopy, to identify this rare lesion. This inversion of the Meckel's diverticulum can then also lead to a complete intussusception of the bowel or to a compromise in blood flow to that bowel, ulceration and then gastrointestinal hemorrhage[6].

Because of the clinical challenge of diagnosing this rare entity, inverted Meckel's diverticulum has been reported in less than 70 cases reported in the English literature. However, as diagnostic modalities have improved the reports of this disease as an occult source of hemorrhage has increased from 40 cases from 1978 to 2005, to 19 cases reported in the last 6 years alone (Table 1). The largest series thus far reported included 18 cases, between 1971-1995 from the Armed Forces Institute of Pathology (AFIP)[7], and the most recent systematic review was in 2005 before the surge in reports[5]. Therefore, our report and review comprehensively reviews all these cases in the literature to further guide clinicians in the approach to the diagnosis and treatment of this readily curable disease (Table 1).

The median age of presentation of inverted Meckel's diverticulum is 27.7, slightly younger than reported by the AFIP, which was 33, with a male to female ratio of approximately 2.33:1. The most common presenting complaint was bleeding in 48 of 59 cases (80%), anemia 47 of 59 cases (78%), and abdominal pain (68%) (Table 1). With the most common presentation being lower gastrointestinal bleeding, it is not surprising that most reported cases included a thorough work up for the source of hemorrhage[5]. In most cases involving bleeding and anemia, patients underwent an upper and lower endoscopy with negative results.

The first reported radiographic description of an inverted Meckel's diverticulum was by Fetterman *et al*[8] in 1968 and there has been a great proliferation of diagnostic modalities available to clinicians[5]. A Meckel's scan, a radionucleotide scan that detects gastric mucosa, can be a useful diagnostic tool, especially in the pediatric population. It has a high sensitivity but low specificity. Only 50% of cases are believed to be associated with ectopic gastric or pancreatic mucosa, but it is seen in 75% of those presenting with symptoms[9]. Meckel scans were reported in only three cases[4,10,11] which were negative. One of these actually had both ectopic gastric and pancreatic mucosa[4]. This strongly suggests that a negative Meckel scan does not rule out the diagnosis, which was exemplified in our case.

Other radiologic diagnostic modalilties include ultrasonography, which demonstrated positive or clinically influential findings in 12 of 13 cases (Table 1). These findings were often non-specific and only prompted surgical exploration in one case of a post-operative bowel obstruction caused by an intussusception from the inverted Meckel's diverticulum which was detected by ultrasound[12]. Some of the nonspecific findings include "eggplant shaped mass within the bowel"[13], fluid filled target[14], and distended loops of bowel with free fluid[15]. The use of a tagged nuclear red blood cell scan was reported in three cases with negative results, and barium enema has been of little use (Table 1). When a patient presents with massive hemorrhage, angiography may be useful, especially in a hemodynamically unstable patient. In a single reported case of angiography used for the diagnosis of Meckel's diverticulum, angiography revealed a vitelline artery centrally located in the ileal lumen[10].

The three most useful tools employed for the diagnosis of inverted Meckel's diverticulum include small bowel follow-through, enteroclysis, and abdominal CT scans[4,13,16]. When the scan reveals a mass, it often is as-

sociated with a central area of fat density[5]. Small bowel follow-through was helpful in 18 of 21 cases. Findings include mass-like lesions, polypoid filling defects, and ulcerations. CT scans have been extremely helpful, especially recently with improving technology. CT scan was used in 24 of the reported cases, and all revealed useful information that ultimately led to an operation. They were especially useful when intussusceptions were found in association with the characteristic "target sign". In adults, intussusceptions with clinical symptoms are a clear indication for operation. In the pediatric population, however, even intussusceptions caused by an inverted Meckel's diverticulum can be treated non-surgically with barium enema reduction[17]. When the small bowel is highly suspected as the source of hemorrhage, enteroclysis has been suggested as the single best study in the diagnosis of inverted Meckel's diverticulum. In 7 reported cases, all seven were useful, as they revealed filling defects and polypoid lesions (Table 1).

In the literature review of the specimens, the average length of the inverted segment was approximately 3.99 cm. Ulceration has been reported in the adjacent ileal mucosa, in the Meckel's segment, and in the tip. There appears to be no direct correlation with the presence or absence of ectopic tissue. The ulceration seen in cases without gastric mucosa may be explained by either ischemia and/or trauma[5]. Fifty-eight percent of the reported cases were associated with ectopic mucosa of either gastric or pancreatic origin (Table 1).

The preferred treatment of any symptomatic Meckel's diverticulum is surgical. Whenever an inverted Meckel's diverticulum is diagnosed either pre-operatively or intra-operatively, the surgical procedure should be segmental resection with reestablishment of bowel continuity. Intussusception was noted in our case preoperatively. In our literature review, twenty three cases documented active intussusception at the time of operation (Table 1). There was one report of an endoscopic mucosal resection which resulted in iatrogenic perforation requiring emergent laparotomy[18]. It has been the general consensus that intussusceptions in the adult should be treated with resection and primary anastomosis[11]. Although most reports have described laparotomy, some minimally invasive techniques have been described in the literature. El-Dhuwaib *et al*[14] and Karahasanoglu *et al*[15] reported exploration and resection laparoscopically for an inverted Meckel's diverticulum. However, others have reported that manual palpation or laparoscopic inspection of the small bowel itself is not enough and may lead surgeons to miss the diagnosis[17]. In our case, we planned on an initial abdominal exploration laparoscopically, and if unsuccessful, had planned on conversion to an open laparotomy with possible intra-operative endoscopy. Fortunately, we were able to find the lesion laparoscopically and performed the resection *via* a mini-laparotomy, which has the potential to provide less morbidity than a larger laparotomy incision. Our approach provided adequate exposure to achieve appropriate margins if the lesion had been found to have been malignant. In cases where even open laparotomy fails to localize the lesion, the successful use of intra-operative endoscopy to localize the lesion and guide treatment of inverted Meckel's diverticulum has been reported[19].

Bleeding seen in inverted Meckel's diverticulum cannot be attributed entirely to ulceration secondary to gastric mucosa, and it may be due to trauma or inversion induced mucosal ischemia[5]. In fact, most cases did not have gastric cells present (Table 1). Trauma, due to its location within the lumen, is likely a primary source of bleeding in most cases reported in association with normal intestinal mucosa[5,13,20-22].

There is some debate as to the treatment of incidentally discovered Meckel's diverticulum in the asymptomatic patient. Resection is generally recommended for patients younger than 40, diverticulum longer than 2 cm, divertula with narrow necks, fibrous bands, ectopic gastric tissue, and/or when the diverticulum appears thickened and inflamed. When a Meckel's diverticulum is discovered as the lead point to an intussusception, it is thought to be a primary pathologic process, and not a secondary process[5,17]. The exact cause of the inversion is not yet understood. Intussusceptions are primarily seen in children under the age of 2, and only 5% of all intussusceptions are seen in adults[1]. In children however, there is no lead point in 95% of the cases[23].

Multiple diagnostic modalities have been described in the diagnosis of inverted Meckel's diverticulum. In instances of lower gastrointestinal hemorrhage, it is appropriate to first exclude an upper gastrointestinal source and a colonic source. Based upon a review of the literature, the studies recommended when inverted Meckel's diverticulum is suspected are CT scans and enteroclysis. However, to make this difficult diagnosis requires a high index of suspicion with an awareness of this important pathologic process and its unique presentation.

Although Meckel's diverticulum is usually an asymptomatic common congenital abnormality of the gastrointestinal tract, it can present with lower gastrointestinal hemorrhage[35]. In the case of inverted Meckel's diverticulum, the bleeding may be due to the presence of ectopic gastric mucosa, but may also be commonly due to trauma or inversion induced ischemia. In a patient presenting with lower gastrointestinal bleeding, upper and lower endoscopy can be used to rule out a source. If these modalities are negative and Meckel's diverticulum is suspected, CT scan or enteroclysis may be more helpful in the diagnosis than other modalities, and its wider use may account for the increase in reports of this rare disease in the literature. Treatment usually provides a complete cure when it entails operative resection, either *via* an open or laparoscopic approach with possible intra-operative endoscopy. Because of the nonspecific presentation of inverted Meckel's diverticulum as an occult source of lower gastrointestinal hemorrhage, it is important for gastroenterologists and surgeons to understand the pathophysiology, appropriate diagnostic approach and therapeutic management of this readily curable disease.

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CASE REPORT

Enterolithiasis-associated ileus in Crohn's disease

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Abstract

Stasis of the flow of the intestinal contents, ingested material and unfavorable composition of the chylus can lead to the formation of enteroliths inside the bowel. Enterolithiasis represents a rare disorder of the gastrointestinal tract that can be associated with intermittent abdominal pain or more serious complications such as bleeding or obstruction. Enterolithiasis in Crohn's disease represents an extremely rare condition and usually occurs only in patients with a long symptomatic history of Crohn's disease. We report an unusual case of enterolithiasis-related intestinal obstruction in a young male patient with Crohn's disease (A2L3B1 Montreal Classification for Crohn's disease 2005) undergoing emergency laparotomy and ileocoecal resection. In addition, we present an overview of the relevant characteristics of enterolithiasis on the basis of the corresponding literature.

Key words: Crohn's disease; Enterolithiasis; Ileus; Inflammatory bowel disease; Obstruction

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INTRODUCTION

As enterolithiasis of the small bowel is a rare condition, only a few cases of enterolithiasis-related small bowel ileus in patients with Crohn's disease have been published. A PubMed database analysis showed 9 results published between 1972 and 2010 when searching for "enterolithiasis + crohn"[1,2]. To date, about 20 cases of Crohn's disease-associated enterolithiasis have been reported in the literature. Here we present the case of a Crohn's disease patient with enterolithiasis-related small bowel obstruction and discuss the clinical and radiological features of this rare entity on the basis of the current literature.

CASE REPORT

A 46-year-old male patient (nonsmoker) presented with acute diffuse abdominal pain, constipation and vomiting at the emergency unit of the department of surgery. His past medical history included laparoscopic cholecystectomy for symptomatic cholecystolithiasis several years ago and a 12-year history of Crohn's disease. At the time of admission the patient was treated with budesonide, enteroclysma and 5-aminosalicylic acid (A2L3B1 Montreal Classification for Crohn's disease 2005). The clinical examination showed no fever, a soft, but distended abdomen without abdominal guarding. Blood investiga-

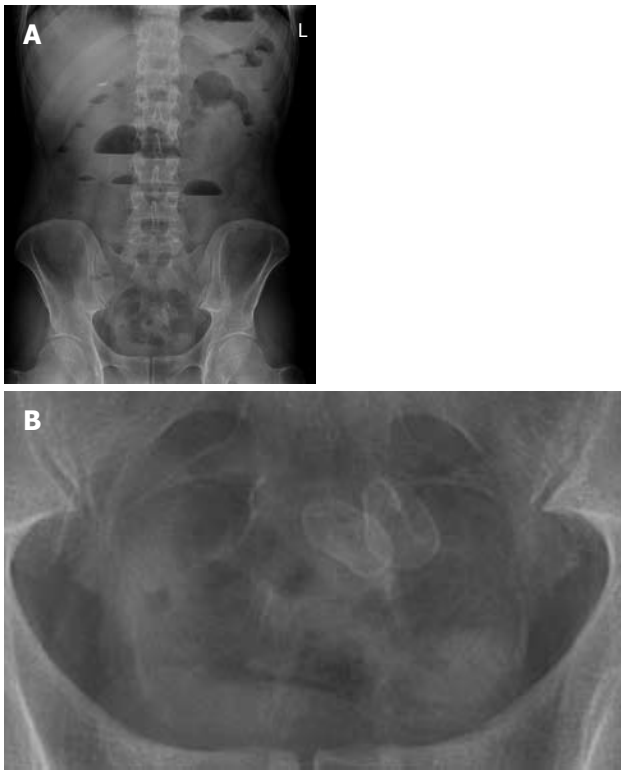

Figure 1 X-ray of the abdomen with two small radiopaque enteroliths in the lower abdomen. A: Overall picture; B: Enlarged detail. L: Left.

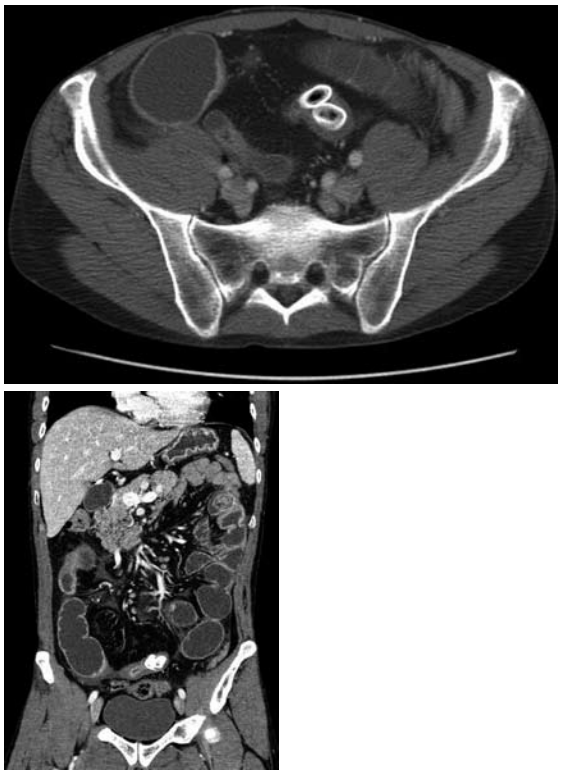

Figure 2 Computed tomography images with two radiopaque enteroliths.

tion revealed a normal leucocyte count and a marginally increased level of C-reactive protein. On the abdominal X-ray, the typical imaging of an incipient small bowel ileus with dilated small bowel loops was apparent (Figure 1A), however, two conspicuous small shadows in the pelvis were also observed (Figure 1B). Consequently, a computed tomography (CT) scan of the abdomen was performed to confirm or exclude a mechanical obstruction. The CT scan showed a significant small bowel stricture and two adjacent intraluminal radiopaque, dense calcified enteroliths with a maximal diameter of 20 mm in the terminal ileum with significant wall thickening, acute inflammatory signs and prestenotic dilatation of the small bowel indicating a mechanical ileus (Figure 2). The patient underwent immediate emergency laparotomy: the intraoperative findings revealed a long (30 cm), dense narrowing of the terminal ileum with irremovable stony palpable masses, poststenotic emptiness and prestenotic dilatation of the bowel (Figure 3). An ileocoecal resection of about 40 cm of the terminal ileum with a side to side ileoascendostomy reconstruction was conducted (Figure 4). Histopathological examination of the resected specimen confirmed the diagnosis of an acute inflammatory episode of Crohn's disease without any sign of malignant transformation or perforation. After an uneventful postoperative stay of 11 d, the patient was discharged home with Crohn's disease specific medication (metronidazole, azathioprine) recommended by the gastroenterologists to prevent postoperative bacterial infection and to control inflammatory activity.

DISCUSSION

The first description of enterolithiasis in the scientific literature can be traced back to 1917, when Pfahler *et al*[3] published a report on the radiological features of enterolithiasis. It was not until 1959 that Atwell *et al*[4] introduced the definition of enteroliths as "endogenous foreign bodies in the gastrointestinal tract". According to a classification by Grettve *et al*[5], enteroliths can be divided into primary and secondary enteroliths. Primary enteroliths always develop inside the bowel, and can be caused by concretions of normal chylus components such as choleic acid, calcium phosphate and calcium carbonate (so called "true" primary enteroliths) or by ingested indigestible material such as hair (trichobezoar), vegetables (phytobezoar) or other exogenous substances such as barium sulfate (so called "false" primary enteroliths). In contrast, secondary enteroliths typically form outside the bowel and then pass into the bowel like gallstones which fistulate into the bowel causing peritonitis and/or mechanical obstruction. The most common localization of enteroliths is the colon including the appendix vermiformis (appendicoliths), however, enteroliths can occur throughout the entire gastrointestinal tract from the stomach to the rectum.

Stasis or decelerated motility of the gastrointestinal tract seems to be the crucial pathogenetic factor in the development of enteroliths, since an unimpaired continuous flow of the gastrointestinal content usually does not allow the formation of enteroliths[5]. However, when stasis occurs, small particles have enough time to crystal-

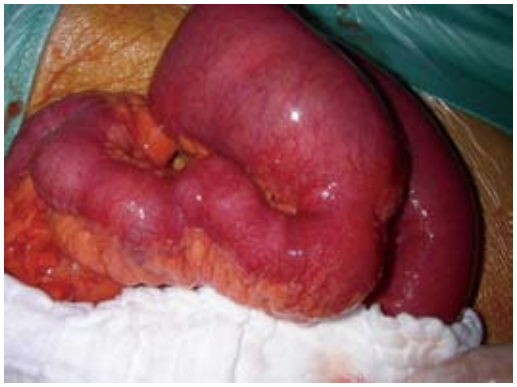

Figure 3 Intraoperative illustration of the small bowel ileus with evident stenosis and prestenotic dilation.

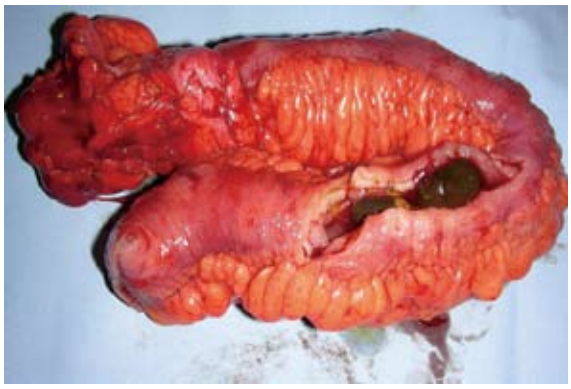

Figure 4 Resected specimen of the cecum and small bowel with two fixed enteroliths causing small bowel obstruction.

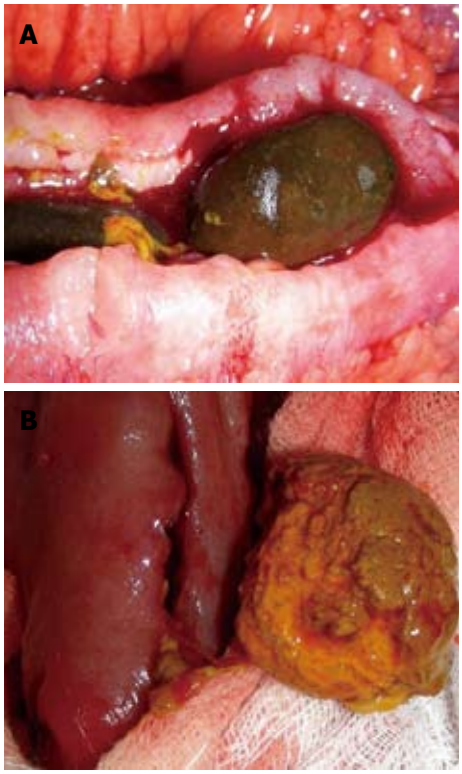

Figure 5 Typical images of enteroliths. A: Long-standing enterolith with blank-looking polished surface; B: Emerging enterolith with a rough and fragile surface.

lize and form accumulating stones. Stasis is always associated with anatomic alterations of the gastrointestinal tract such as harmless anatomic variations (diverticula, duplication cysts)[6,7] or pathologic conditions (strictures) due to bowel diseases such as Crohn's disease or tuberculosis[8-10]. In addition, stasis may also develop after bowel resection with a broad side to side enteroanastomosis with a big blind pouch allowing for continuous deposition of intestinal contents[11]. Enteroliths can be asymptomatic and dissolve spontaneously passing slowly through the gastrointestinal tract until excretion. However, in most cases enterolithiasis is associated with recurrent signs of intestinal obstruction and intermittent abdominal pain. In addition, refractory chronic anemia and perforation have also been reported[4,12]. Interestingly, the patient in our case report did not really suffer from any of these symptoms, although both enterolithiasis and Crohn's disease represent chronic disorders. Obviously, there was a moderate postinflammatory, quite asymptomatic stricture of the terminal ileum which permitted very slow but continuous development of enteroliths without significant alteration of intestinal passage. The actual inflammatory episode then led to complete intestinal obstruction due to thickening of the bowel wall. We suggest that the enteroliths developed primarily on site, since they were almost completely fixed in the diseased bowel segment. Additionally, the enteroliths stood out with a very hard consistency and blank-looking, polished surface (Figure 5A). About 20 cm proximal of the stricture another enterolith was detected in the resected specimen with completely different characteristics: this enterolith was morbid, fragile, rough and easily movable, potentially because there was no stricture (Figure 5B). We hypothesize that this additional enterolith represents the typical status nascendi of an enterolith which is usually not observable, since it is asymptomatic and potentially excreted.

The radiological diagnosis of enteroliths depends on their calcium content[13,14]. In our case, two radiopaque enteroliths were clearly visible on abdominal X-ray, whereas the third mobile enterolith was not detectable. The differential diagnosis of radiopaque enterolith-like alterations includes gallstones, urolithiasis-calcified lymph nodes or pancreatic calcifications. Of note, enteroliths may change their localization on radiographs due to the mobility of the small bowel[9].

Due to its typical strictures impairing the intestinal flow, Crohn's disease offers favorable conditions for the development of enteroliths. Nevertheless, enteroliths are a very rare condition in Crohn's disease, since most patients never become symptomatic or because symptoms are not attributable to enteroliths. Studies have proven that enterolithiasis in Crohn's disease occurs only in patients with a long history of the disorder and long duration of symptoms of between 7 and 40 years (median

15.7 years)[9]. Additionally, a few cases of enterolithiasis-associated adenocarcinomata have been reported in the literature[15]. In summary, there is no evidence for prophylactic treatment of asymptomatic enterolithiasis in Crohn's disease, although enteroliths represent clear indicators of a stenotic condition. Patients with intestinal obstruction require laparotomy with stricturoplasty or segmental bowel resection.

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CASE REPORT

An aortoduodenal fistula as a complication of immunoglobulin G4-related disease

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Author contributions: Sarac M, Marjanovic I and Bezmarevic M operated on the patient;Bezmarevic M took the photos and researched sources for the references;Bezmarevic M, Petrovic S, Sarac M and Marjanovic I were engaged in the postoperative treatment;Sarac M, Bezmarevic M, Zoranovic U and Petrovic S participated in the follow up; Mihajlovic M and Petrovic S helped in the interpretation of photos and helped draft the final version of the manuscript;all authors participated in writing the case report and revising the draft.
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Abstract

Most primary aortoduodenal fistulas occur in the presence of an aortic aneurysm, which can be part of immunoglobulin G4 (IgG4)-related sclerosing disease. We present a case who underwent endovascular grafting of an aortoduodenal fistula associated with a high serum IgG4 level. A 56-year-old male underwent urgent endovascular reconstruction of an aortoduodenal fistula. The patient received antibiotics and other supportive therapy, and the postoperative course was uneventful, however, elevated levels of serum IgG, IgG4 and C-reactive protein were noted, which normalized after the introduction of steroid therapy. Control computed tomography angiography showed no endoleaks. The primary aortoduodenal fistula may have been associated with IgG4-related sclerosing disease as a possible complication of IgG4-related inflammatory aortic aneurysm. Endovascular grafting of a primary aortoduodenal fistula is an effective and minimally invasive alternative to standard surgical repair.

Key words: Abdominal aortic aneurysm; Aortoduodenal fistula; Endovascular repair; Immunoglobulin G4-related disease

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INTRODUCTION

A primary aortoenteric fistula (AEF) is a communication between the aorta and the intestines, and occurs in a setting without prior aortic surgery. It is a rare but potentially lethal condition with an incidence of 0.04% to 0.07%[1]. Primary fistulas; are in most cases (90%), the result of erosion of the bowel wall, caused by an abdominal aortic aneurysm (AAA)[2]. The majority occur between the aorta and the duodenum[3]. Primary aortoduodenal fistulas (ADF) have been reported in the presence of various conditions, including underlying atherosclerotic aortic aneurysm disease, gallstone erosion, foreign body ingestion, and invasive intra-abdominal malignancies[4]. However, it has been reported that an aortic

aneurysm can be a part of a spectrum of immunoglobulin G4 (IgG4)-related sclerosing disease[5]. Most reported cases of IgG4-related inflammatory aortic aneurysm of abdominal aorta (IAAA) have no association with other IgG4-related sclerosing diseases. Without apparent pathological conditions in other organs, confirmation of the existence of IgG4-related IAAA requires histopathological evidence[6-10]. An ADF as a complication of IgG4-related disease has not yet been reported. Endovascular aortic repair is an effective alternative to standard open surgical management of primary aortoenteric fistulae, especially in situations where an open surgical procedure is either difficult or contraindicated[11]. In this article we present the urgent endovascular repair of an ADF in a patient with high serum IgG4 levels.

CASE REPORT

A 56-year-old Caucasian male was admitted to our institution due to severe anemia caused by gastrointestinal hemorrhage. He had chronic fatique and at least one episode of melena every day of one month duration. At admission he had anemia with a red blood cell count (RBC) of 2.8 million per μL, hematocrit (HCT) of 25% and blood pressure of 90/70 mmHg with a heart rate of 110 beats/min. No abdominal pulsating mass was found on physical examination. A digital rectal examination showed signs of melena.

The patient had undergone previous abdominal surgery in another medical institution (9 years before), due to acute generalized abdominal pain. He had no medical documentation, and we were unable to obtain more information about his previous condition.

Urgent upper endoscopy (EGDS) did not show any signs of bleeding. Abdominal ultrasound showed an infrarenal AAA that was verified on computed tomography (CT) angiography (Figure 1). During the same CT evaluation a gas collection in a suspected thrombotic mass in an aneurysmatic sac was noted, suggesting an AEF. The CT findings excluded the presence of autoimmune pancreatitis.

Because there was a suspicion of Meckel's diverticulum, we performed abdominal scintigraphy, without any pathological findings. Colonoscopy findings were normal. In addition to administering blood and blood product substitution therapy, standard therapy was also administered, however, melena persisted. Control EGDS findings were normal. Due to worsening of the patient's general condition [persistent melena followed by anemia; RBC 3.1 million per μL, HCT 27% and C-reactive protein (CRP) 82 mg/L], the case was presented at a meeting of gastroenterologists, vascular and abdominal surgeons, radiologists and anesthesiologists, and it was decided that operative treatment was necessary. We performed an emergency endovascular repair of the AAA using an Excluder Stent Graft (Excluder®, WL Gore and Associates, Flagstaff, Arizona, United States) (Figure 2).

We initiated empiric broad spectrum intravenous

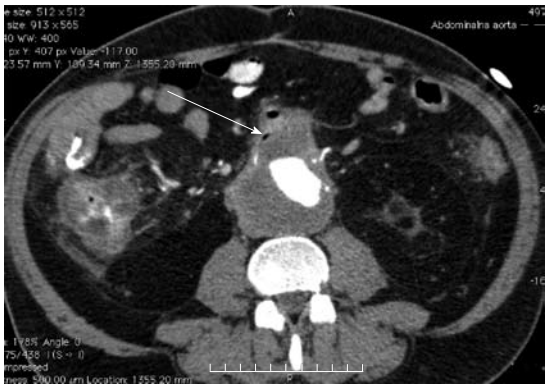

Figure 1 An infrarenal aneurysm (arrow) of the abdominal aorta with gas collection in a suspected thrombotic mass in the aneurysmatic sac, suggesting an aortoenteric fistula.

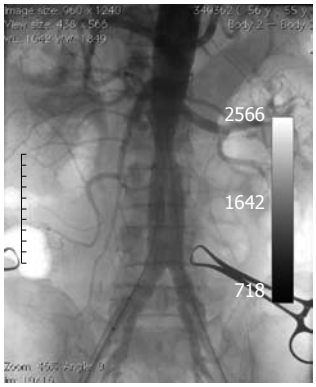

Figure 2 Intraoperative computed tomography angiography after endovascular repair of abdominal aortic aneurysm with excluder stent graft.

antibiotics (imipenem in combination with vancomycin) preoperatively, and continued with the same medication for 4 wk after endovascular repair. The patient then continued taking an oral antibiotic for the next two months (ciprofloxacin 1000 mg per day for 4 wk then doxycycline 100 mg per day for another 4 wk). The postoperative course was without complications and without further blood loss. After the patient was introduced to *per os* food intake, control CT angiography showed no endoleaks and a reduction in thrombotic mass volume in the aneurysm with regression of the aneurysm diameter, however, the gas collection persisted (Figure 3A).

On the tenth day after endovascular repair, RBC and HCT values were normal (RBC 4.2 million per μL and HCT 35%). The patient had improved, but the laboratory tests showed a CRP serum level of 56 mg/L and a high serum IgG level of 20.2 g/L and IgG4 of 4.24 g/L, respectively. Autoantibodies against lactoferrin and carbonic anhydrase Ⅱ were negative, and autoantibodies including anti-nuclear antibodies, antineutrophil cytoplasmic antibodies and rheumatoid factor were not found. Markers for viral hepatitis Bs infection, hepatitis C virus, hepatitis A virus, human immunodeficiency viruses 1 and 2 were negative. Due to lack of histopathological findings, but elevated serum levels of IgG, it could not

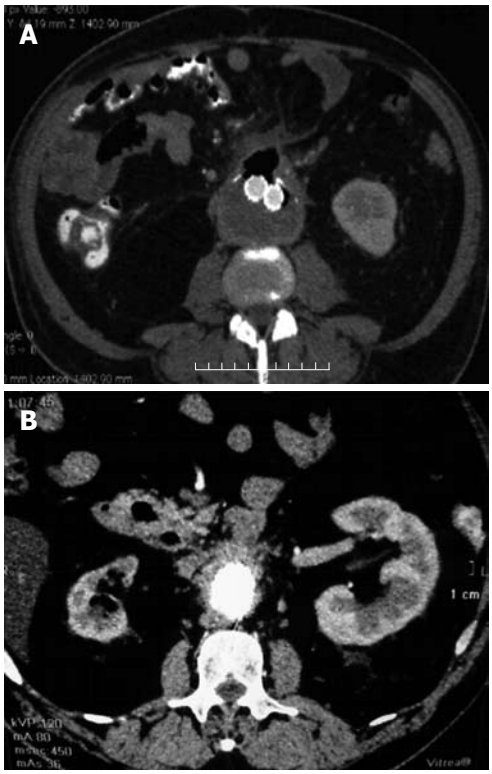

Figure 3 Control computed tomography angiography. A: Control computed tomography (CT) angiography shows no endoleaks and reduction of thrombotic mass volume in the aneurysmatic sac with persistence of gas collection; B: Control CT angiography after 6 mo showed no endoleaks and near total reduction of thrombotic mass in the aneurysmatic sac, without gas collection.

be determined with confidence that this was a systemic disease. It was decided that the patient should start steroid therapy with oral prednisolone at 40 mg per day for 4 wk, and then decrease the dosage by 5 mg every week. Four wk after initiation of steroid therapy, serum levels of IgG (16 g/L) and IgG4 (0.7 g/L) as well as CRP were normal. In the control examination conducted 6 mo after the patient's discharge from hospital (almost 3 mo after the end of steroid therapy), the levels of serum immunoglobulins and CRP were in the normal ranges and control CT angiography showed no endoleaks, near total reduction of the thrombotic mass in the aneurysmatic sac, and no gas collection (Figure 3B).

DISCUSSION

Primary ADF represents a serious condition and is much less frequent than a secondary ADF which occurs as a result of previous aortic aneurysm grafting. The first described case of primary ADF was more than a century ago, and since then about 250 cases have been reported. In the absence of treatment, the mortality rate is almost 100%. With surgical intervention, survival ranges from 18%-93%, but 40% of operated cases develop complications with an overall postoperative mortality rate greater than 30%[12]. Diagnosis of primary ADF is also difficult. Only 33% to 50% of AEF are diagnosed preoperatively[3]. Gastrointestinal bleeding and abdominal pain have been described in all cases, but the classic trio of abdominal pain, palpable mass and gastrointestinal bleeding was found in only 6% to 27.8% of patients[2,13,14]. Our patient only had gastrointestinal bleeding presenting as melena. Diagnostic procedures include EGDS, CT scan, and arteriography. Endoscopy is considered the modality of choice for initial evaluation of upper gastrointestinal bleeding, but has the potential risk of inducing massive hemorrhage by dislodging fresh thrombus in the fistula[15,16]. In our case, EGDS findings were without signs of gastrointestinal hemorrhage. CT angiography was reliable in diagnosing AEF but may be somewhat challenging in unstable patients.

The surgical treatment of ADF consists of repairing the duodenal defect and performing a prosthetic repair of the aorta with graft. Where contamination is present, or in the case of mycotic aneurysm, an extraanatomical aortic graft is preferred and extensive debridement is required[17]. Although the endovascular approach is increasingly utilized in the repair of secondary AEF, only a few primary AEF treated using this method have been reported[4,11,12,18,19]. In the case of any applied vascular procedure, long-term broad spectrum antibiotic therapy is necessary[11,12]. We believe that patients with persistent anemia who undergo laparotomy without diagnosis is hazardous.

Primary ADF is the result of inflammatory destruction of an aortic aneurysm arising from an atherosclerotic AAA. This is an etiological factor in 73% of all primary ADFs, while 26% are caused by traumatic or mycotic aneurysms[2,17,20]. Rarer causes such as radiation, tumors, ulcers and ingestion of foreign bodies account of 1% or less[4,12]. A very small number of AEF occurin the absence of an aortic aneurysm. In comparison to atherosclerotic AAA, IAAA occurs in only 2% to 10% of all AAA, and has different clinical characteristics, such as younger age of patients, non-specific symptoms, and large aneurysm diameter. Recent reports showed a close relationship between IgG4 and sclerosing lesions of various organs [21]. IgG4-related sclerosing lesions, which are also called IgG4-related sclerosing disease, was first reported with regard to autoimmune pancreatitis[22]. The clinical characteristics of IgG4-related sclerosing disease are a frequent occurence in adult male patients, and include elevation of serum IgG4 levels, and steroid sensitivity. In addition to affected organs in IgG4-related disease, elevated IgG4 levels have been described in atopic dermatitis, asthma, some parasitic diseases, pemphigus vulgaris, pemphigus foliaceus and pancreatic cancer[23]. Our patient had no clinical signs of these diseases. The correct diagnosis of IAAA as part of IgG4-related disease is based on histopathological findings[21]. According to these recommendations, the diagnosis of IAAA as part of IgG4-related disease in our patient couldnot be established due to lack of histopathological findings, as endovascular repair of AAA, was successful. If the AAA in our patient was an IAAA, this would be the first case with ADF in IgG4-related sclerosing disease. In addition, the patient had a positive response to steroid therapy. The

question remains as to how it is possible to make a diagnosis of IgG4-related IAAA in such cases where urgent minor surgery is the treatment of choice. In our case, endovascular repair of ADF was successful, and this may be an alternative treatment, especially in patients with high operative risk. Broad spectrum antibiotic therapy is as important as the endovascular treatment in ADF.

In conclusion, primary ADF is a rare complication of aortic aneurysms and is a rare cause of gastrointestinal bleeding. It can be associated with IgG4-related sclerosing disease, whether it occurs as a complication of IAAA or not. CT angiography is a good diagnostic test for an ADF. This case demonstrates that endovascular grafting of primary ADF as an effective, minimally invasive alternative to standard surgical repair. Other supportive therapy such as antibiotics are very important in the treatment of this condition. High serum IgG4 levels and a positive response to steroid therapy suggest the existence of an ADF in IgG4-related IAAA, without histopathological examination.

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CASE REPORT

Should anticoagulants be administered for portal vein thrombosis associated with acute pancreatitis?

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Abstract

Venous complications in patients with acute pancreatitis typically occur as a form of splenic, portal, or superior mesenteric vein thrombosis and have been detected more frequently in recent reports. Although a well-organized protocol for the treatment of venous thrombosis has not been established, anticoagulation therapy is commonly recommended. A 73-year-old man was diagnosed with acute progressive portal vein thrombosis associated with acute pancreatitis. After one month of anticoagulation therapy, the patient developed severe hematemesis. With endoscopy and an abdominal computed tomography scan, hemorrhages in the pancreatic pseudocyst, which was ruptured into the duodenal bulb, were confirmed. After conservative treatment, the patient was stabilized. While the rupture of a pseudocyst into the surrounding viscera is a well-known phenomenon, spontaneous rupture into the duodenum is rare. Moreover, no reports of upper gastrointestinal bleeding caused by pseudocyst rupture in patients under anticoagulation therapy for venous thrombosis associated with acute pancreatitis have been published. Herein, we report a unique case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for portal vein thrombosis associated with acute pancreatitis.

Key words: Pancreatitis; Pancreatic pseudocyst; Portal vein; Venous thrombosis; Warfarin

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INTRODUCTION

The vascular complications of pancreatitis are major causes of morbidity and mortality and are typically related to hemorrhage. Venous complications generally occur as a form of thrombosis in the splenic vein and less commonly in the portal or superior mesenteric vein[1]. Although no randomized controlled trial regarding the use of anticoagulants in acute portal vein thrombosis has been conducted, the use of unfractionated heparin, with subsequent transition to oral warfarin, is the most common approach to anticoagulation[2,3].

Pancreatic pseudocysts are common findings, but spontaneous rupture occurs infrequently. Pseudocyst rupture can occur in the free peritoneal cavity, stomach,

duodenum, colon, portal vein, pleural cavity, and abdominal wall. However, rupture into the duodenum is very rare[4]. Furthermore, no reports of upper gastrointestinal bleeding caused by pseudocyst rupture in patients under anticoagulation therapy for venous thrombosis associated with acute pancreatitis have been published.

We describe herein a very rare and unique case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for portal vein thrombosis associated with acute pancreatitis.

CASE REPORT

A 73-year-old man was transferred to our emergency department for the management of massive hematemesis. On arrival, his systolic blood pressure was 70 mmHg, and his blood hemoglobin level was 6.8 g/dL. He received a transfusion of two units of packed red blood cells. Seven weeks prior, he had initially developed acute alcoholic pancreatitis, and one month prior, he had been diagnosed with acute progressive portal vein thrombosis associated with non-necrotizing acute alcoholic pancreatitis. Since then, he had been receiving anticoagulation therapy. Computed tomography (CT) scans of the abdomen revealed not only thrombosis of the portal vein trunk but also thrombosis of the right and left portal veins (Figure 1A), and low perfusion areas were diffusely observed on the liver parenchyma. A 12 mm × 10 mm cystic lesion was found, suggesting the presence of a pancreatic pseudocyst and extensive fluid collection around the pancreatic head (Figure 1B). Endoscopic ultrasonography also demonstrated the absence of a color Doppler signal and thrombosis of the portal vein, superior mesenteric vein, and distal splenic vein. Arterial portography confirmed an occlusion and thrombosis of the portal vein, superior mesenteric vein, and distal splenic vein. The factors indicating a coagulation defect and tumor markers were normal. We decided to perform anticoagulation therapy because the patient complained of abdominal pain, and serial CT scans showed the progression of the acute portal vein thrombosis. He was treated with intravenous heparin and then switched to warfarin.

Endoscopy and abdominal CT scans were performed to evaluate the hematemesis. Emergency endoscopy (Figure 2A) revealed a 2 cm submucosal tumor-like lesion located just distal to the pylorus. The mucosa showed a central ulceration covered with dark blood clots, which was suggested as a cause of the upper gastrointestinal bleeding. Abdominal CT scans (Figure 1C) revealed a large, walled-in fluid collection of high attenuation around the pancreatic head, indicating a hemorrhagic pseudocyst of the pancreatic head that caused the displacement of the duodenum.

The patient was treated with a high dose of pantoprazole, a proton pump inhibitor, and the warfarin medication was stopped. The patient was hemodynamically stabilized, and no recurrent bleeding occurred. On the seventh day following admission, follow-up endoscopy (Figure 2B) revealed a reduction of the bulging lesion and a fistula opening with clear discharge, which was suspected to be pancreatic fluid. A follow-up abdominal CT scan (Figure 1D) showed a small collapsed cyst without internal hematoma, and a fistulous tract was also observed between the duodenum and the pseudocyst, resulting in air pocket in the cyst. After only conservative treatment, the patient recovered without recurrent bleeding and was discharged without warfarin medication.

DISCUSSION

Pancreatitis may cause a spectrum of venous and arterial vascular complications. Of these complications, venous thromboses are typically reported in the splenic vein and less commonly in the superior mesenteric vein or portal vein[5]. Thrombotic complications have been known to be more common in alcohol-induced, necrotizing, and chronic pancreatitis[6,7]. It has been suggested that the pathogenesis of venous thromboses involves stasis, spasm, and mass effects from the surrounding inflamed pancreas and direct damage of the venous wall by liberated enzymes[5]. Our case was associated with non-necrotizing, alcoholic pancreatitis. The portal vein was mainly involved, although venous thromboses also occurred in the distal splenic vein and superior mesenteric vein.

Specific therapeutic management for portal vein thrombosis seems to be mandatory to resolve portal vein obstruction, thereby preventing the development of chronic portal vein thrombosis and avoiding serious complications, such as portal vein hypertension, mesenteric ischemia, and infarction[2,3]. However, randomized controlled studies on the efficacies of most forms of therapy for portal vein thrombosis are lacking. The use of unfractionated heparin, with subsequent transition to oral warfarin is the most common approach to anticoagulation[2], while Gonzelez *et al*[8] recently reported that recanalization is observed in almost one third of patients, irrespective of whether they receive systemic anticoagulation. The issue of warfarin dose has not been addressed in randomized trials, and the optimal duration of anticoagulation is controversial[2]. What is certain is that the sooner the treatment is given, the better the outcome will be; the rate of recanalization is approximately 69% if anticoagulation is initiated within the first week after diagnosis, while it falls to 25% when initiated during the second week[3].

In our case, anticoagulation therapy was not started at an earlier stage of portal venous thrombosis but began after the confirmation of thrombotic progression, which could explain why the patient achieved only partial portal vein recanalization. Fortunately, the patient did not develop mesenteric ischemia or infarction, and the portal vein thrombosis did not worsen further.

Gastrointestinal bleeding in the setting of pancreatitis arises from vascular complications or coexisting

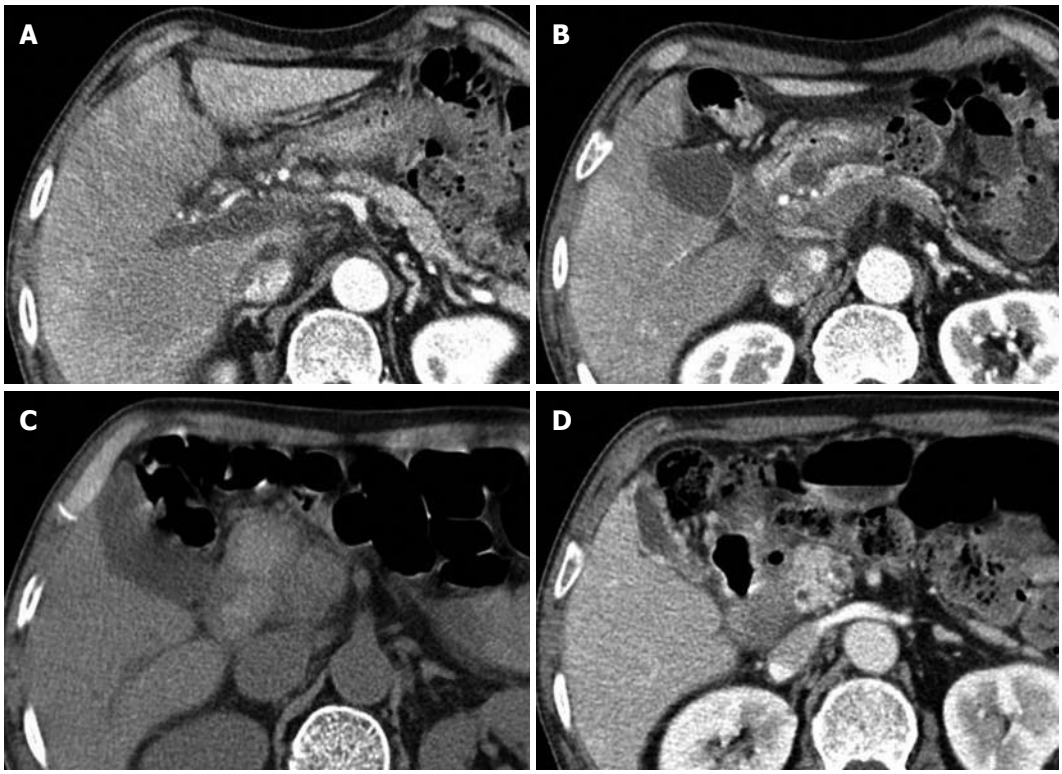

Figure 1 Abdominal computed tomography images. A: A filling defect and intra-luminal hypoattenuation in the portal vein consistent with acute portal vein thrombosis; B: A 12 mm × 10 mm cystic lesion suggestive of a pseudocyst and extensive inflammatory fluid collections around the pancreatic head; C: A walled-in fluid collection with high attenuation, indicative findings of a hemorrhagic pseudocyst around the pancreatic head and compressed duodenum; D: Reduced hemorrhagic fluid collections and an air-filled cyst, indicative of a fistula between the duodenum and the pseudocyst.

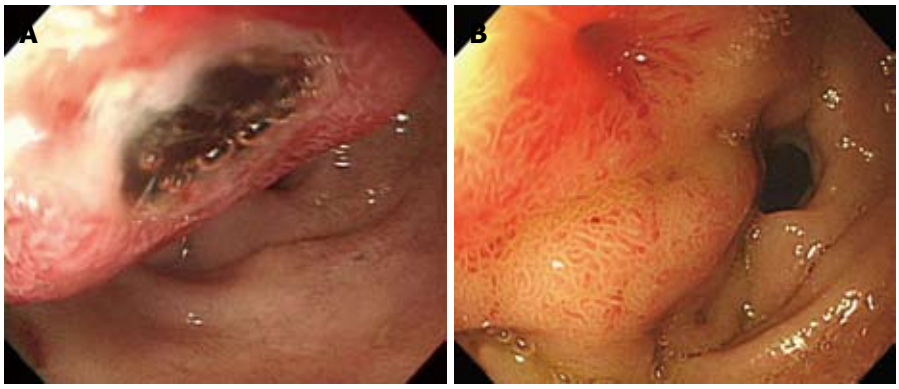

Figure 2 Upper gastrointestinal endoscopy. A: Emergency endoscopy shows a 2 cm submucosal tumor-like bulging mass with central ulceration; B: A follow-up endoscopy image showing a decreased mass size and fistula orifice.

lesions[9,10]. Major gastrointestinal bleeding is considered to be rare, but its exact incidence is not well established. According to a recent report of 1356 acute pancreatitis patients, spontaneous bleeding occurred in 10 patients, and 6 patients had gastrointestinal bleeding[10]. The most frequent cause of severe bleeding in pancreatitis is a ruptured pseudoaneurysm, which accounts for approximately 60% of cases, and a hemorrhagic pseudocyst without a pseudoaneurysm and a capillary, venous, or small vessel hemorrhage each account for approximately 20% of cases[7,11]. In a review of cases from 1987 to 1996, 31 patients were found to have developed vascular lesions either in the form of hemorrhage into a pseudocyst (12 patients) or pseudoaneurysms (19 patients)[12].

In the management of any hemorrhage, the early recognition and investigation of hemorrhagic episodes is imperative because accurate diagnosis and timely radiological interventional procedures can reduce mortality[7]. Dynamic bolus CT and angiography are considered to be the most useful means of finding a hemorrhage[13]. In particular, angiography can play an invaluable role both in locating the source of bleeding and in the embolization of the bleeding vessel[14]. In the present case, the diagnosis of a ruptured bleeding pseudocyst was made

with CT scans and endoscopy, and the ruptured bleeding pseudocyst confirmed the presence of the fistula tract and the walled-in fluid collection with high attenuation around the pancreatic head, providing evidence supportive of a bleeding pseudocyst. Due to the hemodynamically stable clinical conditions and no evidence of pseudoaneurysm formation on CT scans, we did not perform angiography or surgical therapy and decided to pursue a "watchful waiting" policy.

The rupture of a pseudocyst into the gastrointestinal tract either results in no symptoms or leads to melena or hematemesis that typically requires urgent measures[15]. Although rupture into the surrounding viscera is a well-known phenomenon, the spontaneous rupture of a pseudocyst into the upper gastrointestinal tract is very rarely reported[4,16-21], and the spontaneous rupture of a pseudocyst into the duodenum most frequently occurs in the second portion of the duodenum[21]. In the presented case, the first part of the duodenum was involved, and rupture into the duodenum led to hematemesis requiring transfusion.

In our case, the exact pathogenic mechanisms of the bleeding and rupture of the pancreatic pseudocysts were unclear. However, we can suggest some possibilities as to why massive bleeding due to pseudocyst rupture occurred. First, the pseudocyst may have been tensely distended because of intra-cystic bleeding caused by warfarin use and eventually ruptured into the duodenum; Second, the rupture of the pancreatic pseudocyst could have developed first, and severe bleeding of the ruptured duodenal mucosa followed due to the anticoagulation therapy. Consequently, we considered that the warfarin either initiated or aggravated the bleeding into the duodenum. Finally, in this case, it was not clear whether portal hypertension by portal vein thrombosis affected the bleeding risk itself and its severity.

In conclusion, we report a rare case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for acute pancreatitis associated with portal vein thrombosis. Gastroenterologists should consider a pseudocyst rupture into the gastrointestinal tract as a bleeding source in patients with pancreatitis and should also keep in mind that anticoagulants used to manage portal vein thrombosis associated with acute pancreatitis can lead to serious bleeding.

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CASE REPORT

Postoperative retroperitoneal desmoid tumor mimics recurrent gastrointestinal stromal tumor: A case report

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Abstract

Desmoid tumor is a locally invasive, myofibroblastic, nonmetastatic tumor. Its pathogenesis remains unclear and it may involve genetic abnormalities, sex hormones and traumatic injury, including surgery. Postoperative intra-abdominal desmoid tumor is rare, especially in the retroperitoneum. We report a case of postoperative retroperitoneal desmoid tumor that developed 29 mo after the first excision of a gastrointestinal stromal tumor. Sporadic trauma-related intra-abdominal desmoid tumors reported in the English literature are also reviewed. Despite an extremely low incidence, postoperative desmoid tumor should be considered in the differential diagnosis when a recurrent neoplasm is found at least one year after operation. However, it is a clinical challenge to distinguish recurrent malignant neoplasms from desmoid tumors, and surgical resection is the treatment option depending on the anatomic location.

Key words: Desmoid tumor; Gastrointestinal stromal tumor; Recurrence; Retroperitoneum; Surgery

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INTRODUCTION

Desmoid tumor (also known as aggressive fibromatosis or fibromatosis) is an infrequently occurring, locally invasive, nonmetastatic tumor. Desmoid tumors account for 0.03% of all newly diagnosed neoplasms and 3% of all soft tissue neoplasms[1]. These tumors originate from musculoaponeurotic planes and are found intra- and extra-abdominally. The cause of desmoid tumor is unclear and it may be related to trauma, hormonal factors, or genetic associations[2]. The occurrence of a surgery-related retroperitoneal desmoid tumor after the excision of gastrointestinal stromal tumor (GIST) is rare. However, despite its scarcity, desmoid tumor should be differentiated from recurrent neoplasm in diagnosis, especially those occurring near the previous operative site.

CASE REPORT

A 56-year-old man complained of epigastralgia for three weeks without symptomatic improvement after receiving medical treatment at another hospital in 2009, where a deep-seated gastric ulcer had been found by panendoscope. Because there had been no symptomatic relief, he was referred to the in-patient department of our hospital. On physical examination, abdominal fullness with obvious rebounding pain was noticed. A perforated peptic ulcer was suspected and an abdominal computed tomography (CT) scan was performed. The abdominal CT scan revealed a 10-cm gastric mass with perforation (Figure 1A). In May 2009, the patient underwent an emergent explorative laparotomy with debulking of the intraperitoneal tumor and irrigation. A ruptured GIST, of intermediate risk category, with spreading intra-abdominal tumors was diagnosed pathologically (Figure 1B and C). He had no family history of familial adenomatous polyposis (FAP) or colorectal diseases among his close relatives. Because the abdominal CT scan had shown no signs of FAP, colonoscopy was not performed. His clinical course was uneventful and he was discharged on day 8 after admission. After the operation, imatinib (Glivec) 200 mg *b.i.d.* was prescribed. However, due to intractable diarrhea, the target therapy was discontinued. The follow-up abdominal CT scan showed no recurrent tumor two months after the operation. He was then lost to follow-up. The patient revisited our out-patient clinic 12 mo later after the previous operation. The abdominal CT scan showed a 4.7-cm hepatic tumor in segments 6 and 7 and an intra-abdominal 2.5-cm mass located in the upper greater curvature of the stomach (Figure 1D). The impression was metastatic GISTs. He received a second operation with a smooth course in June 2010 and metastatic GISTs were confirmed pathologically (Figure 1E). However, a 1.9-cm tumor between the pancreatic tail and splenic hilum was found in the follow-up abdominal CT scan 3 mo after the second operation. The tumor was located in the incision area of the first debulking operation. The patient refused another operation, and he was managed with target therapy with imatinib (Glivec) 200 mg *b.i.d.*, which was well tolerated. A series of follow-up abdominal CT scans were performed. Fifteen months after the second operation, CT scan revealed progressive enlargement of the splenic hilar tumor from 1.9 cm to 3.2 cm (Figure 1F). The patient continued to take imatinib (Glivec) regularly. Under the impression of a second recurrent GIST, he underwent a third operation in October 2011. Intraoperatively, the tumor was found in the retroperitoneum adhering to the peri-tumor vessels, nerves and the pancreatic tail. This tumor was resected *en bloc* with sacrifice of adjacent vessels and nerves. Grossly, the tumor measured 3.6 cm × 2.5 cm × 1.1 cm and was elastic with grayish-brown cut surface. Histologically, the tumor demonstrated proliferative spindle cells in fibrotic background with keloid-like, glassy, hyalinized collagen fibers, nodular fasciitis-like erythrocyte extravasation and infiltrative growth pattern. Immunohistochemical staining of the tumor cells revealed positive nuclear stains for beta-catenin, but negative stains for CD117 and CD34 (Figure 1G). A desmoid tumor was diagnosed pathologically. The patient's clinical course was uneventful and no recurrent tumor was found 6 mo after the third operation.

DISCUSSION

Although desmoid tumor is locally aggressive with infiltrative growth behavior, it is considered to be a benign neoplasm for its bland cellular appearance, scant mitosis and lack of metastasis. The term "desmoid" originates from the Greek word "demos", meaning band or tendon like, and was first named by Müller[3] in 1838. Desmoid tumor accounts for 0.03% of all neoplasms and 3.0% of all soft tissue tumors[1].

Although the actual cause of desmoid tumor is still being debated, its likely pathogenesis includes genetic abnormalities, sex hormones and trauma, including surgical trauma. Kulaylat *et al*[2] found that 10%-30% of all sporadic abdominal wall desmoid tumors occurred following surgical intervention and half of these tumors developed within 4 years after the surgery. Warren[4] described the criteria for post-traumatic neoplasm, including prior integrity of the tumor site, injury severe enough to initiate reparative cell proliferation, reasonable latent period, and tumor compatible with the scar tissue and anatomic location of the injury. In the present case, the splenic hilar region was incised during the debulking operation for the ruptured GIST of the stomach, and the desmoid tumor occurred 29 mo after the surgery. The present case of postoperative retroperitoneal desmoid tumor was compatible with Warren's criteria[4] and occurred within an appropriate latent period.

The English literature includes 12 cases, including the present case, of sporadic postoperative intra-abdominal desmoid tumor[5-15] (Table 1). The male-to-female ratio was 8:3 (one case showed no gender datum) and the mean age was 49.6 years (range, 27-79 years). Five of the 12 cases were located on the mesentery, which is the most frequent site of this type of desmoid tumor to date. Tumor sizes ranged from 2.8 cm to 18 cm and the mean duration from previous operative insult to excision of desmoid tumor was 2.3 years (range, 11 mo to 7 years). It indicates that a reasonable latent period for this type of desmoid tumor is at least one year. None of the desmoid tumors was diagnosed preoperatively. This denotes the diagnostic challenge of this type of desmoid tumor. Ten of the 12 desmoid tumors were widely excised under the impression of recurrent malignant neoplasm. No recurrence was found in 8 cases followed up between 6 mo to 2 years. Up till now, only 3 postoperative desmoid tumors after resection of gastric GIST have been reported in the literature. Whether GIST is a risk factor for the development of desmoid tumors or just a coincidence should be further elucidated.

Recently, the Wingless/Wnt signaling pathway was hypothesized to be involved in the tumorigenesis of

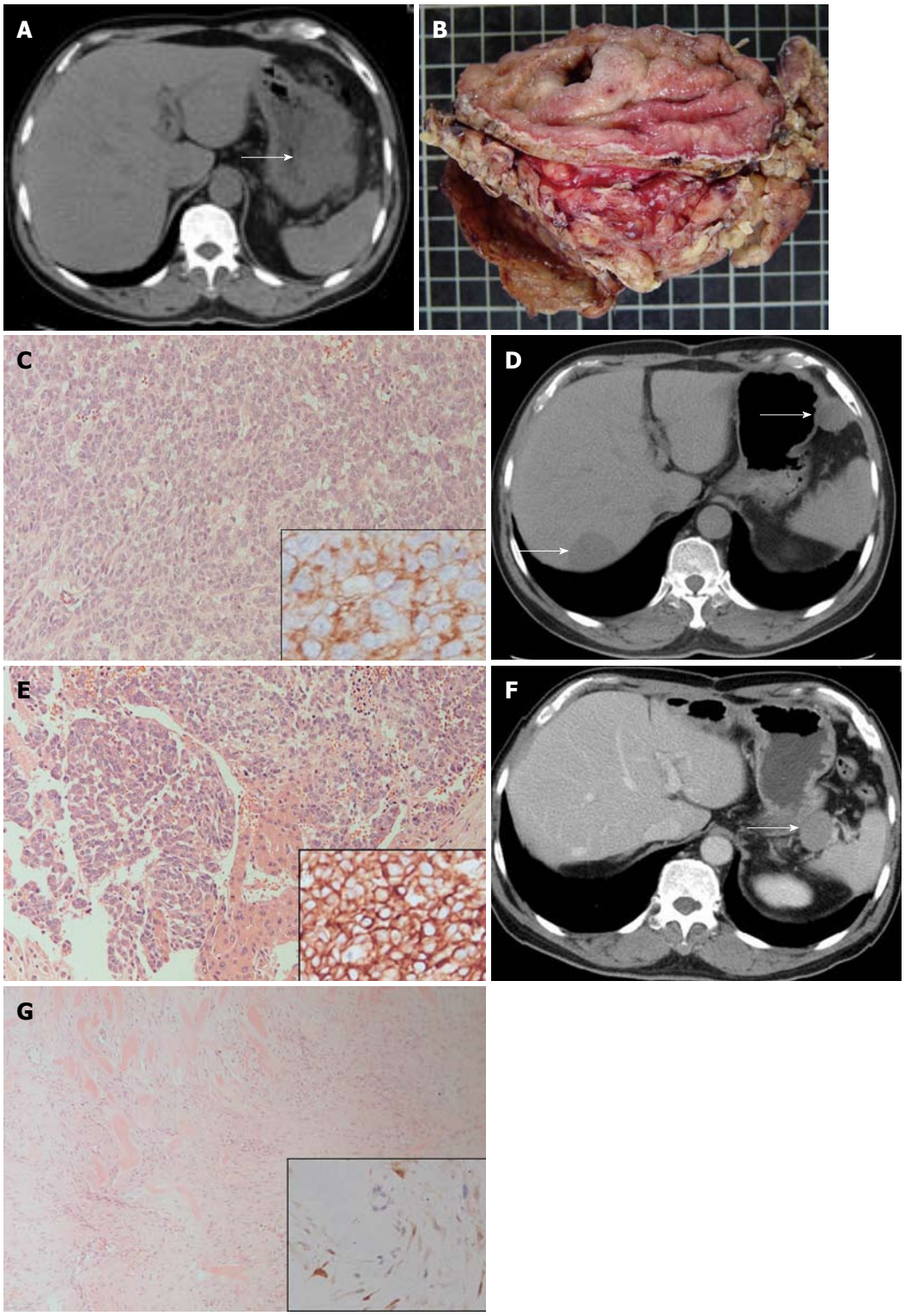

Figure 1 Abdominal computed tomography images and pathologic features of gastrointestinal stromal tumor, metastatic hepatic gastrointestinal stromal tumor and retroperitoneal desmoid tumor. A: Abdominal computed tomography (CT) scan reveals a 10-cm tumor (arrow) located at the greater curvature of the stomach; B: The excised specimen discloses a patch of gastric mucosa with a central deep-seated ulcer and the gastrointestinal stromal tumor adhering to the red yellow omental tissues; C: Histologically, the 10-cm gastric tumor demonstrated sheets of CD117-positive epithelioid cells after immunohistochemical (IHC) staining (right lower inset), hematoxylin and eosin (HE) stain, ×200; D: The follow-up abdominal CT scan shows metastatic tumors in the liver (left lower arrow) and upper greater curvature of the stomach (right upper arrow); E: The histology of metastatic liver tumor demonstrated nests and sheets of CD117-positive epithelioid tumor cells (right lower inset, IHC stain). Entrapped hepatocytes are seen in the middle lower portion, HE stain, ×200; F: Abdominal CT scan discloses a tumor neogrowth (arrow) located in the retroperitoneum between the pancreatic tail and splenic hilar region; G: Histologically, the retroperitoneal tumor demonstrated proliferative spindle cells with keloid-like bundles and erythrocyte extravasation, HE stain, ×200. IHC staining reveals a positive nuclear beta-catenin in spindle cells (right lower inset).

Table 1 Sporadic postoperative intra-abdominal desmoid tumors reported in the literature

No.	Authors	Sex/age	Location	Tumor size(cm)	Diagnosis of previous operation	Type of primary trauma	Duration	Treatment	R/F
1	Mizuno *et al*[5]	M/61	Mesentery near anastomosis	2.8	Ascending colon cancer (pT3N1M0)	Right hemicolectomy	16 mo	Excision with ileum and colon	N/2 yr
2	Liao *et al*[6]	F/79	Left lower abdomen	17 × 14× 13	Encapsulated lipoma	Resection	7 yr	Excision	N/6 mo
3	Khan *et al*[7]	-/37	Mesentery	6 × 4.5 × 4	Gastric GIST	Total gastrectomy	11 mo	Excision	N/8 mo
4	Vendrell *et al*[8]	M/58	Posterior side of stomach	8 × 6 × 4	Gastric GIST	Laparoscopic tumorectomy	2 yr	Wide excision with splenectomy and total gastrectomy	-
5	Komatsu *et al*[9]	M/50	Mesentery in left upper abdominal cavity	10 × 6	Gastric adenocarcinoma (pT3N1M0)	Total gastrectomy	1 yr	Excision with jejunum	N/9 mo
6	Tamura *et al*[10]	F/73	Mesentery of jejunal pouch	6.3 × 5 × 5	Gastric cancer (pT1N1M0)	Total gastrectomy	1 yr	Excision and reconstruction	N/4 yr
7	Lawatsch *et al*[11]	M/27	Retroperitoneum	17 × 13.5 × 8.5	Mixed germ cell tumor of right testis	Retroperitoneal lymph node dissection	2 yr	Excision with ileum and colon	N/10 mo
8	Lai *et al*[12]	M/45	Anterior lower abdomen	-	Mesenteric injury and bowel gangrene	Abdominal surgery	1 yr	Excision with ileum	-
9	Firoozmand *et al*[13]	F/27	Pelvic	17 × 14 × 10	-	Colectomy with ileoanal J pouch anastomosis	4 yr	En bloc resection	-
10	Little *et al*[14]	M/31	Left upper abdomen	12 to 14	Mixed germ cell tumor of right testis	Retroperitoneal lymph node dissection	2 yr	Excision with jejunum	N/18 mo
11	Pasciak *et al*[15]	M/51	Mesentery	18 × 12 × 11	Transitional cell carcinoma of urinary bladder	Radical cystectomy	3 yr	Excision with small bowel	-
12	Shih *et al*, present	M/56	Retroperitoneum	3.6 × 2.5 × 1.1	Gastric GIST	Exploratory debulking	29 mo	En bloc excision	N/6 mo

R: Recurrency; F: Follow-up; N: No; -: Not mentioned; GIST: Gastrointestinal stromal tumor.

desmoid tumors, especially the two key proteins, adenomatous polyposis coli (APC) and beta-catenin[16]. APC is considered to be a tumor suppressor gene and beta-catenin an oncogene. Sporadic desmoid tumors typically present oncogenic mutation in beta-catenin. However, FAP-associated desmoid tumors are associated with germline APC mutation followed by somatic inactivation of the wild-type APC allele[17,18]. Beta-catenin protein level is upregulated in desmoid tumors, due to either APC mutations and subsequent ineffective regulation of beta-catenin activation, or beta-catenin gene mutations that led to stabilization and constitutive activation of the beta-catenin. These pathways indicated that the expression of nuclear beta-catenin may play a role in the differential diagnosis of desmoid tumors from fibroblastic or smooth muscle neoplasms[16].

Primary wide surgical resection with tumor-free margins is the treatment of choice for desmoid tumor. This surgical strategy made it essential to sometimes sacrifice the adhered normal vital tissues such as vessels and nerves. Resections with tumor-positive margins indicate a high risk of recurrence, and secondary resection, chemotherapy or radiotherapy may be performed consequently according to the patient's condition[19]. Garbay *et al*[20] treated 62 patients with cytotoxic chemotherapy for progressive or recurrent desmoid tumors, and 80% of the patients had a clinical benefit (objective response plus stable disease) from the cytotoxic chemotherapy. Anthracycline-containing regimens appeared to be associated with a higher response rate. Radiotherapy for unresectable cases or local tumor control after surgery has been empirically applied in some instances[21]. However, the efficacy of the adjuvant treatment still needs to be further elucidated. Recently, three discrete mutations (ACC41GCC, TCT45TTT and TCT45CCT) in two codons of CTNNB1 exon 3 were reported[22]. Target therapy for desmoid tumor may be feasible and become another treatment option in the future.

Postoperative intra-abdominal desmoid tumor is exceptionally rare and is often overlooked by clinicians. Most clinicians believe that a tumor with locally infiltrative growth behavior in patients with a history of malignancy is a recurrent malignant tumor, and a wide excision with sacrifice of adjacent organs is usually done. However, despite an extremely low incidence, a postoperative desmoid tumor should be included in the differential diagnoses.

In conclusion, postoperative intra-abdominal desmoid tumor is rare and a correct preoperative diagnosis is a clinical challenge. Physicians should keep in mind the possibility that a postoperative desmoid tumor may appear as a so-called "recurrent" neoplasm, especially when the tumor presents at the previous surgical site.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

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